Suppression of ROS generation by 4,4'-diaminodiphenylsulfone in non-phagocytic human diploid fibroblasts

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Abbreviations: CCK-8, Cell Counting Kit-8; DDS, 4,4'-diaminodiphenylsulfone; DiOC6, 3,3'-diethyloxycarbocyanine; DPI, diphenylene iodonium; DPPH, 2,2-diphenyl-1-picrylhydrazyl; HDF, human diploid fibroblast; $\Delta\psi_m$, mitochondrial membrane potentials; NAC, acetyl-L-cysteine; NOX4, NADPH oxidase 4; ROS, reactive oxygen species

Abstract

The action mode of 4,4'-diaminodiphenylsulfone (DDS) is still under debate, although it has long been used in treatment of several dermatologic diseases including Hansen's disease. In this study, we tested the effect of DDS as an antioxidant on paraquat-induced oxidative stress in non-phagocytic human diploid fibroblasts (HDFs). Overall, preincubation of HDFs with DDS prevented the oxidative stress and the resulting cytotoxic damages caused by paraquat in these cells. The specific effects of DDS in paraquat-treated HDFs are summarized as follows: a) reducing the expression of NADPH oxidase 4 (NOX4) by inhibiting paraquat-induced activation of PKC; b) inhibiting paraquat-induced decreases in mitochondrial complex protein levels as well as in membrane potentials; c) consequently, inhibiting the generation of cytosolic and mitochondrial superoxide anions. Taken together, these findings suggest that DDS would suppress the radical generation in non-phagocytic HDFs during oxidative stress, and that DDS might have the extended potential to be used further in prevention of other oxidative stress-related pathologies.

Keywords: antioxidants; leprosy; NADPH oxidase; oxidative stress; paraquat

Introduction

4,4'-Diaminodiphenylsulfone (DDS, Dapsone), synthesized a century ago, continues to be used in the therapy of many skin diseases (Wolf *et al.*, 2002) and has been the mainstay drug for treating patients with Hansen's disease. However, the mode of action of DDS has not been definitively established. Several studies suggested that DDS would act as a pro-oxidant and might cause hemolytic anemia (Bradshaw *et al.*, 1997; Reilly *et al.*, 1999). However, a recent study found little or no risk of hemolysis from topical DDS in 5% gel form (Piette *et al.*, 2008). Furthermore, other studies suggested that DDS would act as an antioxidant rather than as a pro-oxidant (Niwa *et al.*, 1984; Anderson *et al.*, 1987). Thus, question whether DDS acts as a pro-oxidant or an antioxidant has not been clearly answered yet.

To answer the above question we employed paraquat in our experiment, which has been originally developed as an herbicide, and widely used as a source of oxidative stress to cells, since it generates superoxide anions via NADPH-dependent metabolic pathways. PKC is involved in the ligand-initiated assembly of NADPH oxidase (NOX) for the generation of superoxide anions (Martins *et al.*, 2002). Recently, paraquat has been shown to induce ROS through activation of PKC- δ dependent NOX (Miller *et al.*, 2007). In addition, mitochondria are involved in paraquat-induced oxidative stress and toxicity. Mitochondrial complex I (NADH-ubiquinone oxidoreductase) is the major mitochondrial site for superoxide production by paraquat (Cocheme *et al.*, 2008) and paraquat significantly decreases the activity of mitochondrial complex V (Yang and Tiffani-Castiglioni, 2007). Failure of mitochondrial machinery and impairment of mitochondrial complexes by paraquat results in inhibition of electron transport with subsequent increased production of superoxide anion (Boelsterli and Lim, 2007).

