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APE1, the DNA base excision repair protein, regulates the removal of platinum adducts in sensory neuronal cultures by NER

Hyun-Suk Kim[1], **Chunlu Guo**[2], **Eric L. Thompson**[2], **Yanlin Jiang**[3], **Mark R. Kelley**[1,2,3], **Michael R. Vasko**[2], and **Suk-Hee Lee**[1,*]

[1]Department of Biochemistry & Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202, USA

[2]Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, Indiana 46202, USA

[3]Department of Pediatrics and Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, Indiana 46202, USA

Abstract

Peripheral neuropathy is one of the major side effects of treatment with the anticancer drug, cisplatin. One proposed mechanism for this neurotoxicity is the formation of platinum adducts in sensory neurons that could contribute to DNA damage. Although this damage is largely repaired by nuclear excision repair (NER), our previous findings suggest that augmenting the base excision repair pathway (BER) by overexpressing the repair protein APE1 protects sensory neurons from cisplatin-induced neurotoxicity. The question remains whether APE1 contributes to the ability of the NER pathway to repair platinum-damage in neuronal cells. To examine this, we manipulated APE1 expression in sensory neuronal cultures and measured Pt-removal after exposure to cisplatin. When neuronal cultures were treated with increasing concentrations of cisplatin for two or three hours, there was a concentration-dependent increase in Pt-damage that peaked at four hours and returned to near baseline levels after 24 hours. In cultures where APE1 expression was reduced by ~80% using siRNA directed at APE1, there was a significant inhibition of Pt-removal over eight hours which was reversed by overexpressing APE1 using a lentiviral construct for human wtAPE1. Reduction in APE1 expression also altered the expression of the NER proteins RPA70 and XPA in sensory neuronal cultures. Overexpressing a mutant APE1 (C65 APE1), which only has DNA repair activity, but not its other significant redox-signaling function, mimicked the effects of wtAPE1. Overexpressing DNA repair activity mutant APE1 (226+177APE1), with only redox activity was ineffective suggesting it is the DNA repair function

[*]Corresponding author: Suk-Hee Lee, Department of Biochemistry & Molecular Biology, Indiana University Simon Cancer Center, Indiana University School of Medicine, Indianapolis, IN, USA, Tel.: (317) 278-3464; Fax: (317) 274-8046; slee@iu.edu.

Conflict of interest statement

The authors declare that there is no conflict of interest.

of APE1 and not its redox-signaling, that restores the Pt-damage removal. Together, these data provide the first evidence that a critical BER enzyme, APE1, helps regulate the NER pathway in the repair of cisplatin damage in sensory neurons.

Keywords

Sensory neuronal cultures; Cisplatin damage; APE1; Replication protein A; Nucleotide excision repair; Base excision repair

1. Introduction

It is well established that cisplatin, which is a primary therapy in a number of cancers [1–5] cause peripheral neuropathy in a significant number of patents [1, 6–8]. The onset and severity of the neuropathy correlate with the cumulative dosage of the drug administered and with the duration of therapy [1, 9]. The severity of the neuropathy can warrant stopping therapy and in many cases, the toxicity is maintained after therapy is discontinued [8, 10, 11]. To date, there are no therapies available to prevent or reduce cisplatin-induced peripheral neuropathy (CIPN) although a number of agents have been tried [12].

The cellular mechanisms mediating cisplatin-induced neuropathy remain to be determined, but evidence suggests that the neurotoxicity is secondary to a direct action of this drug on the cell bodies of sensory neurons. In patients and in animals receiving cisplatin, the highest concentrations of the drug and of platinum adducts are observed in the cell bodies of sensory neurons and in peripheral sensory neurons [6, 13]. Furthermore, cisplatin caused the formation of intrastrand and interstrand Pt-adducts in rat sensory neurons and the level of these adducts raises with increasing the cumulative dose of cisplatin [13, 14]. The levels of platinum adducts also correlate directly with the ability of cisplatin to cause histopathology, axonal degeneration and/or changes in nerve conduction [14, 15].

Since nuclear excision repair (NER) is the major pathway for removing platinum adducts [16], alterations in the pathway should influence cisplatin-induced neuropathy. Indeed, in mice deficient in *Xeroderma pigmentosum* complementation group A (XPA) or group C (XPC), complexes necessary for NER, there is an increase in platinum adduct formation in sensory neurons and in satellite cells of the dorsal root ganglia compared to wild type mice [17]. Furthermore, in XPC deficient mice, removal of adducts is reduced over time and mice with compromised NER show an increase in symptoms of neuropathy after cisplatin treatment compared with mice with intact NER [17]. These data suggest that the NER pathway may be critical for reducing cisplatin-induced neuropathy.

In previous work from our laboratory, however, we demonstrated that augmenting the base excision repair (BER) pathway by increasing the expression of apurinic/apyrimidinic endonuclease (APE1) is neuroprotective against cisplatin-induced toxicity in isolated sensory neurons [18]. We and others also have shown that cisplatin increases production of reactive oxygen species (ROS) and that oxidative stress may contribute to cisplatin-induced toxicity [18–21]. Since oxidative DNA damage is repaired by BER pathway [7, 21–25], it is interesting to speculate that this pathway contributes to DNA repair after sensory neurons

are exposed to cisplatin. The question remains, whether augmenting the BER pathway affects the ability of the NER pathway to remove platinum adducts from sensory neurons.

To address this question, we used a DNA slot blot method to quantitatively measure platinum adducts after exposure to cisplatin in sensory neuronal cultures with a reduction or overexpression of APE1. We found that reducing expression of APE1 inhibits the repair of cisplatin adducts damage, while adding back APE1 with repair activity, but not the redox-signaling function restores repair of cisplatin damage. Additionally, two proteins involved in early steps of NER, RPA and XPA were affected when APE1 levels were altered. These data support an interaction between the BER and NER pathway that promotes removal of platinum adducts in sensory neurons.

