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Protective effect of 4,4'-diaminodiphenylsulphone against oxidative stress but not to apoptotic stress in human diploid fibroblasts

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Abstract

The antibiotic drug 4,4′-diaminodiphenylsulphone (DDS) is used to treat several dermatologic diseases, including Hansen's disease. This study confirmed the antioxidant nature of DDS in hydrogen peroxide (H_2O_2)-induced oxidative stress and assessed its role in other apoptotic stresses in human diploid fibroblasts (HDFs). Oxidative stress was effectively reduced by DDS in a dose-dependent manner. Moreover, the oxidative stress-induced increases in the levels of the p53 and p21 proteins were inhibited by pre-treatment with DDS. In addition, H_2O_2 and DDS increased the level of cytochrome P450 (CYP450) IIE1 in HDFs, implicating a role for DDS in H_2O_2 scavenging via the activation of CYP450. DDS treatment increased the activity of catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), as well as the GSH/GSSG ratio, indicating activation of the glutathione system against oxidative stress. However, DDS showed no protective effects on HDFs against other apoptotic stimuli, such as thapsigargin and staurosporine, suggesting that DDS would act only against oxidative stress. Therefore, in addition to its antibiotic function, DDS is a potent antioxidant against H_2O_2-induced oxidative stress in HDFs.

Keywords: *4,4'-Diaminodiphenylsulphone, hydrogen peroxide, human diploid fibroblasts, cytochrome P450 IIE1, glutathione, apoptosis*

Abbreviations: *CAT, catalase; CM-H2DCF-DA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; CCK-8, Cell Counting Kit; CYP450, cytochrome P450; DDS, 4,4'-diaminodiphenylsulphone; H_2O_2, hydrogen peroxide; HBSS, Hank's balanced salt solution; HDFs, human diploid fibroblasts; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; NAC, N-acetyl-L-cysteine; PARP, poly (ADP ribose) polymerase; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substances.*

Introduction

4,4'-Diaminodiphenylsulphone (DDS, dapsone), which was first synthesized a century ago, continues to be used in the therapy of many skin diseases [1] and is a mainstay drug for the treatment of Hansen's disease. However, whether DDS acts as a pro-oxidant or an antioxidant remains a subject of debate [2–5].

Hydrogen peroxide (H_2O_2), which is a major reactive oxygen species (ROS), causes lipid peroxidation and DNA damage in various cell types. Oxidative stress has been implicated in the pathogenesis of many conditions [6,7]. Oxidative stress is the consequence of an imbalance between the production of pro-oxidants and the capacity of the antioxidant defenses. Since antioxidants can prevent ROS generation, scavenge ROS and repair ROS-induced damage, the availability of safe and effective antioxidants is important for the prevention of a spectrum of oxidative stress-related pathologies [8,9]. In addition, oxidative stress induces apoptosis, which can be blocked by antioxidants [10,11].

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Of the endogenous antioxidant molecules, reduced glutathione (GSH) has direct ROS-scavenging properties, whereby it is converted into oxidized glutathione (GSSG), which is readily reduced by glutathione reductase (GR). Therefore, the glutathione redox ratio (GSH/GSSG; reduced/oxidized glutathione) is used as an indicator of oxidative stress [12–14]. Glutathione status is stringently controlled through the equilibrium between GSH synthesis, its recycling from GSSG by GR and its consumption by glutathione peroxidase (GPx) or glutathione transferases (GST) [15,16]. N-Acetyl-L-cysteine (NAC) is not only a powerful antioxidant that reacts specifically with several ROS, but it also serves as a precursor for intracellular glutathione synthesis [17].

In the present study, the protective role of DDS against H_2O_2-induced oxidative stress was reassessed. Furthermore, the protective roles of DDS against other apoptotic stresses were evaluated.

Materials and methods

Materials

4,4'-Diaminodiphenylsulphone (DDS, dapsone) was obtained from Taekeuk Pharmaceuticals (Seoul, Korea). Hydrogen peroxide (H_2O_2), N-acetyl-L-cysteine (NAC) and thapsigargin were purchased from Sigma Chemical Co. (St. Louis, MO) and staurosporine from Calbiochem (La Jolla, CA). The Cell Counting Kit (CCK-8) was obtained from Dojindo Laboratory (Japan), Trizol reagent from MRC (Cincinnati, OH) and CM-H2DCF-DA from Molecular Probes (Eugene, OR). The monoclonal antibody against β-actin was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), polyclonal antibodies against CAT, GPx and GR antibodies from AbFrontier (Seoul, Korea) and p-53, p-21, Bcl-2, Bax and PARP from Cell Signaling Technology (Danvers, MA). Other biochemical reagents were supplied by Sigma Chemical Co.

