Aminosalicylic acid reduces ER stress and Schwann cell death induced by MPZ mutations

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Abstract. Mutations in myelin protein zero (MPZ) cause inherited peripheral neuropathies, including Charcot-Marie-Tooth disease (CMT) and Dejerine-Sottas neuropathy. Mutant MPZ proteins have previously been reported to cause CMT via enhanced endoplasmic reticulum (ER) stress and Schwann cell (SC) death, although the pathological mechanisms have not yet been elucidated. In this study, we generated an *in vitro* model of rat SCs expressing mutant MPZ (MPZ V169fs or R98C) proteins and validated the increase in cell death and ER stress induced by the overexpression of the MPZ mutants. Using this model, we examined the efficacy of 3 different aminosalicylic acids (ASAs; 4-ASA, sodium 4-ASA and 5-ASA) in alleviating pathological phenotypes. FACS analysis indicated that the number of apoptotic rat SCs, RT4 cells, induced by mutant MPZ overexpression was significantly reduced following treatment with each ASA. In particular, treatment with 4-ASA reduced the levels of ER stress markers in RT4 cells induced by V169fs MPZ mutant overexpression and relieved the retention of V169fs mutant proteins in the ER. Additionally, the level of an apoptotic signal mediator (p-JNK) was only decreased in the RT4 cells expressing R98C MPZ mutant protein following treatment with 4-ASA. Although 4-ASA is known as a free radical scavenger, treatment with 4-ASA in the *in vitro* model did not moderate the level of reactive oxygen species, which was elevated by the expression of mutant MPZ proteins. On the whole, the findings of this study indicate that treatment with 4-ASA reduced the ER stress and SC death caused by 2 different MPZ mutants and suggest that ASA may be a potential therapeutic agent for CMT.

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Introduction

Myelin protein zero (MPZ) is one of the components of myelin in Schwann cells. It comprises 21% of the protein in the sheath of peripheral nerves, and is responsible for ensheathment and axonal protection by linking adjacent lamellae to stabilize the myelin assembly (1-3). There are >200 known MPZ mutations that cause dominantly inherited peripheral neuropathies, known as Charcot-Marie-Tooth disease type 1B (CMT1B), congenital hypomyelinating neuropathy 2 (CHN2), or Dejerine-Sottas neuropathy (DSN; V169fs) (4,5). CMT1B is a demyelinating neuropathy resulting in distal muscle atrophy and DSN is a more severe form of CMT with a childhood-onset. The symptoms of CHN2 include respiratory difficulty, muscle weakness and incoordination, areflexia and ataxia (6). These mutants also cause heterogeneous neuropathies through diverse pathological mechanisms due to their different gain-of-function traits, which are not yet fully understood (7).

According to previous studies on the pathological mechanisms of MPZ mutations, mutant MPZ proteins commonly cause CMT1B by inducing endoplasmic reticulum (ER) stress, which is mediated by the unfolded protein response (UPR) (8-12). ER stress is initiated when unfolded proteins accumulate and the production of reactive oxygen species (ROS) is induced through oxidative protein folding. Once the concentration of unfolded proteins exceeds the capacity of a chaperone protein, binding immunoglobulin protein (BiP), these surplus proteins activate ER membrane-bound proteins, such as the N-terminal ends of pancreatic ER kinase RNA-like ER kinase (PERK), inositol-requiring enzyme 1α (IRE1α) and activating transcription factor 6 (ATF6) (13). Activated PERK, IRE1α and ATF6 initiate the apoptotic cascade, which is

initially mediated by pro-apoptotic proteins, including C/EBP homologous protein (CHOP), c-Jun N-terminal kinase (JNK) or microRNAs (miRNAs or miRs) (14,15).

Amino derivatives of salicylic acids [aminosalicylic acids (ASAs)] are safe drugs that are commonly used in clinical practice. In particular, 4-ASA, one of the most effective antibiotics, has been used for the treatment of tuberculosis since 1944 (16). In addition, 4- and 5-ASA can be used for the treatment of inflammatory bowel disease owing to their anti-inflammatory properties (17). Further mechanistic studies have indicated that 4- and 5-ASA act to scavenge free radicals that produce inflammation (18,19). A previous study also demonstrated that 3-, 4-, and 5-ASA reduce the ROS concentration, thereby relieving manganese neurotoxicity in dopaminergic human neuroblastoma cells (20).

In this study, we generated *in vitro* models expressing mutant MPZ protein that caused ER stress and Schwann cell death and investigated whether the 3 ASAs can alleviate these pathological effects.

Materials and methods

Cell culture and transfection. Rat Schwann cells, RT4 cells (RT4-D6P2T, CRL-2768, ATCC), were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Biowest) containing 10% fetal bovine serum and 1% penicillin-streptomycin (Biowest) at 37˚C in a 5% CO_2 atmosphere. The MPZ gene was amplified from the pCMV6-entry-MPZ vector (Origene). The amplified PCR product was cloned into the pCMV-Myc or p-EGFP(C1) vector (Clontech). Mutant genes (V169fs, L184fs, R185fs, S226fs and R98C) were generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene). To express wild-type MPZ and mutant MPZ genes, the RT4 cells ($2x10^5$) seeded on 6-well culture plates were transfected with MPZ-containing vectors [pCMV-Myc-MPZ WT/V169fs/R98C and pEGFP(C1)-MPZ WT/V169fs], as well as their control vectors [pCMV-Myc and pEGFP(C1)] using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Based on western blot analysis and immunocytochemistry, the transcription efficiency was >90%. The transfected cells were incubated at 37˚C for 48 h. The RT4 cells ($2x10^5$) were transfected with the MPZ expression vectors and treated with the drugs (1-100 μM) [para(4)-aminosalicylic acid (4-ASA), sodium 4-aminosalicylic acid (s4-ASA) and 5-aminosalicylic acid (5-ASA)] (Sigma-Aldrich), as well as their solvent (PBS) solution as a negative control for 24 h. The direct counting of cell numbers was performed after the collected cells were stained with trypan blue (T8154, Sigma-Aldrich). Ten microliters of cells were mixed with an equal volume of Trypan blue then cells were immediately counted with a hemacytometer (Sigma-Aldrich, Z359626) under a microscopy

Fluorescence-activated cell sorting (FACS) for the measurement of cell death. In order to measure cell death, the RT4 cells were seeded in 6-well culture plates at a density of $2x10^5$ cells per well. The RT4 cells were transiently transfected with wild-type MPZ or mutant MPZ for 24 h, then treated with the ASA compounds (1-100 μM) for 24 h and harvested. Annexin V fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining (BD Biosciences Pharmingen) was performed by incubating the cells in the dark for 15 min at room temperature in binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, at pH 7.4) saturated with Annexin V FITC and PI. Following incubation, the cells were washed with cold PBS, pelleted and analyzed by a FACS VERSE analyzer (BD Biosciences). We determine the number of live, apoptotic and necrotic cells by counting the numbers of Annexin V^-/PI^- cells, Annexin V^+/PI^- cells, and Annexin V^+/PI^+ cells, respectively.