2. Materials and methods

2.1. Materials

Tissue culture supplies were obtained from Invitrogen (Carlsbad, CA), and routine chemicals including cisplatin from Sigma Chemical Company (St. Louis, MO). Normocin was purchased from InvivoGen (San Diego, CA) and nerve growth factor from Harlan Bioproducts for Science, Inc. (Indianapolis, IN). Optiprep was obtained from NYCOMED PHARMA AS (Oslo, Norway). The transfecting reagent, Neuroporter® was purchased from Gene Therapy Systems (San Diego, CA). QIAamp DNA Mini kit was purchase from Qiagen (Valencia, CA), and Western blotting detection ECL system from Amersham GE Healthcare (Pittsburgh, PA). Mouse monoclonal antihuman APE1 antibodies were produced in the Kelley laboratory and are available from Novus Biologicals, Littleton, CO. An anti-1,2-Pt-(GpG) monoclonal antibody was purchased from Oncolyze (Essen, Germany), peroxidase-coupled secondary antibody from Sigma Chemical Company (St. Louis, MO), goat anti-mouse HRP conjugated IgG secondary antibody from Zymed Laboratories Inc. (San Francisco, CA), actin antibodies from Thermo (Fremont, CA), and HA rat monoclonal antibodies from Roche Applied Science (Mannhiem, Germany). The rabbit polyclonal RPA70 (70 kDa subunit of replication protein A, RPA) antibody was from Novus Biologicals (Littleton, CO), while the rabbit polyclonal XPA antibody was from Santa Cruz Biotechnology (Dallas, TX). The Animal Care and Use Committee at Indiana University School of Medicine, Indianapolis, IN approved all procedures used in these studies.

2.2. Cell Culture

Sensory neuronal cultures were prepared for the dorsal root ganglia (DRG) taken from male Sprague Dawley rats (150–175 g) as previously described [26]. Briefly, rats were euthanized by CO_2 asphyxiation and the DRG removed from the length of the spinal column. The DRG were incubated in collagenase, cells mechanically dissociated, and ~60,000 cells were plated into each well of poly-D-lysine and laminin-coated six-well culture plates. Cells were maintained in F-12 media (Invitrogen) supplemented with 10% horse serum, 2 mmol/L glutamine, 100 μg/mL normocin, 50 μg/mL penicillin, 50 μg/mL streptomycin, 50 μmol/L 5-fluoro-2′-deoxyuridine (Invitrogen), 150 μmol/L uridine, and 30 ng/mL of nerve growth factor (Harlan Bioproducts for Science, Inc.) in 3% CO_2 at 37°C. Growth medium was changed every other day.

2.3. Transfection with siRNAs

To reduce APE1 expression in sensory neuronal cultures, on day 3 in culture, cells were exposed to 100 nM APE1-siRNA or a scrambled control (SCsiRNA) for 48 hours as previously described [27, 28]. Briefly, the growth media was replaced with 0.5 mL of Optimem I medium containing 10 μL of the transfecting reagent Neuroporter in the absence or presence of the 21-mer oligonucleotide double-stranded small interfering RNA (siRNA) to rat APE1 (5′-GUCUGGUAAGACUGGAGUACC-3′) or a scramble siRNA (5′-CCAUGAGGUCAGCAUGGUCUG-3′). Fresh medium (0.5 mL) without antibiotics was added after 24 hour of incubation, and after an additional 24 hour, the medium was replaced with normal medium containing antibiotics and cell growth maintained.

2.4. Lentivirus Infection

Lentiviral constructs were produced using the Gateway® LR Clonase™ II (Invitrogen Life Technologies) enzyme mix to move wtAPE1-IRES-EGFP, C65APE1-IRES-EGFP, or 266+177APE1-IRES-EGFP into the lentiviral transfer vector, pCSCGW which we obtained from the Vector Production Facility (Indiana University School of Medicine). In all instances, constructs were derived from human APE1 and had a hemagglutinin epitope (HA) tag on the amino end of the molecule and expressed using the cyclomegalovirus promoter. We used human APE1 since it has a base pair difference for rat APE1 and thus its expression is not inhibited by rat siRNA [29]. The constructs were transformed into STBL3 *E. coli* (Invitrogen Life Technologies) and selected using zeocin resistance and $ccdB^S$ sensitivity. The plasmids were isolated using the EndoFree Plasmid Maxi Kit (IBI Scientific, Peosta, IA) and PCR used to confirm that the destination vector contained the appropriate APE1 insert.

On day one of viral production, T175 flasks were plated with 1.2×10^7 293FT cells in 25 ml medium and grown to 80–90% confluence at 37°C in 5% CO_2 for 24 hrs. On day 2, the transfer vector, pCSCGW with a gene-of-interest insert, in combination with a 3rd generation packaging combo: pMDG-VSVG, pMDLg, pRRE-gag/pol, and pRSV-Rev were transfected into 293FT cells using the Profection Mammalian Transfection System (Promega). The four plasmids, transfer, envelope, packaging, and expression ratios (μg) were 30.4:10.4:15.2:7.6 of each plasmid for a T175 flask. On day 3, the media was changed and on day 4–5 medium was collected and the viral containing supernatant filtered using a 0.45 μM SFCA syringe or flask filter. The virus was concentrated using Centricon Plus-70 Centrifugal Filters (Millipore), collected, aliquoted, and stored at −80°C and the viral titers were determined using QuickTiter™ Lentivirus Quantitation Kit (Cell Biolabs, Inc.) For infection, cells were maintained in culture and 20 μl of the concentrated virus added to the cultures on day 5. The virus was removed on day 7 and the cells maintained in culture for an additional 5 days in regular media.

2.5. Cisplatin treatment

Adult sensory neuronal cultures were grown in culture for 12 days with or without siRNA transfection and/or viral infection. On day 12, the cells were treated with cisplatin at various concentrations, and further incubated for indicated time as described in the results section. Cisplatin was dissolved it in 1-methyl-2-pyrrolidinone (Sigma-Aldrich) or N,N-

dimethylformamide (Sigma-Aldrich) then diluted to the appropriate concentrations with media. The drug was prepared fresh for each experiment.