Availability of the safe and effective antioxidants can possibly help prevent oxidative stress-related pathologies. The experience of long term use of DDS by Hansen's patients stimulated us to clarify the role of DDS possibly as an antioxidant and to study its underlying mechanism. For the study, the effect of DDS on paraquat-induced oxidative stress in non-phagocytic HDFs has been tested, since its

Figure 1. Effect of DDS on viability of paraquat (PQ) treated HDFs. DDS was added to HDFs, and let stand for 3 h before treatment with 1 mM paraquat. Cell viability was assessed after 48 h with a Cell Counting Kit-8. Positive controls were treated with DPI (5 μ M) for 30 min or with NAC (2 mM) for 3 h prior to paraquat exposure. Data are normalized to control and the results are expressed as means $\pm$ SE of three experiments. Results expressed as % of control rate. Marks indicate the significant difference, $P < 0.005$ (#, from untreated control cells (CC); *, from only paraquat treated control cells (C)).

role as an antioxidant during bacterial infection has been proposed in the phagocytic neutrophils (Suda *et al.*, 2005). Through this study, we could suggest that DDS would be the effective antioxidant, which

might modulate ROS generation mainly via regulation of NOX and mitochondrial dysfunction in the non-phagocytic cells.

Results

DDS ameliorates paraquat-induced cytotoxicity

To assess the cellular protective effect of DDS against paraquat-induced cytotoxicity, HDFs were pre-treated with varying concentrations of DDS (0.1, 1, 5, 20, and 50 μ M), diphenylene iodonium (DPI, 5 μ M), or N-Acetyl-L-cysteine (NAC, 2 mM), respectively, followed by treatment of 1 mM paraquat for 48 h. DPI, a flavoenzyme inhibitor including NOX, and NAC, an antioxidant, were used as positive controls. Cell viability was measured with a Cell Counting Kit (CCK-8). Without pre-treatment with DDS, paraquat decreased the cell viability to about 58.8% of the control. In contrast, pre-treatment with DDS improved the cell viability against paraquat toxicity dose dependently up to 90% of the control. DPI and NAC also showed protective effects against paraquat-induced cytotoxicity and recovered the cells upto about 80-90% of the control (Figure 1).

Figure 2. Effect of DDS on paraquat (PQ)-induced cytosolic or mitochondrial superoxide anion generation in HDFs. HDFs were seeded to 96 well plate for 24 h and pretreated with various concentration of DDS (0.1, 1, 5, and 20 μ M), DPI (5 μ M) for 30 min, and NAC (2 mM) for 3 h. The cells were then treated with 1 mM paraquat for 30 min. After paraquat treatment, the cells were incubated with the oxidant-sensitive fluorescent dyes (A) dihydroethidium (DHE) (5 μ M, cytosol specific) and (B) MitoSOX Red (5 μ M, mitochondria specific), respectively, for 15 min at 37°C. Changes in fluorescence were monitored with a multiwell plate reader using an excitation and emission wavelength of 515 and 590 nm (dihydroethidium), 520 and 580 nm (MitoSOX Red), respectively. Results are expressed as % of control rate. The data shown are representative of six experiments. $^{\#}P < 0.005$ relative to CC, $^*P < 0.005$ relative to C.

Figure 3. Effect of DDS on DPPH-radical scavenging and expression of SOD genes. (A) Radical scavenging activities of DDS in cell-free system (*in vitro*). The graph is obtained from studies of various concentrations of DDS and Trolox (400 μ M) against DPPH (200 μ M) radical. (B) Western blot analysis of SOD1 and SOD2 expression in DDS pretreated and paraquat 1 mM treated HDF for 12 h or 48 h.

DDS inhibits paraquat-induced superoxide anion generation

In order to monitor the paraquat-induced superoxide anion generation, we used dihydroethidium to monitor at the level of cytosol and MitoSOX Red to monitor at the level of mitochondria.

Dihydroethidium is oxidized to oxoethidium by superoxide anion, resulting in fluorescence (Miyata *et al.*, 2005). Addition of DDS caused a dose-dependent decrease in superoxide anions as determined by dihydroethidium assay. The positive controls, DPI and NAC, also indicated the reduction in superoxide anion generation (Figure 2A). MitoSOX Red probe does not fluoresce until it enters an actively respiring cell, where it is oxidized to a fluorescent probe, selectively sequestered in the mitochondria (Esposti *et al.*, 1999). DDS-treated HDFs showed a dose-dependent decrease in mitochondrial superoxide anions (Figure 2B). The positive controls, DPI and NAC also inhibited the generation of mitochondrial superoxide anions. These results suggest that paraquat-induced cyto-

toxicity could be ameliorated by DDS via inhibition of generation of cytosolic and mitochondrial superoxide anions.