DCFDA assay. The control experiment involved the treatment of the RT4 cells with Brefeldin A (Sigma-Aldrich) (5 μM) for 48 h. For the experimental groups, the RT4 cells were transfected with MPZ V169fs or R98C mutant vectors for 48 h with the ASA compounds (1-100 μM) or ascorbic acid (A92902, Sigma-Aldrich). To measure ROS levels in the control and experimental groups, the H_2DCFDA assay was performed as per the manufacturer's recommendations (Invitrogen). Initially, the RT4 cells were harvested and incubated with 10 μM H_2DCFDA at 37˚C for 40 min. After being chilled on ice, the cells were pelleted and analyzed with a FACS VERSE analyzer (BD Biosciences). The fluorescence intensity of H_2DCFDA formed by the reaction between H_2DCFDA and intracellular ROS in >10,000 viable cells per sample was analyzed at an excitation wavelength of 488 nm and an emission wavelength of 525 nm using a FACS VERSE analyzer (BD Biosciences). The experiments were repeated at least 3 times, and the most representative histogram data are presented.

Western blot analysis. The expression of MPZ proteins and ER stress markers was confirmed using a standard western blot analysis. Total cells were harvested and lysed with RIPA lysis buffer (Thermo Fisher Scientific). The cell lysates were centrifuged at 13,000 x g for 15 min at 4˚C, and the supernatants were used for quantification using bicinchoninic acid (BCA) method. A total of 20 μg of protein was separated and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were then stained with 0.1% Ponceau S solution (Sigma-Aldrich) to ensure equal loading of the samples, and non-specific binding was blocked with a blocking buffer (Casein blocking buffer 10X; Sigma-Aldrich) for 1 h at room temperature. The membranes were incubated overnight at 4˚C with the following primary antibodies: Anti-Myc-tag (1:2,000; ab9106, Abcam), anti-binding immunoglobulin protein (BiP, #3177), anti-C/EBP-homologous protein (CHOP, #5554), anti-cleaved caspase-3 (Asp175, #9661), anti-phospho-AKT (ser473, #9271), anti-phospho-SAPK/JNK (Thr183/Tyr185, #9251) (all 1:1,000) (all from Cell Signaling Technology) and anti-β-actin (A2228, Sigma-Aldrich). Subsequently, bound antibodies were visualized with anti-mouse (#7076) or anti-rabbit IgG HRP-linked secondary antibodies (#7074) (all 1:2,000) (all from Cell Signaling Technology) by incubating 1 h at room temperature and Western blotting luminol reagent (Santa Cruz Biotechnology) in order to detect proteins. The integrated optical densities of the immunoreactive protein bands were measured using Image J software (https://imagej.nih.gov).

Immunocytochemistry and confocal imaging. For immunofluorescence staining, the RT4 cells ($2x10^4$) were seeded on

coverslips in 24-well culture plates. After being transfected with wild-type MPZ or mutant MPZ (pCMV-Myc-MPZ WT, V169fs, R98C and pEGFP-MPZ WT, V169fs) for 24 h, the cells were treated with the drugs (100 μM of 4-, s4-, or 5-ASA) for 24 h. Subsequently, they were fixed in 4% paraformaldehyde for 20 min, washed with PBS for 5 min 3 times, and blocked with 0.1% Triton X-100 in 5% normal goat serum for 1 h at room temperature. Fixed cells were incubated with the primary antibodies, anti-Myc-tag (1:500; ab9106, Abcam) and anti-protein disulfide isomerase (PDI; 1:100; #2446, Cell Signaling Technology), overnight at 4˚C. After being washed 3 times with PBS for 5 min each time, the cells were incubated with the appropriate secondary antibodies, including Alexa Fluor 488 goat anti-rabbit IgG (1:1,000, A11008) and Alexa Fluor 568 goat anti-mouse IgG (1:1,000, A11001) (Molecular Probes) for 1 h at room temperature. The cells were finally washed and mounted in Vectashield hardset antifade mounting medium (Vector Laboratories) with 4',6-diamidino-2-phenylindole (DAPI) to allow for the visualization of the nuclei. The stained sections were visualized under a laser scanning confocal microscope (CLSM700; Carl Zeiss).

Statistical analysis. All experiments (western blot analysis, FACS, immunocytochemistry and cell quantification) were performed at least 3 independent times. We tested for and found normal distributions and equal variances in our sample distributions. To determine the statistical significance within the multiple groups in western blot analysis and cell quantification, we used one-way ANOVA with a post hoc Tukey's multiple comparison test to confirm whether the F value was greater than F-critical value. For comparisons between the groups (control vs 4-ASA treated cells after transfection with either MPZ-V169fs or R98C vectors), Student's t-tests were used to determine the effects of 4-ASA in FACS analysis of Annexin V^-/PI^- cells, Annexin V^+/PI^- cells, and Annexin V^+/PI^+ cells. $P<0.05$ was considered statistically significant.

Results

MPZ mutations cause Schwann cell death. Previous studies on 2 MPZ mutants (V169fs and R98C) indicated that these mutants induce Schwann cell death and the elevation of ER stress *in vitro* and *in vivo* (10,21). In particular, the R98C mutant has been reported to activate the IRE1 pathway, leading to apoptosis *in vivo* (21). The V169fs mutant has also been shown to induce ER stress and cell death by being retained in the ER compartments of non-Schwann cells *in vitro* (HeLa or 293 cell lines) (10).

To validate the induction of Schwann cell death or ER stress by MPZ mutant proteins, we generated wild-type (WT) and 5 mutant MPZ (V169fs, L184fs, R185fs, S226fs or R98C) expression vectors by site-directed mutagenesis. From western blot analysis and immunocytochemistry, we confirmed the effective expression of MPZ proteins by the transient transfection of WT and mutant MPZ vectors into the RT4 cells with a >90% transfection efficiency (Figs. 1, 2 and S1). In addition, we observed that the levels of ER stress markers, such as BiP and CHOP were altered by either MPZ-V169fs or MPZ-R98C overexpression. The CHOP expression levels were elevated by the overexpression of MPZ-V169fs and MPZ-R98C mutants, while the BiP level was elevated only by the overexpression of MPZ-V169fs mutant (Fig. S1). Thus we proceeded with further experiments using only the MPZ-V169fs and MPZ-R98C mutant.