2.6. Immunoblotting

To confirm knockdown or over expression of wtAPE1 or APE1 mutants, cells were harvested, and an aliquot lysed in 100 μl of lysis buffer (10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.01% Triton X-100). Protein was quantified using Lowey assay and electrophoresed in a 4–10% graded SDS-polyacrylamide gel. After electrophoresis, the gel was transferred to a PVDF membrane, and blocked with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% nonfat dry milk for 1 hour at room temperature while gently agitating. Mouse monoclonal antihuman APE1 antibodies (1:1000), HA antibodies (1:1000), β-Actin monoclonal antibody (1:1000), or rabbit polyclonal antibody to RPA70 (1:1000), or to XPA (1:1000) were added to the blocking solution and incubated overnight at 4°C while gently agitating. In Westerns for endogenous APE1 and HA tagged exogenous APE1 constructs, the blots were first included with HA antibodies then reblotted with APE1 antibody. Antibody binding was detected following appropriate secondary antibody methods using chemiluminescence. The density of the bands was measured using QualityOne® software from Bio-Rad (Hercules, CA) and data expressed as density normalized to actin.

2.7. Measurement of 1,2-Pt-(GpG) adducts on genomic DNA

Platinum adducts were quantitatively measured using DNA slot blot analysis that will be published elsewhere. Briefly, after harvesting cells, genomic DNA was prepared using QIAamp DNA Mini kit (Qiagen) according to the protocol. After adjusting genomic DNA concentration using a spectrophotometer, DNA samples were treated with 0.4 M NaOH and 10 mM EDTA, and incubated at 100°C for 10 min for denaturation of genomic DNA. DNA samples (1.0 μg) were neutralized by adding an equal volume of cold 2 M ammonium acetate, pH 7.0, and applied onto a nitrocellulose membrane pre-wetted with 6× SSC buffer using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad). After washing individual wells twice with SSC, the Bio-Dot SF Microfiltration Apparatus was disassembled, and the membrane was rinsed twice in SSC and air-dried. The membrane was baked under vacuum for 2 hours at 80°C, blocked with 5% BSA in PBS, and incubated with an anti-1,2-Pt-(GpG) DNA adducts antibody (Oncolyze, Germany) and with peroxidase-coupled secondary antibody (Sigma Chemical Co.) for visualization of signals using ECL system (Amersham GE Healthcare). Signals were then quantitatively assessed using Molecular Imager ChemiDoc XRS system (Bio-Rad).

2.8. Data analysis

Slot blot analysis was expressed as the mean ± the standard deviation for three independent experiments from separate harvests. Relative Pt-damage was calculated by manual analysis of slot blot data using Quantity One® analysis software program (Bio-Rad). Analysis of western blots used densitometry normalized to actin. Data are the mean ± standard error of the mean. Statistical significance between groups ($p < 0.05$) was determined using ANOVA with Tukey's post hoc test or Student's t-test as indicated.

3. Results

3.1. Quantitative accumulation of platinum adducts after exposing rat sensory neuronal cultures to cisplatin is concentration dependent

Cisplatin -induced cytotoxicity involves formation of intrastrand and interstrand adducts in DNA [30] and accumulation of these adducts in rat sensory neurons correlates with damage in sensory neurons [14, 15]. To better understand Pt-damage and its repair in sensory neurons, we employed a quantitative assay, where platinum adducts on genomic DNA were detected by a monoclonal antibody that specifically recognizes 1,2-Pt-(GpG) adducts (Oncolyze, Germany). The platinum adduct signals were quantitatively assessed by DNA slot blot using Molecular Imager ChemiDoc XRS system (Bio-Rad). In this assay, DNA bound to blot membrane was also visualized with SYBR-Gold dye which was used as loading control for normalizing the signals. When neuronal cultures were exposed to increasing concentrations of cisplatin for three hours, there was a concentration dependent accumulation of 1,2-Pt-(GpG) adducts in neuronal cells (Figs. 1A and 1B). Although 5 μM cisplatin did not produce detectable adducts, a small but significant accumulation was observed at 10 μM and this increased in an almost linear fashion with higher concentrations of cisplatin. Platinum adduct signals were proportional to the amounts of platinated genomic DNA loaded to each slot blot (Figs. 2A and 2B), indicating that platinum-induced DNA adducts in neuronal cells can be quantitatively measured using DNA slot blot assay. Relative platinum damage was significantly greater when cells were exposed to cisplatin for 3 hours compared to cells exposed for two hours (Fig. 2).

To ascertain the dynamics of the accumulation and removal of 1,2-Pt-(GpG) damage, the major lesion (65%) induced by cisplatin in non-replicating sensory neuronal cultures, cultures were treated with 15 μM cisplatin at 0 hour, then the cells were incubated for various times in the presence of drug before cell harvest and measurement of 1,2-Pt adducts. The relative Pt-damage increased over the first four hours after cisplatin treatment, then the number of adducts declined to barely detectable levels after 24 hours suggesting the repair of adducts is occurring and with a half-life of approximately 12 hours (Fig. 3).

3.2. A targeted reduction in the expression of APE1 in sensory neuronal cultures inhibits the repair of cisplatin damage

We have previously demonstrated that lowering the BER activity through the reduction of APE1 expression exacerbated the neurotoxicity produced by cisplatin treatment, whereas, augmenting the repair activity of APE1 attenuated the neurotoxicity [18, 20, 28]. These data along with the observation that cisplatin produces ROS in sensory neuronal cultures [20, 28] suggest that activating the BER pathway could reverse cisplatin-induced oxidative DNA damage. In addition reducing the expression of APE1 increases the cisplatin-induced phosphorylation of H2AX, a marker for DNA damage [18, 20, 28]. To examine this possibility, we determined whether reducing the expression of APE1 in sensory neuronal cultures could alter the repair of cisplatin damage as measured by platinum adduct removal. When sensory neuronal cultures were exposed to 15 μM cisplatin for three hours, there was a significant increase in 1,2-Pt adducts and the number of adducts was reduced by ~50% eight hours after the cisplatin was removed (Figs. 4A and 4B). Treating the cultures with

SCsiRNA, which did not reduce the expression of APE1 compared to untreated cultures (data not shown) did not alter the amount of adducts produced by cisplatin exposure and also did not alter the repair over eight hours post-dosing (Figs 4A and 4B). In contrast, when neuronal cultures were treated with APE1siRNA which reduced APE1 expression to 21 ± 0.1% of levels after SCsiRNA (Fig. 4C), there was a significant reduction in the ability of the cells to remove the Pt-adducts over time (Figs 4A and 4B). In these cultures the amount of Pt-damage after cisplatin was similar to that observed in control and SCsiRNA treated cells, but 8 hours after cisplatin was removed, the level of damage was similar to that found immediately after a 3 hour exposure to the drug.

