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Silencing of cytosolic NADP$^+$-dependent isocitrate dehydrogenase by small interfering RNA enhances the sensitivity of HeLa cells toward staurosporine

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Abstract
Staurosporine induces the production of reactive oxygen species, which play an important causative role in apoptotic cell death. Recently, it was demonstrated that the control of cellular redox balance and the defense against oxidative damage is one of the primary functions of cytosolic NADP$^+$-dependent isocitrate dehydrogenase (IDPc) by supplying NADPH for antioxidant systems. The present report shows that silencing of IDPc expression in HeLa cells greatly enhances apoptosis induced by staurosporine. Transfection of HeLa cells with an IDPc small interfering RNA (siRNA) markedly decreased activity of IDPc, enhancing the susceptibility of staurosporine-induced apoptosis reflected by DNA fragmentation, cellular redox status and the modulation of apoptotic marker proteins. These results indicate that IDPc may play an important role in regulating the apoptosis induced by staurosporine and the sensitizing effect of IDPc siRNA on the apoptotic cell death of HeLa cells offers the possibility of developing a modifier of cancer chemotherapy.

Keywords: *Antioxidant enzyme, siRNA, apoptosis, redox status*

Introduction

Anti-cancer drugs exert their cancer chemopreventive effects by modulating various biochemical and molecular pathways leading to the apoptotic cell death of cancer cells. Thus, induction of apoptosis in cancer cells has become an indicator of the cancer treatment response and reduction of mortality in cancer patients [1]. Reactive oxygen species (ROS) have been implicated in cell death regulation [2]. Not only can apoptosis be induced by exposing cells to exogenous oxidants [3], but also many chemical and physical agents capable of inducing cell death are also known to generate ROS [4]. Apoptosis is induced in HeLa cells by a variety of anti-cancer drugs such as the anthracycline doxorubicin, etoposide, antinomycin D and the bacterial alkaloid staurosporine induce apoptosis in various tumour cells and elicit ROS formation [5–8]. Therefore, oxidative stress and apoptosis can be induced by several anti-cancer agents and it can be enhanced by decreasing the ability of a cancer cell to remove ROS.

Biological systems have evolved an effective and complicated network of defense mechanisms which enable cells to cope with lethal oxidative environments. These defense mechanisms involve antioxidant enzymes, such as superoxide dismutases (SOD), which catalyse the dismutation of O_2^- to H_2O_2 and O_2 [9], catalase and peroxidases which remove hydrogen peroxide and hydroperoxides [10]. The isocitrate dehydrogenases (ICDHs; EC1.1.1.41 and EC1.1.1.42) catalyse oxidative decarboxylation of isocitrate to α-ketoglutarate and require either NAD$^+$ or NADP$^+$, producing NADH and NADPH,

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respectively [11]. NADPH is an essential reducing equivalent for the regeneration of GSH by glutathione reductase and for the activity of NADPH-dependent thioredoxin system [12,13], both are important in the protection of cells from oxidative damage. Therefore, ICDH may play an antioxidant role during oxidative stress. We recently reported that both cytosolic ICDH (IDPc) and mitochondrial ICDH (IDPm) are involved in the supply of NADPH needed for GSH production against oxidative damage [14,15]. Although the relationship between the IDPc and its mitocondrial counterpart is unknown, it is likely that these two $NADP^+$-dependent isoenzymes are derived from two different genes, based on the lack of cross-immunoreactivity [16], different chromosomal loci and gene sequences [17,18], different tissue-specific expression [19] and different subcellular localization [19,20].

In the present report, we demonstrate that suppression of IDPc activity in HeLa cells regulates apoptosis induced by staurosporine. We report here that decreased expression of IDPc by a small interfering RNA (siRNA) enhances staurosporine-induced ROS generation and also significantly increases nuclear condensation, DNA fragmentation and apoptotic cell death. These results suggest that IDPc may play an important role in regulating the apoptosis induced by staurosporine, presumably, through acting as an antioxidant enzyme. Furthermore, the sensitizing effect of IDPc siRNA on the apoptotic cell death of HeLa cells offers the possibility of developing a modifier of cancer chemotherapy.

Materials and methods

Materials

β-$NADP^+$, isocitrate, NADPH, Hoechst 33258, staurosporine, N-acetylcysteine (NAC) and anti-rabbit IgG tetramethylrhodamine isothiocyanate (TRITC) conjugate conjugated secondary antibody were obtained from Sigma Chemical Co. (St. Louis, MO). Dihydrorhodamine 123, *t*-butoxycarbonyl-Leu-Met-7-amino-4-chloromethylcoumarin (CMAC), 2′,7′-dichlorofluoroscin diacetate (DCFH-DA) and rhodamine 123 (Rh 123) were purchased from Molecular Probes (Eugene, OR). Manganese (III) tetrakis(N-methyl-2-pyridyl)porphyrin (MnTMPyP) was obtained from Calbiochem (La Jolla, CA). Antibodies were purchased from Santa Cruz (Santa Cruz, CA) or Cell Signaling (Beverly, MA). Antibody against IDPc was prepared from IDPc-immuized rabbit and the antibody was purified by Protein A affinity chromatography.

Cell culture and cytotoxicity

HeLa cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mm glutamine and 100 units/ml penicillin/streptomycin. The cells were incubated at 37°C in a 90% humidified atmosphere containing 5% CO_2. Cytotoxicity was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazo-lium, inner salt (MTS) assay [21].