To determine whether the overexpression of two MPZ mutants (V169fs and R98C) affects Schwann cell viability, we transfected 2 MPZ mutant-overexpressing vectors into the RT4 cells. Within 48 h, the detachment of transfected RT4 cells from the bottom of the dish was observed. Manual cell counting following Trypan blue staining indicated that the overexpression of the V169fs or R98C mutant in the RT4 cells caused 65 or 32% significant cell death compared to the expression of wild-type protein, respectively (Fig. 1A). To validate this result, the number of Annexin V^- and PI-positive RT4 cells following transfection with the MPZ mutants were measured by FACS analysis. Expectedly, the percentage of live RT4 cells indicated a 38% significant decrease following the expression of the V169fs mutant compared to wild-type MPZ (Fig. 1B). This cell death was driven by the significant induction of apoptosis. On the other hand, the expression of the R98C mutant resulted in a 46% significant reduction in the late apoptotic/dead cell population; however, the number of live cells was not significantly altered. This result was further confirmed by western blot analysis. The expression of the V169fs mutant led to a 2-fold ($P<0.05$) increase in cleaved caspase-3 levels 48 h following transfection compared to the wild-type control (Fig. 1C).

ER stress and ROS levels are elevated in Schwann cells by the expression of MPZ mutants. As previously reported, selected MPZ mutant proteins (S22_W28 deletion, V169fs, 550del3insG, S63del and R98C) induce ER stress *in vitro* or *in vivo* (7,10,21-23). In this study, to determine whether Schwann cell death is associated with ER stress in the mutant MPZ protein (V169fs or R98C)-expressing cells, the levels of BiP and CHOP were measured in cell lysates of transfected RT4 cells (Fig. 2A and Fig. S1). The BiP level exhibited a 1.3-fold significant increase in the MPZ V169fs-expressing RT4 cells, whereas the overexpression of the R98C mutant did not markedly alter the BiP levels. The level of CHOP was significantly increased in the cells overexpressing either MPZ mutant (V169fs or R98C) compared to the cells overexpressing the wild-type control.

To link the elevation of ER markers to the accumulation of mutant protein in the ER, we assessed the localization of MPZ (wild-type/mutant) proteins in RT4 cells. We detected MPZ with an anti-Myc antibody and marked the ER with anti-PDI in transfected RT4 cells (Fig. 2B). Wild-type and R98C mutant proteins were found throughout the cytosol, whereas V169fs mutant protein co-localized with PDI.

As the retention of unfolded proteins in the ER possibly causes an elevation of oxidative stress levels in the ER (24,25), we also determined the extent of induction of ROS levels using DCFDA, which detects intracellular ROS levels through the fluorometric measurement of DCF oxidation. Using FACS analysis, the ROS levels underwent a statistically significant increase in the cells expressing the V169fs and R98C mutants, respectively (Fig. 2C). On the