3.3. Augmenting APE1 repair, but not its redox activity, restores the removal of Pt-adducts in sensory neuronal cultures with a reduced expression of APE1

The data presented above suggest that reducing the expression of APE1 blunts the ability of the NER pathway to remove Pt-adducts, but does not prevent adduct formation. To ascertain whether the lowered repair activity observed in cultures with reduced APE1 expression was selectively caused by the reduction in APE1, we performed add-back experiments, where wtAPE1 was overexpressed in neuronal cultures using lentivirus in the absence or presence of APE1 knockdown. For these studies, cultures were exposed to SCsiRNA or APE1siRNA on days 3–5 in culture and treated with lentivirus on days 5–7 as routinely performed in our laboratories [20, 27, 28]. Five days after lentiviral infection (Day 12 in culture), sensory neurons were exposed to vehicle or 15 μM cisplatin for 3 hours and platinum adducts measured at 0, 4, 8, and 16 hours after cisplatin treatment. As observed in the previous experiments (Fig. 4), reducing the expression of APE1 by >70% significantly delayed the removal of Pt-adducts in cultures when compared to cultures treated with SCsiRNA (Figs. 5A and 5B). For example at 16 hours, the Pt-damage in cultures treated with SCsiRNA was only ~20% of the level observed immediately after drug treatment, whereas DNA damage in cells treated with APE1siRNA was still 77% of the initial value (Fig. 5B). When neuronal cultures treated with APE1siRNA were infected with a lentiviral vector, the removal of Pt-adducts was compromised in a manner similar to that seen with APE1 knockdown alone (Figs. 5A and 5B). In contrast, when cells were infected with lentivirus containing the construct for wtAPE1, there was a significant increase in removal of Pt-adducts such that at 16 hours the relative Pt-damage was reduced to the same level as cultures treated with SCsiRNA (Figs. 5A and 5B). In duplicate wells of cells from the same harvest used to determine Pt-adducts, exposure to wtAPE1 lentivirus resulted in an expression of APE1 that was similar to that observed in control cultures plus HA tagged APE1, whereas exposing cultures to the viral vector did not increase APE1 expression (Fig. 5C).

The data from Fig. 5 show that augmenting the expression of APE1 in neuronal cultures after reducing expression reverses the effects of loss of the protein on NER-mediated DNA repair. The question remains, however, whether it is the DNA repair or the redox activity of APE1 that affects the NER pathway. The repair and redox activities of APE1 are located in two functionally distinct regions within the protein [24, 31, 32]. Consequently, by mutating specific regions of APE1, and overexpressing mutated APE1 molecules in cells with a reduced expression of endogenous APE1, we can ascertain whether APE1's functions in DNA repair, redox or combined actions are crucial for removal of Pt-adducts in neuronal

cells. The mutation of Cys 65 to Ala (C65 APE1) removes the redox, but not repair function of APE1 [24, 31, 33, 34], whereas a combined mutation of Arg 177 to Ala and Asn 226 to Ala (226+177APE1), drastically reduces the repair function but leaves the redox function intact [27].

As observed in previous experiments, when neuronal cultures treated with APE1siRNA were infected with a lentiviral vector, there is minimal reduction in Pt-damage at four and eight hours after a three hour exposure to 15 μM cisplatin (Figs. 6A and 6B). When cultures with reduced APE1 expression are infected with lentivirus containing the construct for 226+177APE1 which only exhibits redox activity, the removal of Pt-adducts is small and similar to that observed in cells with reduced APE1 expression infected with the viral vector (Figs. 6A and 6B). In contrast expression of the redox-defective, repair competent C65 APE1 reverses the loss in NER activity that occurs in APE1-knockdown cells as indicated by a significant reduction in Pt-damage at four and eight hours after cisplatin exposure (Figs. 6A and 6B). As can be seen in a representative Western blot in Fig. 6C, exposing duplicate wells of cells from the same harvests to lentiviral constructs of C65 APE1 or 226+177APE1 results in an expression that is similar to that observed for wtAPE1 in control cultures (Fig. 4C). Together, these data suggest that APE1's DNA repair function not the redox signaling activity is essential for its role in the removal of Pt-damage in sensory neuronal cultures.

3.4. Reducing APE1 levels in sensory neuronal cultures alters expression of the NER proteins, RPA70 and XPA

In light of our findings of retained Pt adducts following knockdown of APE1 in neuronal cultures, we initiated studies to determine if there is a link between BER and NER. We treated sensory neuronal cultures with cisplatin at 10 or 50 μM for 24 hours following transfection with SCsiRNA or rat APE1-siRNA as before. Using Western blot analysis, protein expression of RPA70 was significantly reduced when APE1 expression was decreased, however RPA70 expression rebounded when cultures were exposed to cisplatin (Figs. 7A and 7B). In contrast, XPA, another NER protein involved in the early recognition step of Pt-DNA adducts, was elevated in both the controls and following 10 μM of cisplatin, but not after 50 μM cisplatin (Figs. 7A and 7C). These data clearly demonstrate that altering APE1 levels in sensory neuronal cultures dramatically affect two early NER recognition proteins, albeit in opposite directions and in a different response pattern than in the untreated controls.