DDS itself cannot scavenge radicals

To understand how DDS protects the cells from oxidative stress, we first tested its effect on DPPH, a stable free radical. DDS did not exert any ROS scavenging activity on DPPH *in vitro*, in comparison with Trolox, used as a positive antioxidant control, which showed a strong radical scavenging activity (Figure 3A). Since most antioxidants increase the oxidative defense functions with activation or induction of radical scavenging enzymes, we assumed that DDS also might induce or activate ROS scavenging enzymes. We determined the levels of superoxide dismutase 1 (SOD1) and superoxide dismutase 2 (SOD2) in HDFs in presence or absence of several concentrations of DDS. Interestingly, DDS did not increase, rather decreased the levels of SOD1 and SOD2

Figure 4. Effect of DDS on paraquat-induced NOX4 mRNA. (A) HDFs were pre-exposed to different concentrations of DDS for 3 h, after which cells were exposed to 1 mM paraquat for different time periods. NOX4 mRNA levels were determined using a RT-PCR. (B) Results are expressed as % of control rate (paraquat untreated). The data shown are representatives of three experiments from 3 h DDS pretreatment, followed by 3 h paraquat treatment. [#] $P < 0.05$ relative to CC, ^{*} $P < 0.05$ relative to C.

Figure 5. Effect of DDS on paraquat (PQ)-induced calcium dependent PKC activation in HDFs. (A) Effect of DDS on paraquat-induced changes in calcium levels in HDFs. HDFs were seeded to 96 well plate for 24 h and pretreated with various concentration of DDS and 5 μ M DPI for 30 min. The cells were incubated with the intracellular calcium-indicator Fluo-4 AM at 37°C for 30 min, and then exposed to 1 mM paraquat for several minutes. The data of 3 min paraquat treatment are representatives of three independent experiments. (B) Paraquat-induced PKC phosphorylation was determined using western blotting. HDFs were first treated with DDS and then exposed to 1 mM paraquat for 1 min or 5 min. PKC phosphorylation was determined using phosphorylation-specific anti-PKC antibody. The data of PKC-pan phosphorylation (specifically serine 660) graphed is representative of three independent experiments. [#] $P < 0.05$ relative to CC, ^{*} $P < 0.05$ relative to C.

(Figure 3B), which might implicate the lesser requirement for radical scavenging activity in DDS-treated cells. These results suggest strongly that DDS would be related rather with suppression of ROS generation than with induction of ROS scavenging capacity.

DDS suppresses NOX4 expression

NOX4 is the predominant form of NOX genes in skin fibroblasts (Park *et al.*, 2005; Rossary *et al.*, 2007), and expression of NOX4 is associated directly with ROS generation. Down-regulation of NOX4 would result in reduction of ROS generation (Lambeth, 2004). Therefore, NOX4 has been monitored in this study. After treating cells with 1 mM paraquat, we subjected HDFs to RT-PCR

analysis of NOX4 and GAPDH. An increased level of NOX4 was observed in paraquat-treated HDFs at several indicating times (1, 3, and 6 h) with a peak induction at 3 hours. However, pretreatment of DDS decreased NOX4 gene expression in a dose dependent manner (Figures 4A and 4B).

DDS modulates PKC activation

It has been demonstrated that DDS can adjust intracellular calcium levels (Suda *et al.*, 2005). In order to confirm the role of DDS in paraquat-induced calcium status, we measured calcium by Fluo-4 fluorescence in varying conditions. DDS decreased paraquat-induced calcium levels dose dependently (Figure 5A).