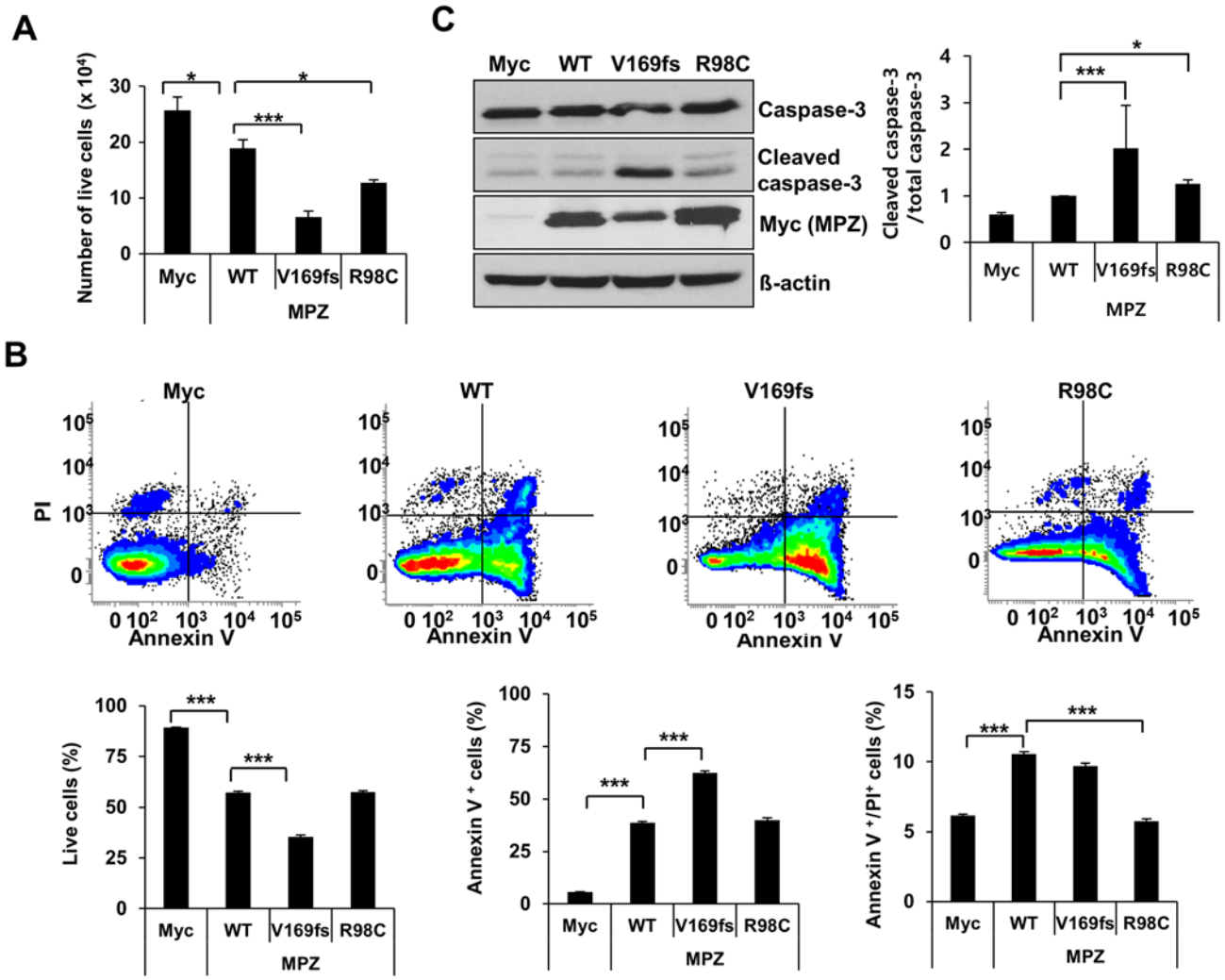

Figure 1. Schwann cell death is induced by MPZ mutant overexpression. (A) Following the overexpression of mutant MPZ proteins (V169fs and R98C) in RT4 cells for 48 h, the number of live rat Schwann cells was reduced (n=3; *P<0.05; ***P<0.001). (B) FACS analysis showing the quantities of live cells, apoptotic cells (Annexin V^{+} cells), and late-stage apoptotic/necrotic cells (Annexin V^{+} and PI^{+} cells) by Annexin V and PI staining (n=3; ***P<0.001). (C) Western blot analysis indicating the levels of cleaved and total caspase-3 following the overexpression of MPZ mutant. Quantification of the western blot analysis data indicating an increase in the level of cleaved caspase-3 following the overexpression of MPZ mutant (n=3; *P<0.05; ***P<0.001). MPZ, myelin protein zero.

whole, the protein localization and ER stress levels within the Schwann cells varied depending on the particular MPZ mutation, yet both the V169fs and R98C mutations resulted in Schwann cell death.

ASAs reduce the cell death induced by MPZ mutants. To examine whether ASAs reduce Schwann cell death, we treated the MPZ-transfected RT4 cells with 3 different concentrations (1, 10, or 100 μM) of 4-, s4-, or 5-ASA for 24 h. This treatment resulted in the distinctively less detachment of transfected RT4 cells. Quantitative analysis of the live cells indicated that the number of live cells was significantly increased following treatment with 100 μM of ASAs, except for the case of 5-ASA treatment in the R98C MPZ mutant proteins (Fig. 3A). To verify whether the enhancement of cell viability was dependent on the dose of ASAs, we used one-way ANOVA with a post hoc Tukey's multiple comparison test to compare the numbers of live cells following treatment with various concentrations of each ASA (Fig. 3A). Although the ASA-treated group exhibited a significant enhancement of cell viability compared to an experimental control group, this increment was not dose-dependent. FACS analysis of the Annexin V and PI indicated that 4-ASA treatment significantly enhanced the number of live RT4 cells overexpressing either the V169fs (1.24-fold increase from control group) (Fig. 3B) or R98C mutant (1.28-fold increase from control group) (Fig. 3C). The increase in cell survival occurred by virtue of a significant reduction in both apoptosis and necrosis (V169fs: 10.8% reduction of apoptosis and 51.5% reduction of necrosis; R98C: 32.4% reduction of apoptosis and 13.4% reduction of necrosis). Treatment with s4- and 5-ASA also increased the number of live cells via a reduction in both apoptosis and necrosis (data not shown). The reduction of the apoptosis of the mutant-overexpressing RT4 cells following 4-ASA treatment (100 μM) was also determined by using cleaved caspase-3 staining (Fig. 3D). Taken together, the results indicated that 4-, s4-, and 5-ASA