4. Discussion

The work presented here demonstrate for the first time a potential interaction between the two major DNA repair pathways in sensory neurons; BER and the NER and that compromising the BER pathway by reducing APE1 expression interferes with the ability of NER to remove Pt adducts from sensory neuronal cultures. In this study, we employed the DNA slot blot assay that quantitatively measures kinetics of accumulation and removal of cisplatin-DNA lesions in genomic DNA of neuronal cell cultures and demonstrate a correlation between the cisplatin concentration and the accumulation of Pt-damage in our measurement of Pt-adducts in sensory neuronal cultures. These findings confirm and expand on previous work showing that cisplatin binds effectively neuronal DNA and causes high

levels of Pt-DNA adduct formation in DRG neurons [13, 15]. However, the most intriguing aspect of the current study is the observation that reducing the expression of APE1 in neuronal cultures significantly diminished the removal of platinum damage (1,2-Pt-GpG), implicating a central BER enzyme to be involved in NER. When wtAPE1 or DNA repair competent-redox negative APE1 (C65-APE1) was overexpressed in cells that had reduced expression of endogenous APE1, the suppression of platinum removal was reversed, confirming that APE1, and specifically its DNA repair, but not its redox-signaling function is involved in the removal of 1,2-Pt-GpG probably via modulating the NER pathway. This is a novel function of APE1 in Pt-damage repair as a number of transcription factors that are regulated by APE1 redox function are involved in the expression of DNA repair proteins across all pathways [32].

We focused our studies on isolated sensory neurons in culture since these neurons appear to be a primary target of cisplatin which can cause CIPN. Indeed, previous studies have shown that platinum adducts accumulate in the dorsal root ganglia in rodents after systemic administration of cisplatin to a higher degree than other peripheral tissues [13, 14] and cause significant damage to sensory neurons [1, 35, 36]. Cisplatin administration in animals also mimics symptoms of CIPN in humans, including changes in nerve conduction velocity [37, 38] and in sensory perception, especially nociception [35, 39]. One limitation of the current work, however, is that it is possible that our alterations in platinum adduct removal involves other cell types since the cultures of adult sensory neurons contain some non-neuronal cells. As far as we know, APE1 is expressed in all cell types that would be found in our cultures including sensory neurons [24, 27, 40–47]. Because we measure Pt adducts from all the cells in culture, and used a CMV promotor to over express APE1, we do not know whether the effects we observe occur in all the cells in culture. However, this seems unlikely since the kinetics of removal of adducts in mitotic cells appears to be faster than in post-mitotic cells (unpublished observations), which not only supports the previous finding that cell cycle activation and DNA replication is necessary for NER DNA repair [48], but also raises a possibility that a lowered NER activity in post-mitotic cells could be a contributing factor for CIPN. Furthermore, in our previous studies using cisplatin, over expressing APE1 reversed a specific endpoint of sensory neuronal function, the release of the transmitter CGRP which is only occurring in neuronal cells, not support cells [28]. In addition, when we used ionizing radiation to cause DNA damage and to alter sensory neuronal function, we showed that the effects of the treatment were neuronal specific as lentiviral constructs with a neuronal specific promoter driving the transgenes were used [27].

Although the mechanisms for the interaction of APE1 with NER remains to be determined, our data suggest that it is the DNA repair activity of APE1 rather than the redox signaling function that mediates the interaction. When a mutant APE1 that is redox deficient, but repair competent (C65 APE1) is overexpressed it reverses the compromise in platinum adduct removal that occurs after depletion of endogenous APE1, whereas overexpressing a repair deficient, but redox competent mutation of APE1 (266 + 177 APE1) does not reverses the effect of depleting endogenous APE1. These findings relating APE1's DNA repair, but not its redox function as the important component of its action in protecting DRG neuronal cells from platinum DNA induced damage are similar to our studies showing APE1 response to platinum induced ROS and oxidative DNA damage repair [20, 27, 28]. While the precise

mechanism of APE1 and NER interaction requires further study, it is possible that a physical interaction of APE1 with specific NER proteins, such as RPA70 and/or XPA [7, 27, 49, 50] could be the crucial step. Indeed, we demonstrated that altering the expression of APE1 in sensory neurons changes the levels of both RPA70 and XPA although the effects are in opposite directions. Recognition of DNA damage is a critical step in the early stage of NER repair, where RPA and XPA as well as other factors can independently bind to damaged DNA [51, 52]. Both XPA and RPA preferentially bind to cisplatin-damaged DNA [52, 53] and may also play a role in subsequent steps in NER through interaction with other repair proteins [54]. RPA has been found to interact with APE1 [55], although to date this interaction has not been observed in sensory neurons nor in the context of cisplatin repair in neurons. Given our findings demonstrating APE1 knockdown altering Pt adduct removal and alterations in RPA and XPA, it will be interesting to see whether increasing damage recognition ability by overexpressing damage recognition factors such as XPA and RPA can actually enhance repair of platinum damage and how altering APE1 levels influences this DNA repair response. It also is possible that APE1 is interacting with XPC, a NER protein involved in global genomic NER since deficiencies in either XPA or XPC result in enhanced Pt- adducts in sensory neurons [17].

Based on our studies and previous work, it seems likely that DNA damage contributes to the malfunction of sensory neurons. This damage could occur secondary to the formation of platinum adducts, such as increased ROS production and subsequent oxidative DNA damage [20, 27, 28]. While there is a correlation between the amounts of adducts produced and the degree of apoptosis [13, 15], there is also a correlation of increased platinum adduct formation and reduced activity of sensory neurons as measured by alterations in conduction velocities [17]. Evidence from our previous work, however, demonstrates a direct relationship between compromise of the BER pathway and cisplatin-induced neurotoxicity [20, 28]. When APE1 expression is reduced in sensory neuronal cultures, there is an increase in cisplatin-induced cell death and in the ability of the drug to reduce release of the neurotransmitter CGRP [28]. In contrast overexpression of wtAPE1 or the redox deficient mutant C65 APE1 reverses the cisplatin-induced neurotoxicity, decreased DNA damage and ROS production [28]. In general, the BER pathway is involved in oxidative or alkylating DNA damage [20, 23, 49] so altering its activity was not expected to affect cisplatin-induced neurotoxicity. However, as previously demonstrated [20, 27, 28], APE1 does protect neuronal cultures from cisplatin and oxaliplatin ROS induced oxidative DNA damage [20, 27, 28]. Cisplatin exposure resulted in a concentration-dependent decrease in cell viability that was significantly greater with reduced expression of APE1 [20, 28]. A significant loss of viability was observed with cisplatin concentration used in this study [28]. Based on these data and our current work, we demonstrate that cisplatin produces both platinum adducts and oxidative DNA damage and the repair of these insults to DNA are mitigated by APE1 in the BER pathway and interactions, as yet unidentified, with the NER pathway.