PKC isoforms are implicated in NOX activation

Figure 6. Effect of DDS on paraquat (PQ)-induced mitochondrial change. (A) HDFs were pretreated with DDS (3 h), DPI (30 min), and NAC (3 h) and then with paraquat 1 mM (24 h). The treated HDFs were harvested for analysis of mitochondrial complex proteins, and immunoblotting with individual antibodies that recognize Complex I (20 kDa ND6 subunit), Complex II (30 kDa FeS, non-heme iron protein, SDHB), Complex III (47 kDa core protein 2), Complex IV (18 kDa subunit IV, and Complex V (55 kDa subunit an ATP synthase), as described in the Methods. (B) The change of mitochondrial membrane potential ($\Delta\psi_m$) was examined by DiOC6. DDS, DPI, and NAC pre-treated and then paraquat 1 mM (24 h) treated HDF cells were stained 40 nM DiOC6 for 15 min. The result analyzed by Cary Eclipse fluorescence spectrophotometer (Varian, California) with excitation at 480 nm and emission at 520 nm. (C) Mitochondria morphological change analysis used MitoTracker Red and confocal microscopy. HDFs growing on glass coverslips were exposed to paraquat and DDS (or DPI) as described in Methods. Cell cultures were incubated with 1 mM paraquat for 24 h. First, Control HDFs, second, HDFs incubated with only paraquat, third, Cell cultures co-treated with DDS 20 μ M and 1 mM paraquat, and last, Cell cultures co-treated with DPI 5 μ M and 1 mM paraquat. (A, B, C, and D: $\times 4,000$) Value are means $\pm$ SEM ($n = 3$). [#] $P < 0.005$ relative to CC; ^{*} $P < 0.005$ relative to C.

(Inoguchi *et al.*, 2003). The conventional (cPKC) or calcium-dependent isoforms such as PKC α , β I, β II, and γ require calcium for activation (Coppi *et al.*, 2000). In this study with HDFs, it is confirmed that paraquat could activate only PKC β II but not PKC α and PKC δ . For the analysis of PKC dependent activation of NOX, we examined the effect of DDS on paraquat-induced PKC phosphorylation by western blot analysis. Paraquat could activate PKC β II (specifically via phosphorylation of ser 660) in HDFs, which was reduced by DDS (Figure 5B). Phospho-PKC pan antibody could detect serine 660 phosphorylation of PKC β II, but

Phospho-PKC α/β antibody could detect PKC α/β , phosphorylated at threonine 638/641.

These results implicate that DDS would suppress paraquat-induced cellular calcium signaling, followed by lowering of PKC activation with subsequent suppression of NOX4 activation, which might result in reduction of ROS generation.

DDS adjusts paraquat-induced mitochondrial dysfunction

Mitochondrial dysfunction is characterized by superoxide generation, membrane potential decrease,

mitochondrial DNA (mtDNA) damage, and morphological changes. To understand how DDS is involved in restoring mitochondrial dysfunction, we examined the effect of DDS on paraquat-induced cell damages.

We monitored the effect of DDS on mitochondrial complex protein levels by western blot analysis. DDS restored the protein levels of all mitochondrial complexes, which had been decreased by paraquat treatment (Figure 6A). We then assessed the changes in mitochondrial membrane potentials by DiOC6 fluorescence. Mitochondrial membrane potential in HDFs decreased to 76.8% of the control after treatment with 1 mM paraquat for 24 h. Pre-treatment with several concentrations of DDS for 3 h or 5 μ M DPI for 30 min significantly protected HDFs from paraquat-induced decrease in mitochondrial membrane potential (Figure 6B).

In normal HDFs, mitochondria exhibit elongated patterns (Figure 6C, first). Cells are defined MitoTracker Red positive (MT+), if well defined fluorescent points corresponding to mitochondria are observed, and MitoTracker Red negative (MT-) when a diffuse cytoplasmic staining is observed (Matylevitch *et al.*, 1998). HDFs, incubated with 1 mM paraquat for 24 h, showed spotting pattern on staining, and the disappearance of the MT (+) cell morphology (Figure 6C, second). When HDFs were co-treated with DDS (or DPI) and paraquat, there was a partial recovery of original mitochondrial morphology (Figure 6C, third and fourth). Because MitoTracker Red dye could be used for monitoring the mitochondrial membrane potential (McCarthy *et al.*, 2004), these results also support the effect of DDS on paraquat-induced mitochondrial membrane potential collapses.

Discussion

Long-term administration of DDS at the standard dose (100 mg/day) in patients with Hansen's disease usually results in little or no clinically significant side effects. Administration of DDS, 100 mg/day, for 6-28 days to patients results in 1-20 μ M of plasma DDS concentration (Zone, 1991). A recent study evaluating the risk of hemolysis reported that the topical use of DDS at 5% gel would be safe, and DDS levels of plasma could reach about 5 ng/ml (20 μ M) (Piette *et al.*, 2008).