Cell viability measurements and calculation of IC_{50} values

Primary HDFs were isolated from the foreskins of newborns, as described previously [18]. HDFs were maintained in DMEM that contained 10% foetal bovine serum and antibiotics. Cell viability was assessed using a Cell Counting Kit (CCK-8), as described in the Dojindo Laboratory technical manual. HDFs were cultured for 24 h in 96-well plates before DDS treatment. HDFs were maintained in various concentrations of DDS (100–800 μM; dissolved in DMSO) for 48 h; 10 μl of CCK-8 were directly added to each well and the plates were incubated for 3 h at 37°C in 5% CO_2. Control HDFs were incubated in culture medium alone. The wells that contained only culture medium were used as blanks. To calculate the IC_{50} values (concentrations that produced a 50% inhibitory effect on cell proliferation), the results were transformed to percentages of the control values. The percentage inhibition of a given extract against all the cell lines was calculated using the following formula:

$$\% \text{ Cell survival} = \frac{(At - Ab)}{(Ac - Ab)} \times 100$$

where *At* is the absorbance of the test well, *Ab* is the absorbance of the blank (medium) and *Ac* is the absorbance of the control (control).

$$\% \text{ Cell inhibition} = 100 - \% \text{ Cell survival}$$

The effects of the extracts are expressed as IC_{50} values calculated from the dose–response curves.

Cell culture and measurement of intracellular ROS

Intracellular ROS levels were measured by DCF-DA assay method, in which the fluorescent probe, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCF-DA, Molecular Probes Inc., Eugene, OR) is converted by intracellular esterases to 2′,7′dichlorodihydrofluorescin. This is then oxidized by intracellular ROS to the highly fluorescent 2′,7′dichlorofluorescein (DCF), which becomes highly fluorescent on oxidation by intracellular H_2O_2. After H_2O_2 and DDS treatment, cells were washed with Hank's balanced salt solution (HBSS) buffer and then incubated in the dark for 30 min in HBSS buffer containing 2 μM CM-H2DCF-DA. DCF fluorescence was measured using a Varian Cary Eclipse fluorescence spectrophotometer (excitation 480 nm, emission 520 nm).

Lipid peroxidation measurement

Level of cellular lipid peroxidation products was monitored by thiobarbituric acid-reactive substances (TBARS) method by Morliere et al. [19]. TBARS concentration was measured using a Bio-Rad microplate spectrophotometer at 532 nm. Malondialdehyde (MDA) was used as the standard. TBARS values were normalized by HDF protein content. All data are expressed as the means ± SD of triplicate measurements.

Western blot analysis

Lysates of DDS and 500 μM H_2O_2-treated HDFs were boiled in sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 0.14 M 2-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue) and separated by electrophoresis. After transfer to nitrocellulose membranes, western blotting was carried out with antibodies to p53, p21 (for H_2O_2-induced stress

response molecules), CAT, GR, GPx (for scavenger enzymes), Bcl-2, Bax, PARP (for apoptosis inducers) and β-actin, respectively, using horseradish peroxidase-conjugated secondary antibodies (Zymed) and ECL (Pierce), followed by exposing blots to autoradiographic film (Image Reader, LAS-3000, Fujifilm, Japan).

RT-PCR and real-time PCR detection of CYP450 IIE1

Total RNA samples from HDFs were prepared using Trizol reagent (MRC, Cincinnati, OH) and RTPCR for CYP450 IIE1 and GAPDH was performed with Biometra T Gradient PCR (Biometra, Göttingen, Germany) using the following specific primer pairs: for CYP450 IIE1, 5′-ACCTGCCCCATGAAGCAACC-3′ (forward) and 5′GAAACAACTCATGCGAGCC3' (reverse); and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′ACCACAGTCCATGCCATCAC3' (forward) and 5′TCCACCACCCTGTTGCTGTA3' (reverse). PCR-amplified DNA was separated by 1.2% agarose gel electrophoresis. Quantitative PCR (qPCR) analysis of CYP450 IIE1 expression was performed using a real-time PCR System (Bio-Rad) with the forward primer 5′-CGCAAGCATTTTGACTAC3' and reverse primer 5′-TTCCTTCTCCATTTCCA3′, which were designed based on the published human CYP450 IIE1 sequence [20]. Each reaction mix contained 10 µl SYBR-Green Master Mix (Applied Biosystems), 0.5 µl of oligonucleotides (10 pmol each of the forward and reverse primers) and 2 µl cDNA. qPCR analysis of GAPDH expression was performed as an endogenous control with the forward primer 5′-CCGTCTAGAAAAACCTGCC-3' and reverse 5′-GCCAAATTCGTTGTCATACC-3' human GAPDH sequence. The PCR conditions were 95°C for 10 min, followed by 50 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min. The reactions were set up in 96-well plates with iCycler iQ™ optical caps (Bio-Rad).

Activity determination of catalase, glutathione reductase and glutathione peroxidase

Catalase (CAT) activity was assayed by monitoring the degradation of H_2O_2 by absorbance reduction at 240 nm for 1 min with a UV spectrophotometer [21]. The assay contained 50 mM potassium phosphate buffer (pH 7.0), 88 mM H_2O_2 and ~ 50 µg protein. The reaction was initiated by addition of H_2O_2. CAT activity was calculated using the extinction coefficient of H_2O_2 at 240 nm.