The implications of the results presented points to a more complicated DNA repair response to cisplatin induced DNA damage in post-mitotic cells than the activation of a single pathway and that understanding the interaction of various repair pathways has the potential to provide novel targets for treating CIPN. This is imperative in cisplatin therapy since 30–40 % of patients receiving the drug develop symptoms of neuropathy [8, 56, 57] and in a

significant portion of these the neuropathy persists and can even be worse after stopping therapy [10, 58]. These clinical observations imply there is a semi-permanent to permanent alteration in the function of sensory neurons which could be a direct downstream result of DNA damage in sensory neurons. Indeed, in post-mitotic cells, the DNA damage could manifest as changes in expression of proteins or in mutated proteins that alter the function of the neurons that lead to CIPN. Thus, it appears DNA damage and the appropriate DNA repair pathway or multiple pathway response seems critical for maintaining the genome to express normal proteins at levels necessary for homeostatic function.

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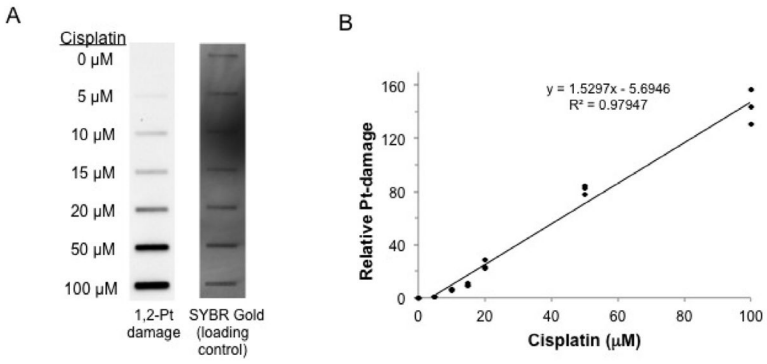

Figure 1. Exposing sensory neuronal cultures to increasing concentrations of cisplatin augments the formation of platinum adducts

Neuronal cultures were treated with various concentrations of cisplatin for 3 hours, then the cells were harvested for genomic DNA followed by slot blot measurement of cisplatin adducts. **A:** A representative slot blot measuring the immunoreactivity of 1,2-Pt-(GpG) DNA adducts and a SYBR gold loading control after exposure to various concentrations of cisplatin for 3 hours as indicated. **B:** Each point represents the mean ± the SD for 3 experiments of the Pt-damage relative to untreated cultures as measured by Molecular Imager ChemiDoc XRS using Quantity One® analysis software program (BioRad).

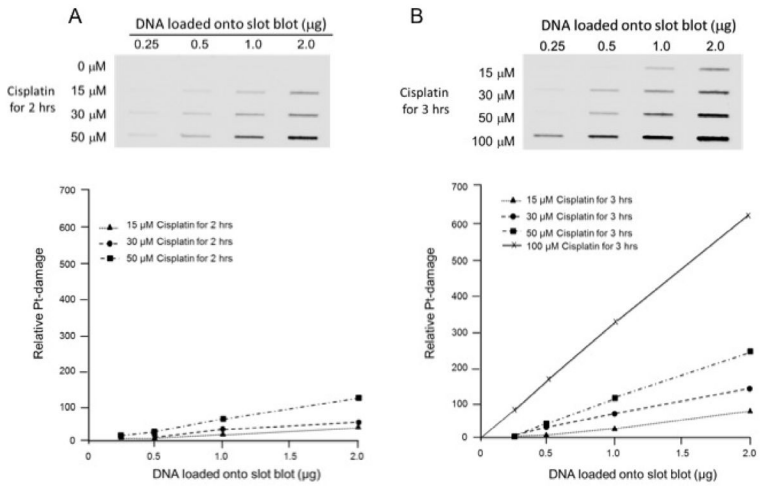

Figure 2. Levels of platinum adducts detected by slot blot analysis are proportional to the amount of genomic DNA loaded

Following treatment of neuronal cells with indicated amounts of cisplatin for 2 (panel A) or 3 hours (panel B), cells were harvested for genomic DNA preparation. The top panels show slot blots after loading different amounts of DNA from cells treated with various concentrations of cisplatin as indicated. The bottom panels show the plots of the relative Pt-damage versus the amount of DNA loaded for the various concentrations of cisplatin after a 2 hour (panel A) or a 3 hour (panel B) exposure from the data obtained in the slot blots.

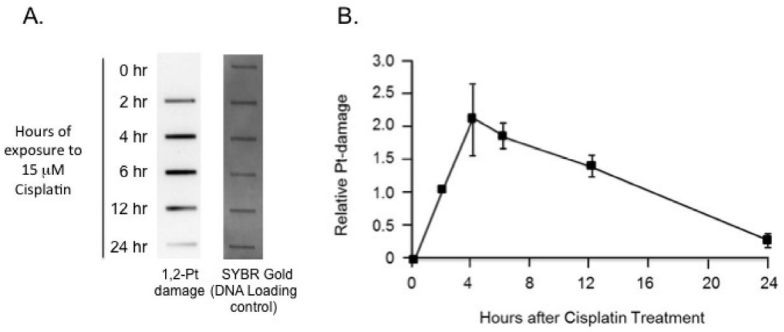

Figure 3. Time course for the accumulation and removal of platinum adducts in sensory neuronal cultures exposed to cisplatin

Untreated sensory neuronal cultures (time 0) or cultures treated with 15 μM of cisplatin for 2, 4, 6, 12, or 24 hours were harvested and genomic DNA isolated. Two μg of genomic DNA were loaded onto each slot blot for quantitative measurement of cisplatin adducts. **A:** A representative slot blot measuring the immunoreactivity of 1,2-Pt-(GpG) DNA adducts and a SYBR gold loading control after exposure to 15 μM cisplatin for various times as indicated. **B:** Each point represents the mean ± the SD for 3 experiments of Pt-damage relative to untreated cultures (0 hr) as measured by Molecular Imager ChemiDoc XRS using Quantity One® analysis software program (BioRad).