Recently it has been reported that DDS suppresses the production of $O_2^{\cdot-}$ in phagocytic neutrophils (Suda *et al.*, 2005). Thereby, we asked the question whether its effect as an antioxidant could be reproduced in non-phagocytic cells. ROS generation in non-phagocytic cells is much lower

than in phagocytic cells, and its implication might be distinct from bacterial killing. For the study on the cellular effect of DDS in non-phagocytic HDFs, we adopted the paraquat-induced oxidative stress cytotoxic model, since the mechanism of paraquat-induced cytotoxicity has been well associated with oxygen radical generation.

In this study, we tried to test the possibility that DDS might act as an effective blocker of radical generation, not only in phagocytic cells but also in non-phagocytic cells. Initially, the genuine radical scavenging activity of DDS has been tested on DPPH, a stable free radical (Dinis *et al.*, 1994; Wang *et al.*, 1998). Antioxidants react with DPPH and convert it to 1,1-diphenyl-2-(2,4,6-trinitrophenyl) hydrazine, indicating their scavenging potentials. But in this study, DDS itself could not scavenge DPPH radicals *in vitro*, in contrast to the strong scavenging activity of Trolox, a positive control for radical scavenging (Figure 3A). Then the effect of DDS on expression levels of radical scavenging enzymes, SOD1 and SOD2 has been assessed. Cytosolic copper-zinc SOD (SOD1) and mitochondrial manganese-SOD (SOD2) reduce superoxide anion to hydrogen peroxide (H_2O_2) and molecular oxygen (Zelko *et al.*, 2002). Interestingly, DDS could reduce the expression of both enzymes in HDFs rather than their induction (Figure 3B). These data strongly suggest that the antioxidant function of DDS would not be directly related to the radical scavenging system, neither by direct radical scavenging activity nor by induction of radical scavenging enzymes.

Therefore, we focused on the role of DDS in modulation of radical generation rather than in radical scavenging. Paraquat-induced oxidative stress, mainly mediated by activation of the NOX, but not by activation of xanthine oxidase (Miller *et al.*, 2007), is accompanied by mitochondrial dysfunction (Thakar *et al.*, 1988; Tomita, 1991). NOXs utilize molecular oxygen and NADPH as substrates and FAD as a coenzyme. NOX enzymes are involved in regulation of a wide range of physiological functions including cellular survival, differentiation, proliferation, Ca^{2+} signaling and migration (Lambeth, 2004). NOX4, the major NOX form present in skin fibroblasts, generates substantial amounts of ROS (Park *et al.*, 2005; Rossary *et al.*, 2007) and is increased by TGF β stimulation (Cucoranu *et al.*, 2005; Sturrock *et al.*, 2006) and by angiotensin stimulation of mesangial cells in the kidney (Gorin *et al.*, 2003). In this study, NOX4 mRNA level was found to be increased by paraquat treatment, which was suppressed by DDS (Figure 4).

DDS was suggested to be involved in calcium modulation (Suda *et al.*, 2005). However, no data

are available about its effect on paraquat-modulated calcium levels in HDFs. In this study, it was confirmed that paraquat would induce the intracellular calcium and that this increase could be reversed by DDS (Figure 5B). The increased calcium might activate the calcium-dependent events such as PKC activation, required for NOX activation. The association of paraquat-induced superoxide anion generation with activation of PKC is well established (Martins *et al.*, 2002; Miller *et al.*, 2007). Therefore, it was asked whether DDS could modulate PKC activation. Our results indicated that DDS could inhibit paraquat-induced PKC β II phosphorylation at serine 660 in HDFs (Figure 5B). Taken together, these findings suggest that DDS would restore the intracellular Ca²⁺ levels, followed by inhibition of PKC-mediated activation of NOX, resulting in suppression of superoxide anion generation in paraquat-treated HDFs.

Paraquat-induced mitochondrial dysfunction is characterized by high superoxide production, reduced membrane potential, mitochondrial DNA damage and morphological changes. Paraquat is used in experimental models of Parkinson disease to generate mitochondrial oxidative damage (Thiruchelvam *et al.*, 2000). Thereby, antioxidants are investigated for possible regulators of mitochondrial superoxide anion generation and consequently for treatment of neurodegenerative diseases. Our study demonstrated that DDS might attenuate the paraquat-induced mitochondrial abnormalities in HDFs. Not unexpectedly, DPI, a large-spectrum inhibitor of electron transporters including various NOX enzymes and mitochondrial oxidase (Li *et al.*, 1998), inhibited paraquat-induced mitochondrial toxicity. DDS thus emerges as a possible novel efficient inhibitor of paraquat toxicity.