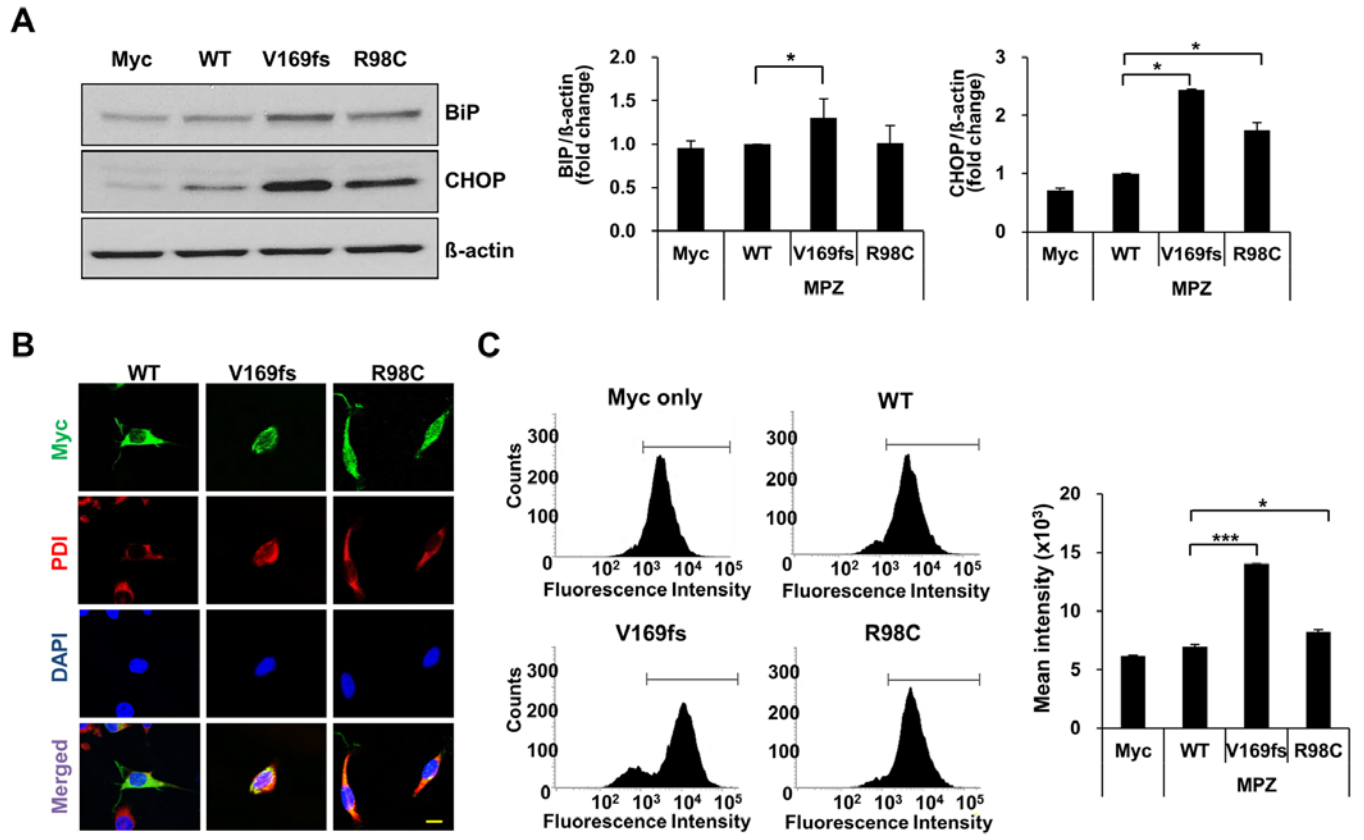

Figure 2. ER stress in Schwann cells is induced by MPZ mutant overexpression. (A) Western blot analysis demonstrating changes in the levels of ER stress markers (BiP and CHOP) following transfection with wild-type, V169fs and R98C mutants of MPZ in RT4 cells. Quantitative analyses of the western blot analysis data were performed for BiP and CHOP normalized to ß-actin (n=3; *P<0.05). (B) Immunocytochemistry for overexpressed wild-type MPZ and the mutants (Myc), and an ER marker (PDI) indicating the localization of MPZ proteins in relation to the ER in RT4 cells (n=3; scale bar, 10 μm). (C) DCFDA assay showing that expression of both mutants resulted in an augmentation of ROS levels in RT4 cells. Expression of the MPZ V169fs and R98C mutants resulted in statistically significant 2- and 1.2-fold increases in ROS levels, respectively (n=3; *P<0.05; ***P<0.001). MPZ, myelin protein zero.

significantly increased Schwann cell viability through a significant reduction in both apoptosis and necrosis.

4-ASA reduces ER stress induced by MPZ mutant proteins. 4- and 5-ASA are used clinically as anti-inflammatory drugs, particularly for patients with inflammatory bowel disease (26). 4-ASA is recognized as an antibiotic for the treatment of tuberculosis (27). Apart from these known effects of the ASAs, a previous study demonstrated the drug repositioning of ASA in reducing manganese-induced ROS generation and cell death in human neuroblastoma cells (20). In this study, we examined whether 4-, s4-, or 5-ASA can alleviate ER stress in Schwann cells induced by the presence of mutant MPZ proteins. We measured the level of ER stress by detecting BiP and CHOP expression at 24 h following treatment with the drugs. The BiP levels in the V169fs-transfected RT4 cells were significantly decreased in response to treatment with 100 μM 4-ASA (23% decrease from control) (Fig. 4A). However, the s4- and 5-ASA-treated cells did not exhibit a significant decrease in BiP levels. Similarly, the CHOP level was significantly decreased only in the 100 μM 4-ASA-treated V169fs mutant-expressing RT4 cells (37% decrease from control). On the other hand, the level of neither ER stress marker was significantly decreased in the R98C-transfected RT4 cells following treatment with 4-ASA in comparison with the control, apart from the BiP level in the 5-ASA (10 μM)-treated group (Fig. 4B). As ER stress in V169fs mutant-transfected RT4 cells occurred due to the retention of mutant MPZ proteins within the ER compartment, we observed the localization of MPZ V169fs protein, which is tagged with EGFP. GFP fluorescence was observed in the cytosol of RT4 cells following treatment with 100 μM of ASAs (Fig. 4C). Treatment of the RT4 cells expressing V169fs mutant protein with 4-ASA resulted in a cellular phenotype similar to that of wild-type protein-expressing RT4 cells, in which MPZ protein was distributed throughout Schwann cell processes (yellow arrowheads) rather being restricted to the soma or ER compartment (PDI-positive). Treatment with s4-ASA and 5-ASA also resulted in GFP expression in Schwann cell processes, which were shorter than those of the 4-ASA-treated RT4 cells. Taken together, these results indicated that treatment with 100 μM of 4-ASA ameliorated the cellular phenotypes and the ER stress caused by MPZ V169fs mutant protein.

In R98C mutant-expressing cells, no significant changes were observed in the BiP or CHOP levels following treatment with any of the 3 ASAs, apart from the decrease observed in BiP expression with 10 μM 5-ASA (Fig. 4B). This result was similar to that of a previous study, which indicated that the ablation of CHOP did not rescue the phenotype of R98C transgenic mice and that the R98C mutant activates the IRE1 pathway of the UPR (21). Subsequently, in order to eludicate the mechanism of apoptosis that results from the overexpression