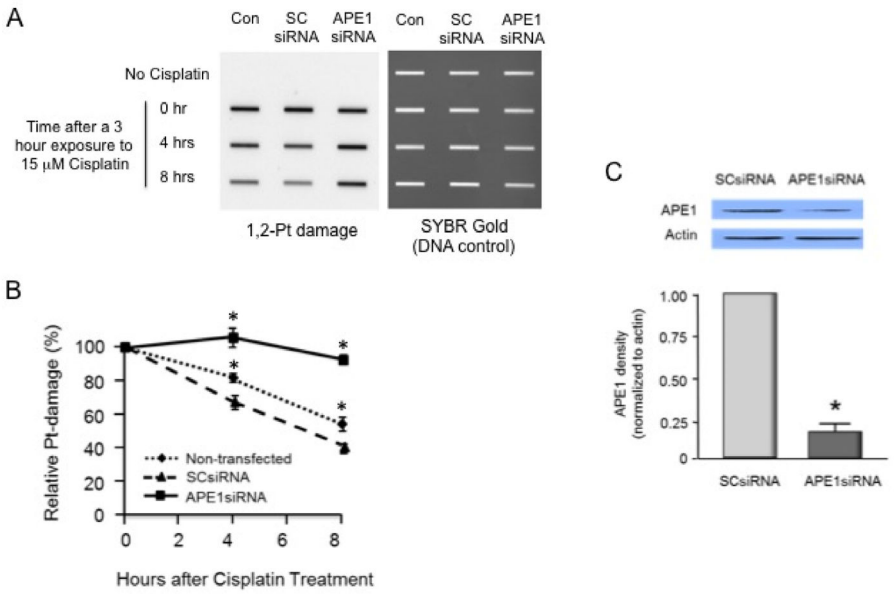

Figure 4. A targeted reduction of APE1 expression in sensory neuronal cultures significantly reduces removal of Pt-damage

Neuronal cultures were treated with 100 nM scramble siRNA (SCsiRNA) or 100 nM APE1siRNA on days 3–5 in culture or were not treated with siRNAs (Con) and on day 12 exposed to 15 μM cisplatin for 3 hours, then genomic DNA isolated at 0, 4, or 8 hours after cisplatin treatment. Two μg of genomic DNA were loaded onto each blot for quantitative measurement of cisplatin adducts. **A:** A representative slot blot measuring the immunoreactivity of 1,2-Pt-(GpG) DNA adducts and a SYBR gold loading control at various times after exposure to 15 μM cisplatin for 3 hours in the absence or presence of siRNA treatment as indicated. No cisplatin represents cultures in the absence or presence of siRNAs, but not treated with the anticancer drug. **B:** Each point represents the mean ± the SD for 3 experiments of the percent of Pt-damage normalized to untreated wells of cells after a 3 hour exposure to cisplatin as measured by Molecular Imager ChemiDoc XRS using Quantity One® analysis software program (BioRad). An asterisk indicates a statistically significant difference compared to cultures treated with SCsiRNA using ANOVA with Tukey's post hoc test. **C:** The top panel shows a representative Western blot of APE1 and actin (as a loading control) from neuronal cells treated with either 100 nM SCsiRNA or 100 nM APE1-siRNA. In the lower portion each column represents the mean ± SEM of the density of the APE1 bands normalized to actin in cultures exposed to scramble or APE1 siRNAs. An asterisk indicated a significant reduction in APE1 expression compared to cells treated with SCsiRNA using ANOVA with Tukey's post hoc test.

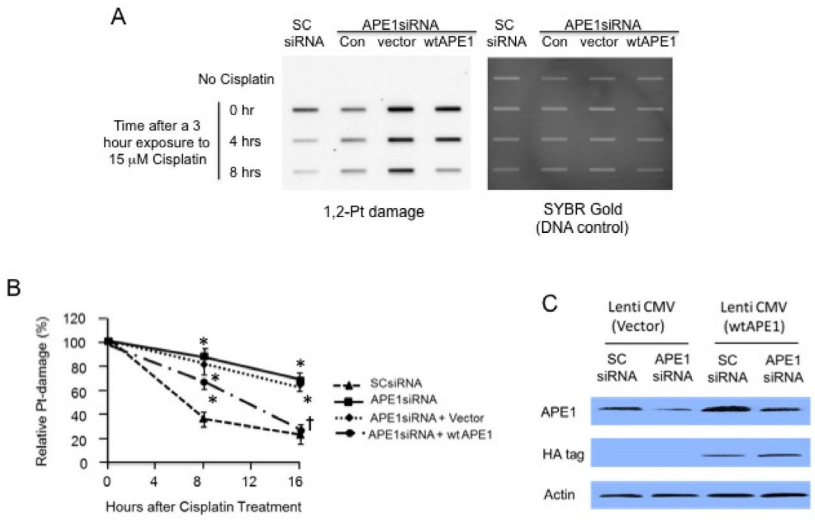

Figure 5. Overexpression of wtAPE1 restores removal of 1,2-Pt-GpG adducts in sensory neuronal cultures treated with APE1-siRNA

Cultures were treated with 100 nM scramble siRNA (SCsiRNA) or 100 nM APE1siRNA on days 3–5 in culture and lentiviral constructs containing the CMV promoter and EGFP (vector control) or CMV-wtAPE1-IRES-EGFP on days 5–7 in culture. On day 12 in culture cells were exposed to 15 μM cisplatin for 3 hours, then genomic DNA isolated at 0, 8, or 16 hours after cisplatin treatment. Two μg of genomic DNA were loaded onto each blot for quantitative measurement of cisplatin adducts. **A:** A representative slot blot measuring the immunoreactivity of 1,2-Pt-(GpG) DNA adducts and a SYBR gold loading control at various times after exposure to 15 μM cisplatin for 3 hours in cultures treated with SCsiRNA or APE1siRNA and either no virus (Con), viral vector, or virus containing the human wtAPE1 construct as indicated. No cisplatin represents cultures in the absence or presence of siRNAs exposed to vehicle and not the platinum drug. **B:** Each point represents the mean ± the SD for 3 experiments of the percent of Pt-damage normalized to cells treated with SCsiRNA but not virus after a 3 hour exposure to cisplatin as measured by Molecular Imager ChemiDoc XRS using Quantity One® analysis software program (BioRad). An asterisk indicates a statistically significant difference compared to cultures treated with SCsiRNA, whereas a cross indicated a significant difference in cells treated with the lentiviral vector versus those treated with lentivirus for wtAPE1 using ANOVA with Tukey's post hoc test. **C:** A representative Western blot APE1, HA, and actin (as a loading control) in sensory neuronal cultures infected with lentiviral vector or wtAPE1 after treatment with SCsiRNA or APE1siRNA as indicated.