In summary, we have shown here that DDS inhibits paraquat-induced oxidative stress and mitochondrial toxicity in HDFs through inhibition of NOX4 activation by modulation of Ca²⁺ level and the consequent regulation of PKC activity. Further studies are required to confirm the effectiveness of DDS in prevention of other oxidative stress-related pathologic conditions in general. In the mean time, it might be tentatively proposed that DDS would be the novel potential agent for mitigating or minimizing oxidative stress via its ability to suppress the generation of oxygen radicals.

Methods

Chemicals

The following are the sources of various chemicals and

reagents used: 4,4'-diaminodiphenylsulfone (DDS, dapsone) was from Taekeuk pharmaceuticals (Seoul, Korea); Paraquat, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox, diphenylene iodonium (DPI), and N-Acetyl-L-cysteine (NAC) were from Sigma (St. Louis, MO); Cell Counting Kit (CCK-8) was from Dojindo Laboratory (Japan), and MS601 (a Total OXPHOS Complex Detection Kit, antibody) was from Mitoscience (Eugene, OR). SOD1 and SOD2 antibodies were from AbFrontier (Seoul, Korea); Trizol reagent was from MRC (Cincinnati), and dihydroethidium, MitoSOX Red, Fluo-4 AM, MitoTracker Red CMXRos, and 3, 3-dihexyloxycarbocyanine (DiOC6) were from Molecular Probes (Eugene, OR).

Cell culture and assessment of cell viability

Primary HDFs were isolated from newborn foreskins, as described (Boyce and Ham, 1983). Cells were maintained in DMEM containing 10% FBS and antibiotics. Cell viability assessment was performed using Cell Counting Kit (CCK-8) as required in Dojindo Laboratory technical manual. HDFs were seeded and grown for 24 h in 96-well plates prior to any treatment. HDFs were pre-treated with DDS for 3 h, and then treated with paraquat (1 mM) for 48 h and 10 μ l CCK-8 were directly added to each well, and incubated for 3 h at 37°C under 5% CO₂. The results were expressed relative to the control values specified in each experiment and were subjected to statistical analysis.

Determination of radical scavenging activity

The radical scavenging activity was determined by reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol. Free radical scavenging effect of DDS was determined as previously described (Mellors and Tappel, 1966), using Trolox as a reference antioxidant. In brief, 0.5 ml of methanol containing different concentrations (0.1-100 μ M) of DDS and Trolox (400 μ M) were mixed in the test tube with 2.5 ml methanol containing 200 μ M DPPH, respectively. DPPH is a stable free radical with a deep purple color in alcohol solution and has a peak absorbance at 517 nm, but becomes pale yellow when trapped by an antioxidant. The reaction mixture was kept in dark at room temperature for 30 min and the absorbance was monitored at 517 nm. The free radical scavenging activity was approximated using the following equation: % inhibition = [(absorbance of control-absorbance of test sample)/absorbance of control] $\times$ 100%.

RT-PCR detection of NOX and GAPDH

Total RNA of HDFs was prepared with Trizol reagent (MRC Cincinnati), and RT-PCR for NOX4 and GAPDH were performed with Biometra T Gradient PCR (Biometra, Goettingen, Germany) using specific primers: 5'-GGTCCT-TTTGGAAGTCCATTGAGG-3' (forward primer) and 5'-CACAGCTGATTGATTCCGCTGAG-3' (reverse primer) for NOX4; 5'-ACCACAGTCCATGCCATCAC-3' (forward primer) and 5'-TCCACCACCTGTTGCTGTA-3' (reverse primer) for GAPDH. PCR-amplified DNA was separated by 1.2% agarose gel electrophoresis.