Glutathione reductase (GR) activity was assayed by determining NADPH oxidation at 340 nm using a UV spectrophotometer for 1 min. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA, 0.15 mM NADPH, 0.5 mM GSSG and enzyme extract. The reaction was initiated by addition of NADPH [22]. GR activity was calculated using the extinction coefficient of NADPH at 340 nm. Glutathione peroxidase (GPx) activity was determined using 15 mM cumene hydroperoxide as a substrate. Absorbance was read at 340 nm for 1 min. Activity is expressed as nmole/min/mg protein.

GSH/GSSG ratio assay

The ratio of reduced (GSH) to oxidized glutathione (GSSG) was measured utilizing the Bioxytech GSH/GSSG-412 kit (Oxis Research, Portland, OR), adapted for HDFs. Briefly, H_2O_2- and DDStreated HDFs were washed with PBS and harvested into a GSH assay buffer. After homogenization, they were centrifuged (10 000 *g*, 20 min). Then, 5% metaphosphoric acid was added to the supernatant. After centrifugation (1000 *g*, 10 min), the supernatant was divided into two samples, one for GSH and the other for GSSG measurements. The GSH/GSSG ratio was determined in the presence of DTNB and NADPH; the change in absorbance at 412 nm over 3 min was measured using a spectrophotometer for both samples and standards (0–1.5 µM GSSG). Using a standard curve and the sample reaction rates, the GSH/GSSG ratio was calculated. The GSH/GSSG ratio was normalized to total protein content.

Assessment of cell viability and apoptosis

Apoptosis responses of HDFs toward thapsigargin, staurosporine and H_2O_2 were determined using cell viability assessments with the Cell Counting Kit (CCK-8). HDFs were seeded and grown for 24 h in 96-well plates prior to any treatment. HDFs were pretreated with DDS for 3 h and then treated with H_2O_2 (1 mM) for 24 h. Then, 10 µL CCK-8 was added to each well and incubated for 3 h at 37°C under 5% CO_2. Absorbance was determined at 450 nm. The results were expressed relative to the control values specified in each experiment and were subjected to statistical analysis. We monitored Bcl2 and Bax protein level changes as well as poly (ADPribose) polymerase (PARP) cleavage to assess apoptosis response. HDFs were treated with thapsigargin and staurosporine for the indicated time and HDFs were also treated with or without DDS. PARP cleavage was analysed by Western blotting using an anti-PARP antibody.

Statistical analysis

Statistical comparisons for continuous variables were performed using Student's *t*-test or analysis of variance (ANOVA), followed by the Tukey test. The SPSS

software version 15.0 (SPSS Inc., Chicago, IL) was used for all statistical analyses.

Results

Protective effect of DDS against hydrogen-peroxide-induced oxidative stress

The IC_{50} value of DDS was examined in HDFs. For an incubation period of 48 h, the IC_{50} of DDS was determined as 711 μM for HDFs. Since plasma concentrations of DDS after administration to human patients are within the range of 1–20 μM, all the following experiments were carried out using DDS concentrations within this range.

To evaluate the H_2O_2-scavenging capacity of DDS, CM-H2DCF-DA (DCF) fluorescent dye was used. To observe short-term (30 min) effects of scavenging, H_2O_2 was used at 500 μM. In all the groups, cell viability was unchanged (data not shown). As shown in Figure 1B, the DCF fluorescence intensity significantly increased when HDFs were treated with 500 μM H_2O_2 ($p < 0.01$). However, when HDFs were pre-treated with different concentrations of DDS (0.1, 1, 5, 20 and 50 μM), the intensity of DCF fluorescence was significantly decreased in a dose-dependent manner, as compared with HDFs that were treated with H_2O_2 alone (Figure 1B). This H_2O_2-scavenging effect is similar to that seen with the antioxidant NAC (2 mM). Furthermore, H_2O_2-induced lipid peroxidation (thiobarbituric acid-reactive substances formation, TBARS) was also effectively reduced by DDS (Figure 1C).

Effects of DDS on oxidative stress-induced increases in the levels of p53 and p21 proteins

DDS treatment inhibited H_2O_2-induced expression of p53 and p21 in HDFs. When HDFs were treated with 500 μM H_2O_2 for 48 h, the p53 and p21 protein levels increased. Statistically significant reductions in the levels of p53 and p21 were observed when HDFs were pre-treated with DDS prior to H_2O_2 treatment (Figure 2). As a positive control, NAC (2 mM) effectively regulated the H_2O_2 responses.