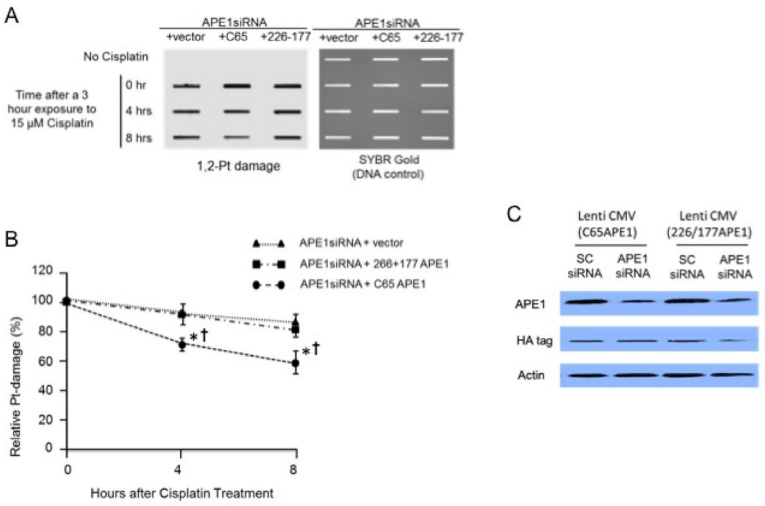

Figure 6. Overexpression of the redox-defective mutant APE1, but not the repair- defective mutant in the presence of APE1 knockdown enhances removal of 1,2-Pt-GpG adducts in sensory neuronal cultures treated with APE1-siRNA

Cultures were treated with 100 nM APE1siRNA on days 3–5 in culture and lentiviral constructs containing the CMV promoter and EGFP (vector control) or CMV-C65APE1-IRES-EGFP or CMV-226–177APE1-IRES-EGFP on days 5–7 in culture. On day 12 in culture cells were exposed to 15 μM cisplatin for 3 hours, then genomic DNA isolated at 0, 4, or 8 hours after cisplatin treatment. Two μg of genomic DNA were loaded onto each blot for quantitative measurement of cisplatin adducts. **A:** A representative slot blot measuring the immunoreactivity of 1,2-Pt-(GpG) DNA adducts and a SYBR gold loading control at various times after exposure to 15 μM cisplatin for 3 hours in cultures treated with APE1siRNA and viral vector, virus containing the C65APE1 construct or the 226–177APE1 construct as indicated. No cisplatin represents cultures in the absence or presence of siRNAs exposed to vehicle and not the anticancer drug. **B:** Each point represents the mean ± the SD for 3 experiments of the percent of Pt-damage normalized to cells treated with APE1siRNA and viral vector after a 3 hour exposure to cisplatin as measured by Molecular Imager ChemiDoc XRS using Quantity One® analysis software program (BioRad). An asterisk indicates a statistically significant difference between cultures treated with the lentiviral vector versus those treated with with lentivirus for C65APE1, whereas a cross indicates a statistically significant difference between cultures treated with the lentivirus containing 226+177APE1 versus those treated with lentivirus for C65APE1 using ANOVA with Tukey's post hoc test. **C:** A representative Western blot APE1, HA, and actin (as a loading control) in sensory neuronal cultures infected with lentiviral vector viral vector, virus containing the C65APE1 construct or the 226–177APE1 construct after treatment with SCsiRNA or APE1siRNA as indicated.

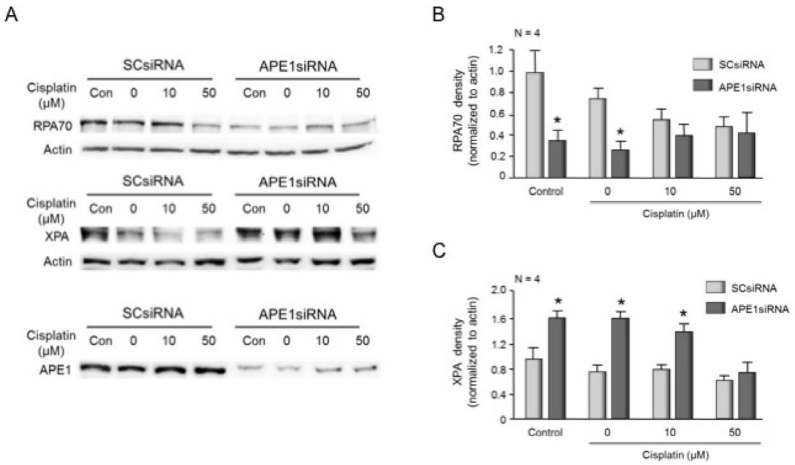

Figure 7. A targeted reduction of APE1 expression in sensory neuronal cultures significantly alters expression of RPA70 and XPA

A: Representative Western blot for RPA70 (top panel), XPA (middle panel), and APE1 (bottom panel) in control sensory neuronal cultures or those treated with 10 μM or 50 μM cisplatin for 24 hours following transfection with 100 nM SCsiRNA or APE1-siRNA as indicated. The Western blots were probed for RPA70, actin and APE1 then reprobed for XPA. **B and C.** Columns represent the mean ± SEM from cultures transfected with SCsiRNA (control; light columns) or APE1 siRNA (dark columns) then not treated or treated with cisplatin (10 μM or 50 μM for 24 hours in 4 independent harvests. An asterisk indicates a statistically significant ($p < 0.05$) comparing SCsiRNA and APE1siRNA treated cultures using Student's *t*-test.