Monitoring of the intracellular superoxide anion

HDFs were seeded to 96 well plate and were subjected to pretreatment with various concentration of DDS, DPI (5 μ M, 30 min), and NAC (2 mM) for 3 h respectively. HDFs were then treated with 1 mM paraquat for 30 min, and then incubated with 5 μ M dihydroethidium for 15 min at 37°C in dark. The cells were rinsed twice with DPBS, and then ethidium-DNA fluorescence was immediately measured with a Cary Eclipse fluorescence spectrophotometer (Varian, California) with an excitation wavelength at 515 nm and an emission wavelength at 590 nm.

Detection of mitochondrial ROS

Mitochondrial ROS production was detected using MitoSOX Red. HDFs were treated with 1 mM paraquat for 30 min under each condition (DDS, DPI, and NAC pre-treated), and then MitoSOX Red was added at a final concentration of 5 μ M. After incubation at 37°C for further 15 min, the cells were washed twice with DPBS. The fluorescence was immediately measured with a Cary Eclipse fluorescence spectrophotometer (Varian, California) with an excitation wavelength at 515 nm and an emission wavelength at 590 nm.

Western blot analysis for PKC and mitochondrial complexes

Lysates of DDS and 1mM paraquat 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 were subjected to electrophoresis. After transfer to nitrocellulose, western blots were carried out with the respective antibodies to MS601 (Total OXPHOS Complexes Detection Kit, MitoSciences, containing antibodies against SDH30 subunit of complex II (5 μ g/ml), Core2 subunit of complex III (0.2 μ g/ml), COXII subunit of complex IV (2 μ g/ml), and subunit α of F1-ATPase of complex V (0.2 μ g/ml)), PCKpan-p (β II ser660), PCK α / β -p (Thr638/641), PKC δ -p (Phospho-PKC antibody Sampler Kit, Cell Signaling Technology), and β -Actin (Sigma). Detection was carried out using HRP-conjugated secondary antibodies (Zymed) with ECL (Pierce), visualized by exposing blots to autoradiographic film (Image Reader, LAS-3000, Fujifilm, Japan).

Measurement of intracellular Ca^{2+}

Intracellular free Ca^{2+} levels were assessed semi-quantitatively by loading cells with 5 μ M of the cell-permeate fluorescent calcium indicator Fluo-4 AM at 37°C for 30 min and at the end of incubation, cells were further incubated for additional 30 min at room temperature to allow complete deesterification of intracellular Fluo-4 AM. After rinsing cells once with 20 mM HEPES (pH7.4), 100 μ l HBSS was added into each well. The fluorescent signal was measured at the indicated times using a multi-well fluorescence plate reader (Spectrafluor Plus, Tecan, Switzerland) with an excitation wavelength at 494 nm and an emission wavelength at 516 nm (Minta *et al.*, 1989). Data were presented by relative fluorescence units

(RFUs).

Assay for mitochondrial membrane potential

Changes in mitochondrial membrane potential ($\Delta\psi$ m) were determined by staining the cells with mitochondrial specific fluorescent cationic dye, DiOC6, with fluorescence spectrophotometer (Quillet-Mary *et al.*, 1997; Hishita *et al.*, 2001). 40 nM DiOC6 was added to the incubation medium for 15 min before the end of the treatment at 37°C. Then after washing, the cells were analyzed by Cary Eclipse fluorescence spectrophotometer (Varian, California) with excitation at 480 nm and emission at 520 nm.

MitoTracker Red staining

HDFs, plated in 24-well plates and treated as above for 24 h, were incubated with the mitochondrial dye MitoTracker Red CMXRos (50 nM) for 30 min at 37°C. These dye labels mitochondria with intact transmembrane potential and thus the level of staining would serve as an index of mitochondrial function. After this incubation and rinsing with PBS, the cells were fixed with 4% paraformaldehyde overnight at 4°C. After washing with PBS, the cells were incubated for 1 h at room temperature with blocking solution (2% bovine serum albumin in PBS). The HDFs were then incubated for 10 min at room temperature with DAPI. After further washes, coverslips were applied, and the cells were visualized under a fluorescent microscope.

Statistical analysis

The statistical comparisons for continuous variables were performed using the t-test or analysis of variance (ANOVA) test where appropriate. SPSS software version 15.0 (SPSS, Inc., Chicago, IL) was used for all statistical analyses.

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