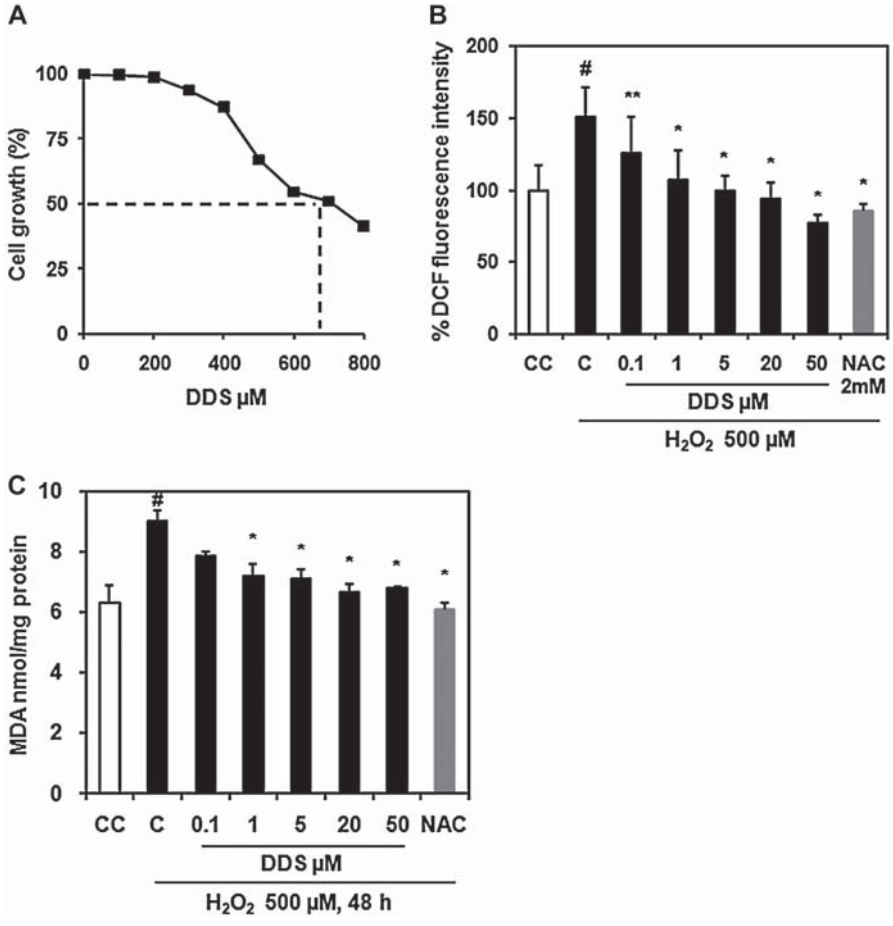

Figure 1. Protective effects of DDS against hydrogen peroxide-induced oxidative stress. (A) The IC50 values were determined for HDFs treated with 100-800 μM DDS. (B) Intracellular ROS was measured using a CM-H2DCFH-DA probe, as described in Materials and methods. The data shown are the mean fluorescence intensities (% of control rate.) ± SD, calculated from six independent experiments ($^{\#}p < 0.01$ vs. H2O2-untreated control cells (CC); $^{*}p < 0.01$, vs. H2O2-alone-treated cells (C); $^{**}p = 0.018$, vs. H2O2 alone). (C) TBARS assay of HDFs treated with H2O2 and DDS, as described in Materials and methods. Values shown represent means ± SD calculated from three independent experiments.

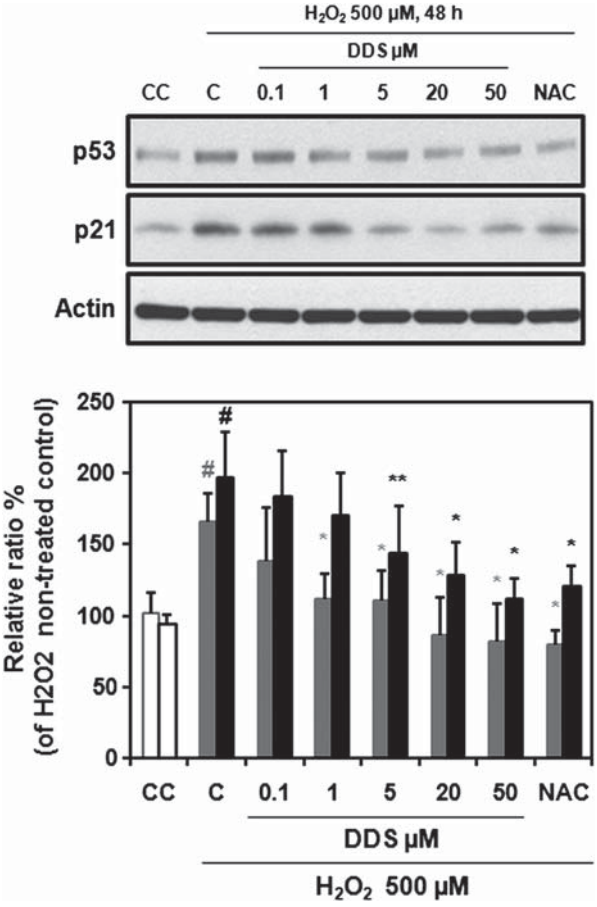

Figure 2. Effects of DDS on oxidative stress-induced increases in the levels of p53 and p21 proteins. HDFs in the presence or absence of various concentrations of DDS were treated with 500 μM H2O2 for 48 h. The levels of p53 and p21 were analyzed by immunoblotting; the graph shows the mean ± SD values from three separate experiments. $^{\#}p < 0.05$, vs. CC; $^{*}p < 0.05$, vs. C.