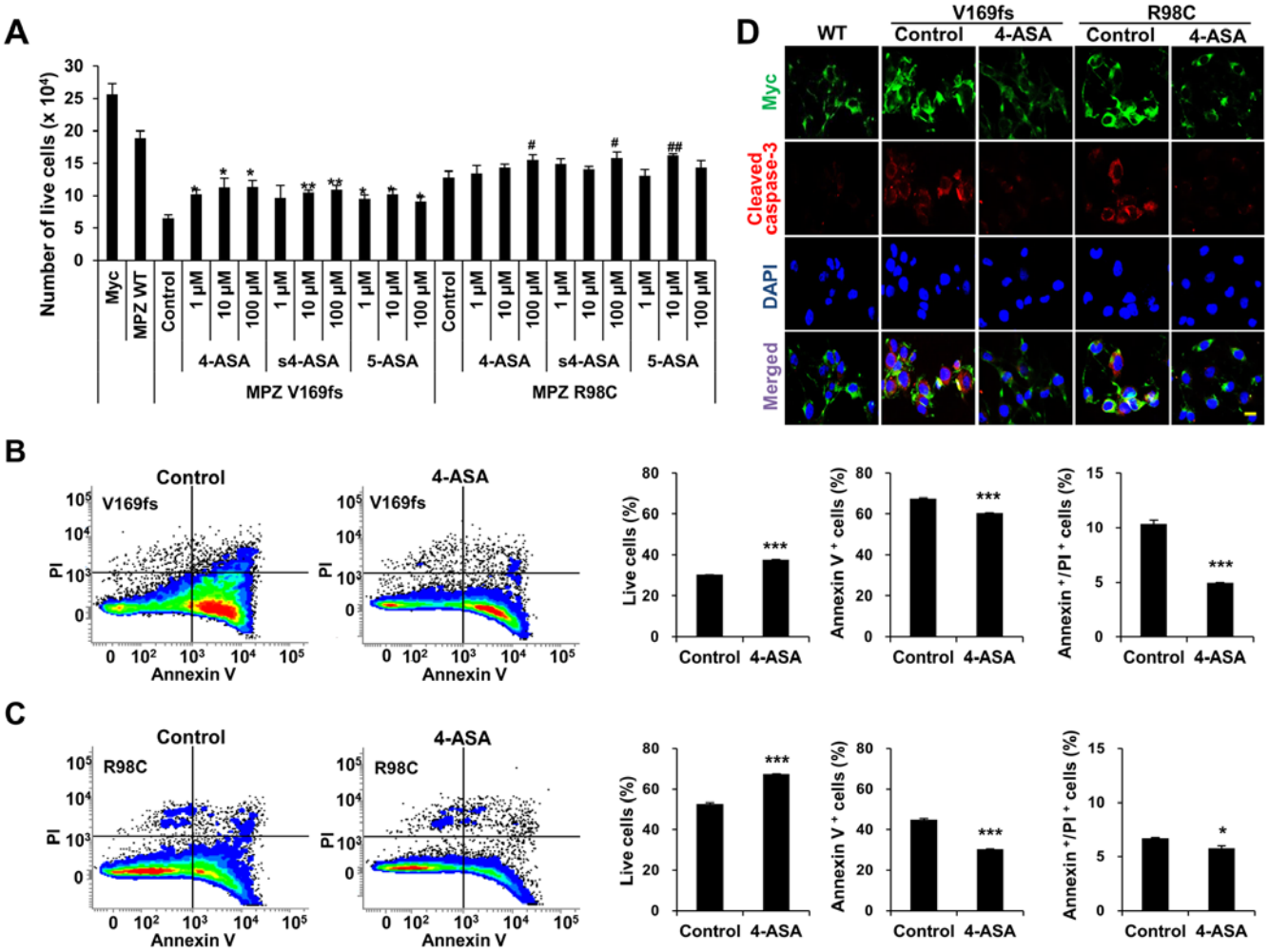

Figure 3. Effects of ASAs on cell death resulting from MPZ mutant expression. (A) Single cell counts indicating that treatment with 4-, s4- or 5-ASAs increased the number of live RT4 cells expressing the V169fs or R98C mutants. An asterisk (*) indicates statistical significance vs. MPZ V169fs control. A pound sign (#) indicates statistical significance vs. MPZ R98C control (n=3; $^{*}P<0.05$; $^{**}P<0.01$; $^{\#}P<0.05$; $^{\#\#}P<0.01$). (B and C) FACS analysis also showing the quantities of live cells, apoptotic cells (Annexin V^{+} cells), and late-stage apoptotic/necrotic cells (Annexin V^{+} and PI^{+} cells) by Annexin V and PI staining of MPZ V169fs (B) or R98C (C) overexpressing RT4 cells (n=3; $^{*}P<0.01$; $^{***}P<0.001$). (D) RT4 cells expressing the wildtype or mutants (V169fs or R98C) were stained with cleaved caspase-3 for detecting the apoptosis of the RT4 cells (n=5; scale bar, 20 μm). MPZ, myelin protein zero; ASA, aminosalicylic acid.

of MPZ R98C protein in Schwann cells, we detected the levels of phosphorylated JNK (p-JNK) as a marker of ER stress-induced apoptosis (28). As expected, the RT4 cells treated with 100 μM 4-ASA and expressing the R98C mutant exhibited a 64% significant reduction in the p-JNK levels (Fig. 4E), while no significant changes were observed in the cells expressing the V169fs mutant (Fig. 4D). Therefore, 4ASA treatment reduced the level of UPR-mediated pro-apoptotic markers in RT4 cells expressing the R98C MPZ mutant, while ER stress was not affected.

4-ASA does not affect ROS levels induced by MPZ mutant proteins. Increased ER stress may also results in ROS accumulation (29), which is caused by the overexpression of mutant MPZ proteins in RT4 cells. Thus, in this study, we examined whether 4-ASA treatment can alleviate ROS levels. RT4 cells expressing each MPZ mutant were treated with 3 different concentrations of 4-ASA, and the ROS level was measured by DCFDA assay. Notably, none of the 4-ASA-treated groups exhibited any marked changes in ROS levels (Fig. 5A and B). We also used ascorbic acid (600 μM) to confirm the results of the DCFDA assay with the MPZ V169fs mutant, as ascorbic acid potently inhibits ROS production (30,31). Treatment with ascorbic acid resulted in a 26.5% significant reduction in ROS levels (Fig. 5C). These data suggest that 4-ASA does not affect the ROS levels, which are elevated by ER stress caused by the expression of mutant MPZ proteins.

Discussion

Currently, there is no treatment for CMT that affects the course of the disease. In this study, we investigated whether amino derivatives of salicylic acids (4-, s4-, or 5-ASA) may be effective treatments in rat Schwann cells overexpressing 2 MPZ mutations, V169fs and R98C, which cause DSN and CMT1B, respectively (4,32). We initially generated *in vitro* models overexpressing the mutant proteins in rat Schwann cells, and found that only treatment with 100 μM 4-ASA exhibited significant drug efficacy for those 2 MPZ mutations: i) 4-ASA treatment increased the viability of Schwann cells which overexpressed MPZ V169fs or MPZ R8C mutant proteins; ii) 4-ASA reduced the level of ER stress markers (BiP and CHOP) in MPZ