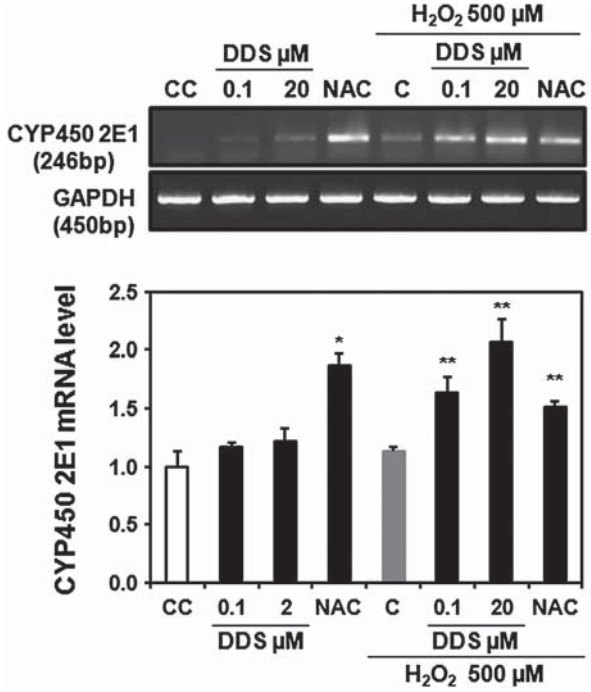

Figure 3. Modulation of CYP450 IIE1 by hydrogen peroxide and DDS treatment. Expression of CYP450 IIE1 mRNA in HDFs pretreated with different concentrations of DDS or NAC, and stimulated with 500 μM H2O2. RT-PCR (A) and real-time PCR analyses (B) using specific primers for CYP450 IIE1 and GAPDH. Values shown represent arbitrary ratios of the level of expressed mRNA for each factor relative to that of GAPDH. The graph shows the mean ± SD values from three separate experiments. $^{*}p < 0.05$, vs. CC; $^{**}p < 0.05$, vs. C.

Modulation of CYP450 IIE1 by H_2O_2 and DDS treatment

To analyse the mechanism underlying the DDS effect on H_2O_2-induced oxidative stress, changes in the level of the DDS-metabolizing enzyme CYP450 IIE1 were measured using RT-PCR and real-time PCR. The expression of CYP450 IIE1 was increased in HDFs in relation to the concentration of DDS applied, to a greater extent than with H_2O_2 treatment alone. Interestingly, the level of CYP450 IIE1 was increased to a greater extent in HDFs treated with both DDS and H_2O_2 than in HDFs treated with DDS alone (Figure 3), which suggests a synergistic action of these two drugs for the induction of xenobiotic metabolism.

Effects of DDS on radical-scavenging enzymes and glutathione status

To monitor the effect of DDS on oxygen radical scavenging, the activities of CAT, GR and GPx were measured from H_2O_2 short-term (30 min) treated HDFs. DDS increased CAT activity in a concentration-dependent manner (Figure 4A), confirmed protein level by Western blot analysis (Figure 4B). DDS treatment also increased the GR and GPx activities (Figure 4A). GR and GPx play pivotal roles in the maintenance of the intracellular redox state by adjusting the GSH/GSSG ratio and scavenging free radicals. DDS treatment restored effectively the GSH/GSSG ratio ($p < 0.05$) in HDFs that were treated with H_2O_2 (Figure 5), probably through the induction of radical-scavenging enzymes and the maintenance of glutathione status.

Effect of DDS on apoptotic stress

Since oxidative stress-induced cell death can be blocked by antioxidants [10,11], DDS protection against H_2O_2-induced apoptosis was assessed. HDFs were pre-treated with various concentrations of DDS (0.1, 1, 5, 20 and 50 μM) and NAC (2 mM), followed by treatment with 1 mM H_2O_2 for 24 h. In the absence of pre-treatment, H_2O_2 decreased cell viability to ~72% of the control level. In contrast, pre-treatment with DDS improved cell viability in the presence of H_2O_2 to ~90% of the control level. NAC also showed protective effects against H_2O_2-induced cytotoxicity (Figure 6A). In addition, DDS prevented

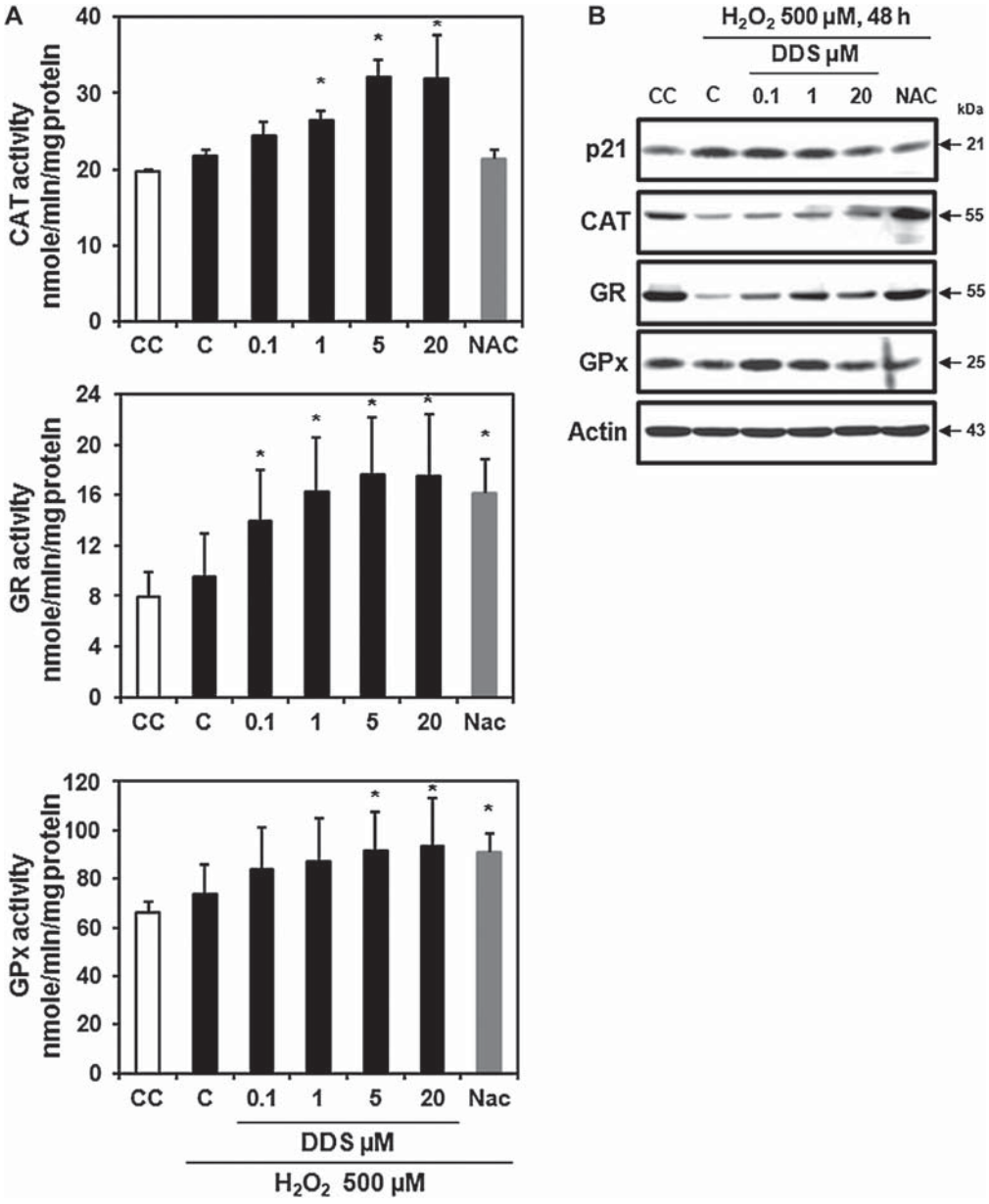

Figure 4. Effects of DDS on the expression and activities of catalase, glutathione reductase, and glutathione peroxidase. (A) The activities in H2O2 (500 μM, 30 min)- and DDS-treated HDF homogenates were analyzed as described in Materials and methods. Each enzymatic activity is expressed in nmole/min/mg protein. The graphs are representative of three separate experiments ($^{*}p < 0.05$, vs. C). (B) Immunoblot analysis showing the levels of CAT, GR and GPx after treatment of HDFs with 500 μM H2O2 and different concentrations of DDS for 48 h.

the reduction of Bcl-2 and the induction of Bax by oxidative stress (Figure 6B). In contrast, DDS had no effect on either thapsigargin-induced or staurosporine-induced apoptosis, as evidenced by the unchanged levels of PARP cleavage in HDFs (Figure 6C).

Discussion

Long-term administration of DDS at standard doses (100 mg/50 kg body weight per day) in patients with Hansen's disease usually results in few or no clinically significant side-effects [23] and results in a DDS plasma concentration that ranges from 1–20 μM [24]. The dosages used in the present study were optimized based on the principle that, at an appropriate concentration, DDS has minimal toxicity for HDFs. Since the IC_{50} of DDS for HDFs is ~ 711 μM (Figure 1A), a dose range close to the plasma concentrations observed in patients treated with DDS appears to be safe. Recently, a 5% DDS gel, which achieves a plasma concentration of ~20 μM, was reported to be safe and effective for long-term treatment [25]. Despite its therapeutic effects, the capacities of DDS, either as a pro-oxidant [2,3] or antioxidant [4,5,26,27], remain controversial. However, most studies on the pro-oxidant effects of DDS have been based on its use at high concentrations [28,29]. In human dermal

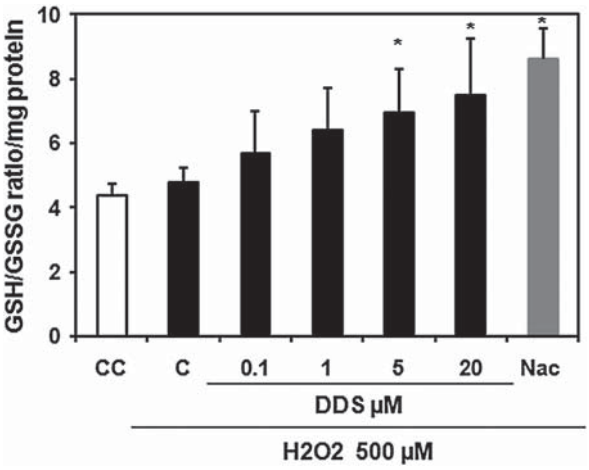

Figure 5. Effect of DDS on cellular glutathione status. The GSH/GSSG ratio of H2O2- and DDS-treated HDFs was assessed using colorimetric assays and the GSH reductase-DTNB recycling procedure, as described in Materials and methods. Data shown are the mean ± SD values of the relative GSH/GSSG ratios/mg proteins from three independent experiments ($^{*}p < 0.05$, vs. C).