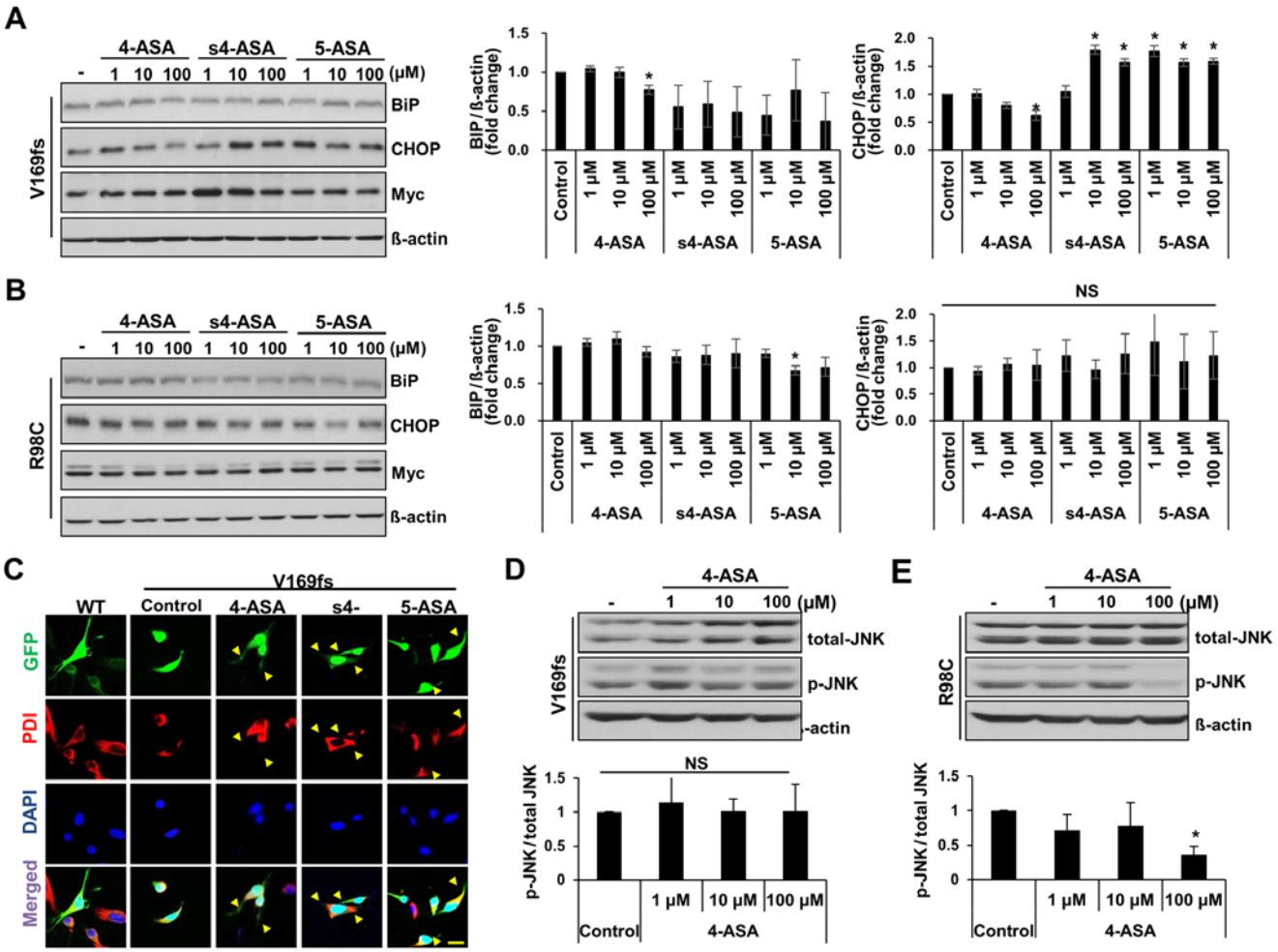

Figure 4. Effects of ASAs on the reduction of ER stress induced by MPZ mutant expression. (A and B) Western blot analysis indicating changes in ER stress markers (BiP and CHOP) following treatment with 3 different concentrations of each ASA in RT4 cells expressing MPZ (A) V169fs and (B) MPZ R98C. Quantitative analyses of western blot analysis data were performed for BiP and CHOP (n=3; *P<0.05; NS, not significant vs. control). (C) Immunocytochemistry to detect an ER marker (PDI) in cells overexpressing GFP-tagged wild-type MPZ and the V169fs mutant, which indicates that ASA treatment relieves the retention of MPZ V169fs in the ER compartment in RT4 cells (n=3; scale bar, 20 μm). (D and E) Western blot analysis showing changes in phosphorylated JNK and total JNK levels following treatment with 3 different concentrations of 4-ASA in RT4 cells expressing either (D) V169fs or (E) R98C mutant MPZ. Quantitative analyses of western blot analysis data were performed for the ratio of phosphorylated JNK to total JNK in MPZ V169fs or R98C (n=3; *P<0.05; NS, not significant vs. control). MPZ, myelin protein zero; ASA, aminosalicylic acid.

V169fs-expressing RT4 cells, but not in MPZ R98C-expressing cells; iii) 4-ASA released V169fs protein retained in the ER; and iv) 4-ASA decreased the level of p-JNK, which is involved in apoptosis, in R98C protein-expressing RT4 cells, but not in MPZ V169fs-expressing cells.

The structure of MPZ proteins is favorable to be retained in the ER, thus triggering the unfolded protein response (UPR), which can be assessed by UPR markers (BiP and CHOP) (7). Our data support previous evidence that both the V169fs and R98C mutants similarly induced the UPR and ER stress *in vitro* and *in vivo*, respectively (10,21). Furthermore, the expression of the V169fs mutant significantly elevated the levels of both ER stress markers compared to expression of wild-type protein, whereas the expression of the R98C mutant caused a significant increase only in the level of CHOP. We hypothesized that the pathological mechanism of V169fs mutant protein involves the retention of mutant proteins in the ER causing an elevation of BiP, which consequently activates downstream effectors (CHOP) and eventually leads to Schwann cell apoptosis. On the other hand, the R98C mutant did not result in this particular cellular phenotype. Based on the evidence, the 2 mutants led to protein structural changes during post-translational modification and caused ER stress in a different manner.

Both MPZ mutants do not seem to share the apoptotic mechanism. According to previous studies, the expression of R98C and S63C mutant proteins did not cause the recovery of Schwann cell apoptosis in the absence of CHOP expression *in vivo*, although CHOP is important for the demyelination of MPZ mutant nerve cells (21,23). It was also previously demonstrated that R98C mutant protein induced Schwann cell death and UPR activation in the ER via activation of the IRE1α/JNK pathway instead of the PERK/CHOP pathway (21). On the other hand, there is no evidence regarding the pathological mechanisms of action of MPZ V169fs protein in Schwann cells, except that patients carrying this frameshift mutation develop more severe neuropathy phenotypes than CMT (10,33). Based on the data of this study, the two different mutations cause different patterns of ER stress and different mechanisms for apoptosis. Although 4-ASA treatment (100 μM) resulted in