fibroblasts, a high dose of DDS (1.5 mM) induced oxidative stress and glutathione depletion [28], while in rat livers, DDS administration at a dose of 30 mg/kg body weight resulted in oxidative stress [29]. Therefore, the debate regarding the nature of DDS as a pro-oxidant or antioxidant appears to reflect the dosage effect.

As a major component of ROS, H_2O_2 has been extensively used as an inducer of oxidative stress in *in vitro* models [30]. H_2O_2 treatment enhanced ROS formation in the HDFs and this was significantly reduced in a dose-dependent manner by pre-treatment with DDS (Figure 1B). H_2O_2 also causes lipid peroxidation in various cell types. When TBARS, which is a major product of lipid peroxidation, was monitored, DDS was found to reduce H_2O_2-induced MDA generation effectively (Figure 1C). A similar trend was detected for the intracellular levels of products of lipid peroxidation. Increased MDA alters the structure and function of the cellular membrane and blocks cellular metabolism, leading to cytotoxicity [31]. In addition, a sufficiently high concentration of H_2O_2 causes cell death via apoptosis, an effect that may be blocked by antioxidants. In the present study, we demonstrated that treatment of HDFs with H_2O_2 leads to cell death via apoptotic processes; these results are consistent with previous reports showing

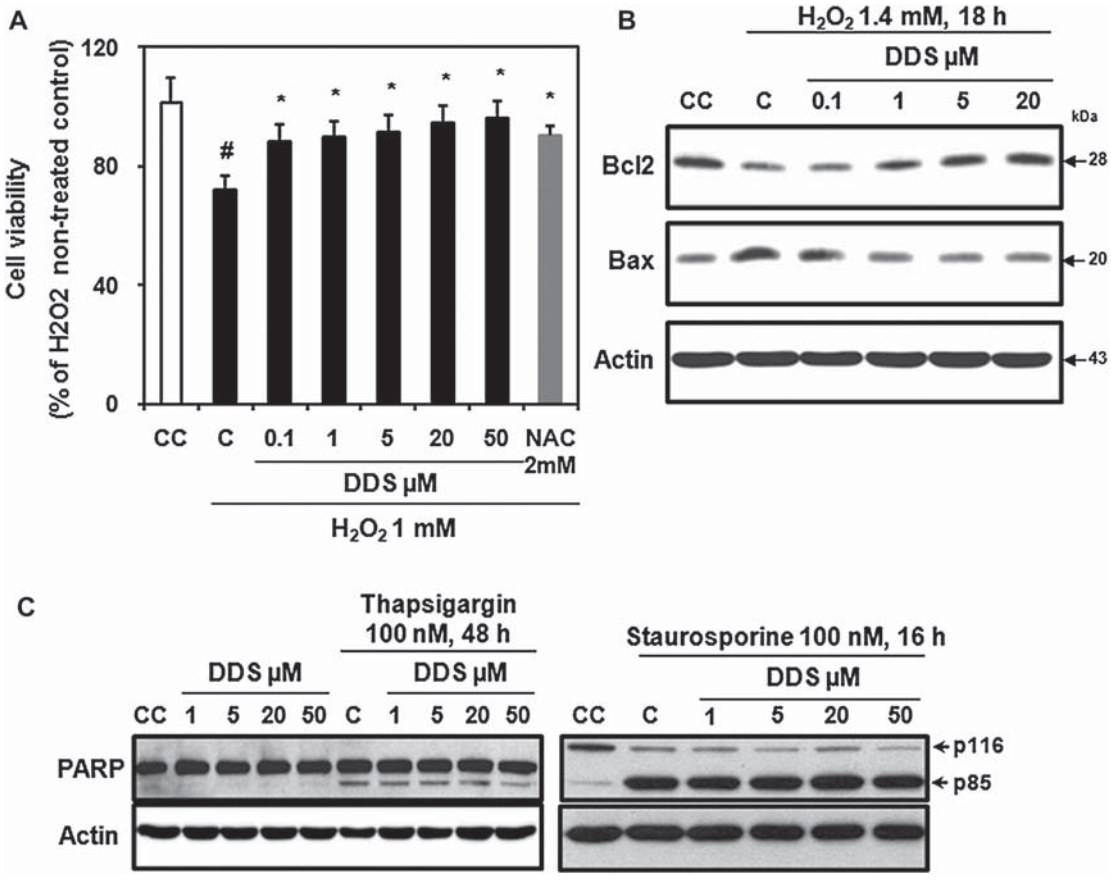

Figure 6. Effects of DDS on cell viability and apoptotic responses. (A) Cell viability was assessed for H2O2- and DDS-treated HDFs using a Cell Counting Kit-8, as described in Materials and methods. Data are normalized to the controls, and the results are expressed as mean ± SD calculated from four independent experiments. Results shown are percentages of the control rate. $^{\#, *}p < 0.005$. (B) HDFs were treated with 1.4 mM H2O2 and different concentrations of DDS. Changes in the levels of the Bcl2 and Bax proteins were monitored by Western blotting. (C) HDFs were treated with 100 nM thapsigargin (for 48 h) or 100 nM staurosporine (for 16 h), and PARP cleavage was analyzed by immunoblotting with an anti-PARP antibody.

apoptotic death processes in various types of cells treated with H_2O_2 [11,30,32]. Antioxidants that inhibit the production of ROS or that enhance cellular antioxidant defenses can prevent apoptosis and protect against the damaging effects of oxygen radicals [10,11,33]. Consistent with these reports, DDS exhibited high anti-apoptotic activities in the H_2O_2-treated HDFs. Therefore, we hypothesized that the mechanism underlying the anti-apoptotic activities of DDS might be related to its antioxidant capacity to inhibit the production of ROS. Pre-treatment with DDS improved cell viability (Figure 6A). These results suggest that DDS acts as a strong antioxidant, similar to NAC. Furthermore, H_2O_2 induces an increase in the level of the p53 tumour suppressor protein, thereby inducing apoptosis in a p53-dependent manner [34], and leads to the activation of $p21^{Cip1/WAF1}$, a cyclin-dependent kinase inhibitor [30,35]. After treatment with H_2O_2, the levels of p53 and p21 were significantly increased in the HDFs, while pre-treatment with DDS prevented these increases (Figure 2).

Since the H_2O_2-induced apoptotic HDFs showed decreased levels of Bcl-2, which is a survival factor, and increased levels of Bax protein, which is a death factor, these apoptotic markers were monitored. DDS was found to influence the regulation of the Bcl2 and Bax proteins (Figure 6B). However, DDS did not affect the activities of other pro-apoptotic stimuli, such as thapsigargin, which disrupts endoplasmic reticulum (ER) Ca^2+ stores [36], or staurosporine, which causes caspase-3-dependent apoptosis [37], as evidenced by the lack of changes in PARP cleavage levels in the HDFs despite DDS treatment (Figure 6C). These results confirm the specific role of DDS in oxidative stress rather than other apoptotic stresses.

DDS is N-hydroxylated by several cytochrome P-450s, such as CYP IIIA, IIEl and IIC in the rat and CYP450 IIC9, IIEl and IIIA4 in humans [38,39]. DDS toxicity may be initiated by the formation of a hydroxylamine metabolite, catalysed by CYP450. To understand the observed DDS effect on H_2O_2-induced oxidative stress, the levels of CYP450 IIE1 were monitored. Interestingly, the levels of CYP450 IIE1 in HDFs were increased to a greater extent by combined treatment with DDS and H_2O_2 than by treatment with DDS alone (Figure 3), suggesting a synergistic action of these agents on xenobiotic metabolism, which warrants further study.

We next investigated whether the protective effects of DDS would act directly as a ROS scavenger or indirectly by modulating antioxidant enzyme systems. Since we reported that DDS did not directly scavenge DPPH radicals *in vitro*, in contrast to Trolox, which is a positive control for radical scavenging [27], we assumed that the antioxidant effects of DDS occurred indirectly through modulation of the scavenger enzymes and the redox status of the HDFs. Antioxidants have been shown to induce the activity of CAT, GR and GPx in mammalian cells [40]. Moreover, these stress responses have been linked to increased tolerance to subsequent insults and resistance to apoptosis [41]. We observed that DDS increased the activity of the ROS-scavenging enzyme CAT (Figure 4A). Furthermore, DDS induced the GR and GPx activities, suggesting positive regulation of the cellular redox status. Moreover, DDS also prevented the H_2O_2 dependent reduction of ROS-scavenging enzymes. Previously it was demonstrated that the enzymes for scavenging oxidative radicals could be induced by chemical agents, such as 1,2-dithiole-3-thiones and 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid-methylamide (CDDO-MA) via a nuclear factor E2-related factor 2 (Nrf2)-mediated antioxidant response element-driven transcriptional mechanism [42,43]. The molecular mechanism underlying DDS-mediated elevation of radical scavenging system such as CAT, GR and GPx activities might be linked also to the above Nrf2 mediated system, but it requires to be confirmed. The GSH/GSSG ratio was higher in HDFs treated with H_2O_2 and DDS (Figure 5), indicating an active role of DDS in adjusting the glutathione status. These data suggest that DDS protects cells against oxidative stress through modulation of both GSH redox status and antioxidative enzymes. Previously, we reported that DDS would effectively suppress the ROS generation by inhibiting the NADPH oxidase system in paraquat treated HDFs [27]. Moreover, DDS has been clinically used safely by Hansen's patients for several decades.

Taken together, DDS can be suggested as one of the safe antioxidants, blocking not only ROS generation but also ROS scavenging by activation of antioxidative enzymes. Therefore, DDS could be recommended for the prevention or modulation of oxidative stress-associated pathologies and probably of ageing processes.

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