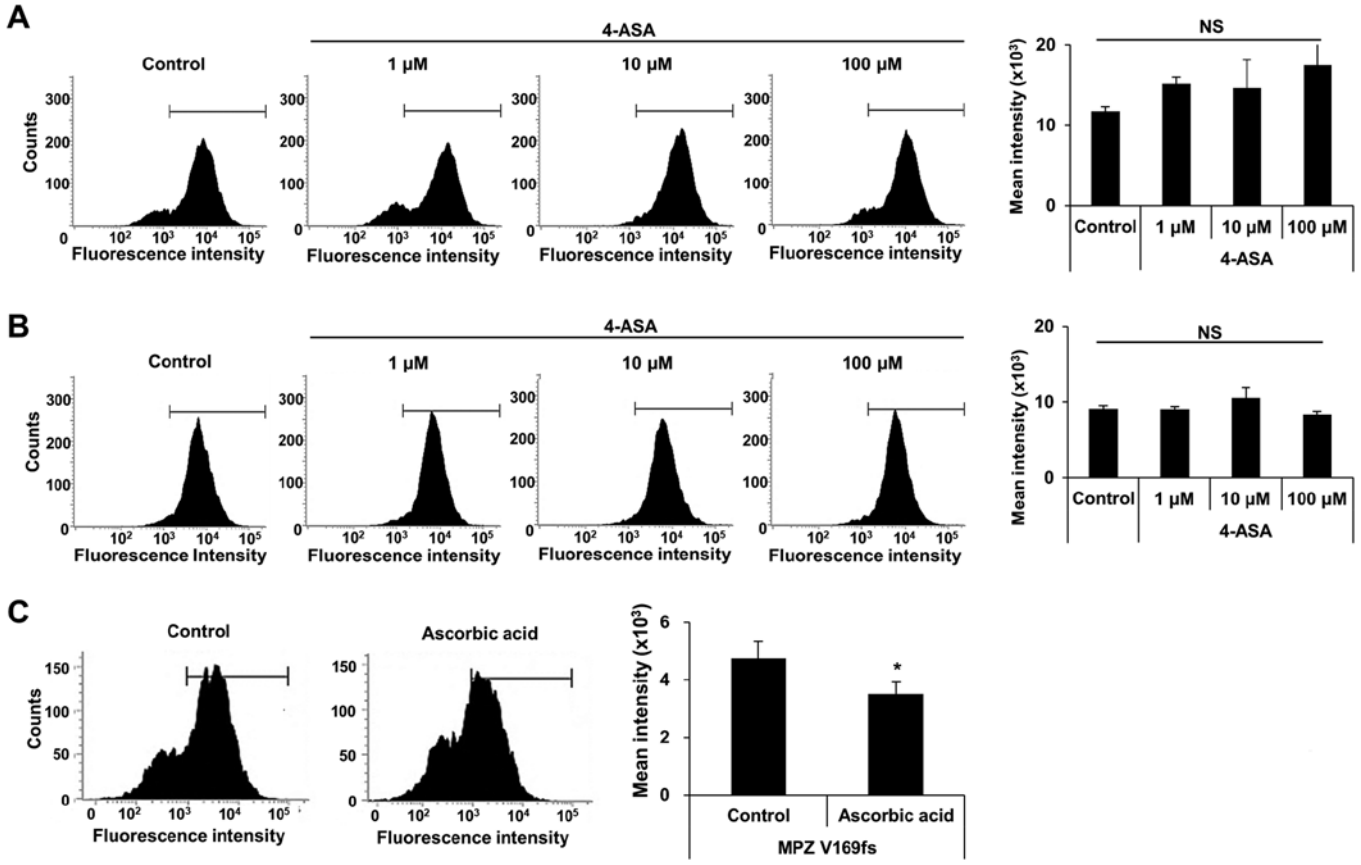

Figure 5. Effects of ASAs and ascorbic acid on the increased ROS levels induced by expression of mutant MPZ proteins. (A) Treatment with 3 different concentrations of 4-ASA in MPZ V169fs-overexpressing RT4 cells did not alter the intracellular ROS levels (n=3; NS, not significant). (B) DCFDA assay indicating that treatment with 3 different concentrations of 4-ASA in RT4 cells overexpressing MPZ R98C did not alter intracellular ROS levels (n=3; NS, not significant). (C) Treatment of MPZ V169fs-overexpressing RT4 cells with ascorbic acid resulted in a significant reduction of intracellular ROS levels (n=3; $^{*}P<0.05$). MPZ, myelin protein zero; ASA, aminosalicylic acid; ROS, reactive oxygen species.

significant reduction of apoptosis determined by using cleaved caspase-3 staining, both the BiP and CHOP levels were only significantly reduced in V169fs mutant-expressing cells and p-JNK levels were only reduced in the R98C mutant-expressing cells. Owing to the diverse function of each MPZ mutant protein in Schwann cells, it is necessary to elucidate the pathological mechanisms of individual mutations in order to devise treatment strategies for patients with each pathology as unique gain-of-function mechanisms are involved in CMT and DSN.

Recently, curcumin has been suggested as a promising therapeutic candidate for the treatment of CMT (34), as it allows misfolded proteins to traverse within the ER to the plasma membrane, thereby reducing cytotoxicity (35-37). However, it must be modified for clinical use due to its instability, low efficacy and insolubility (33). From the view of the drug development process, the repositioning of an established drug is a strong alternative option. ASAs are used clinically as anti-inflammatories and antibiotics. In this study, we evaluated the efficacy of ASAs and found that only 4-ASA treatment was effective in alleviating ER stress and reducing apoptosis. Although treatment with the other ASAs (s4-ASA and 5-ASA) resulted in similar outcomes in terms of increasing Schwann cell viability, they did not bring about a reduction in the CHOP level, which was elevated by expression of mutant MPZ proteins. As MPZ mutants elevated ER stress by increasing ROS levels, we measured tje intracellular ROS levels following treatment with 4-ASA; 4-ASA is known to reduce the concentration of free radicals in a manner mediated by nuclear factor-κB inhibition (18,19). Although 4-ASA reduced the intracellular ROS levels induced by manganese neurotoxicity (20), it seems that 4-ASA could not suppress the MPZ mutant-driven ROS level. These data suggest that 4-ASA may be involved in other pathways of the apoptotic cell death that was caused by the UPR-mediated ER stress in Schwann cells.

In conclusion, in this study, we demonstrated that treatment with 4-ASA reduced the ER stress and SC death caused by 2 different MPZ mutants. However, the treatment efficacy was limited and the mode of action was not clearly revealed. Thus, ASAs need to be further developed to enhance the therapeutic efficacy for the treatment of CMT or DSN. In addition, enhancing the *in vitro* and *in vivo* model by the generation of point mutations at the endogenous MPZ using CRISPR/Cas9 technique may be helpful for the better understanding of the MPZ mutations-mediated pathogenesis and the mode-of-action of ASAs. Thus far, there is no treatment available that affects the course of progression of CMT. With an eye towards identifying small molecules or drugs for CMT treatment, 4-ASA warrants further investigationr as a treatment option for CMT or DSN.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

EHC and WMM designed and performed the experiments, interpreted the data, and wrote the manuscript. HMD and JSL collected and analyzed data. HTP analyzed and interpreted data. BOC and YBH generated the study concept and design, drafted the manuscript, and provided study supervision. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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