Knockdown of IDPc by siRNA

IDPc siRNA and control (scrambled) siRNA were purchased from Samchully Pham (Seoul, Korea). The sequences of the dsRNAs of IDPc and control used in the current experiments are as follows. For IDPc, sense and antisense IDPc siRNA are 5′-GGA CUUGGCUGCUUGCAUUdTdT-3′ and 5′-AAUG CAAGCAGCCAAGUCCdTdT-3′. For scrambled control, sense and antisense siRNAs are 5′-CUGAU-GACCUGAGUGAAUGdTdT-3′ and 5′-CAUUCA-CUCAGGUCAUCAGdTdT-3′, respectively. HeLa cells were transfected with 40 nm oligonucleotide by using Lipofectamine (Invitrogen) in serum-free conditions according to the manufacturer's protocol. After incubation for 2 days, the cells were washed and supplemented with fresh medium containing 10% FBS.

ICDH activity assay

Cells were collected at 1000 × g for 10 min at 4°C and were washed once with cold PBS. Briefly, cells were homogenized with a Dounce homogenizer in sucrose buffer (0.32 m sucrose, 10 mm Tris-Cl, pH 7.4). Cell homogenate was centrifuged at 1000 × g for 5 min and the supernatants further centrifuged at 15 000 × g for 30 min. The supernatants were used to measure the activity of IDPc. The precipitates were washed twice with sucrose buffer to collect mitochondria pellet. The mitochondrial pellets were resuspended in 1X PBS containing 0.1% Triton-X100, disrupted by ultrasonication (4710 Series, Cole-Palmer, Chicago, IL) twice at 40% of maximum setting for 10 s and centrifuged at 15 000 × g for 30 min. The supernatants were used to measure the activity of IDPm. The protein levels were determined by the method of Bradford [22] using reagents purchased from Bio-Rad. The fraction was added to 1 ml of 40 mM Tris buffer, pH 7.4, containing $NADP^+$ (2 mM), $MgCl_2$ (2 mM) and isocitrate (5 mM). Activity of ICDH was measured by the production of NADPH at 340 nm at 25°C. One unit of ICDH activity is defined as the amount of enzyme catalysing the production of 1 μmol of NADPH/min.

Immunoblot analysis

Proteins were separated on 10–12.5% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and subsequently subjected to immunoblot analysis

using appropriate antibodies. Immunoreactive antigen was then recognized by using horseradish peroxidase-labelled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

RT-PCR Analysis of IDPc

RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instruction. The cDNA template was then amplified by quantitative RT-PCR using the following specific primers: IDPc, 5′-GCT TCA TCT GGG CCA GTA AAA ACT ATG-3′ (forward) and 5′-GTA AAC CTT TAA TGC TAG CAG CCA AGT CC-3′ (reverse); and actin, 5′-TCT ACA ATG AGC TGC GTG TG-3′ (forward) and 5′-ATC TCC TTC TGC ATC CTG TC-3′ (reverse). The amplified DNA products were resolved on a 1% non-denaturing agarose gel, which was stained with ethidium bromide.

Cellular redox status

Intracellular peroxide production was measured using the oxidant-sensitive fluorescent probe DCFH-DA with confocal microscopy. Cells were grown at 2×10^6 cells per 100-mm plate containing slide glass coated with poly-L-lysine and maintained in the growth medium for 24 h. Cells were treated with 10 μM DCFH-DA for 15 min and cells on the slide glass were washed with PBS and a cover glass was put on the slide glass. 2′,7′-Dichlorofluorescein (DCF) fluorescence (excitation, 488 nm; emission, 520 nm) was imaged on a laser confocal scanning microscope (DM/R-TCS, Leica) coupled to a microscope (Leitz DM REB). The intracellular GSH level was determined by using a GSH-sensitive fluorescence dye CMAC. HeLa cells were incubated with 5 μM CMAC cell tracker for 30 min. The images of CMAC cell tracker fluorescence by GSH were analysed by the Zeiss Axiovert 40 CFL inverted microscope at fluorescence DAPI region (excitation 351 nm; emission 380 nm) [23]. 8-Hydroxy-2'-deoxyguanosine (8-OH-dG) levels of HeLa cells were estimated by using a fluorescent binding assay as described by Struthers et al. [24].

Mitochondrial redox status and damage

To evaluate the levels of mitochondrial ROS cells in PBS were incubated for 20 min at 37°C with 5 μM DHR 123 and cells loaded with the fluorescent probes were imaged with a fluorescence microscope. Mitochondrial membrane permeability transition (MPT) was measured by the incorporation of Rh 123 dye into the mitochondria, as previously described [25].

Assessment of apoptosis morphology

Cells were collected, washed once with ice-cold PBS, fixed with 1 ml of 4% paraformaldehyde for 20 min and washed once with ice-cold PBS. Then, the cells were incubated in 1 ml PBS containing 10 μmol/l Hoechst 33258 at 37°C for 30 min, washed twice and observed using fluorescence microscopy.

DNA fragmentation

To determine the degradation of chromosomal DNA into nucleosome-sized fragments, a 500 μl aliquot of the lysis buffer (100 mM Tris-HCl, pH 8.5, EDTA, 0.2 m NaCl, 0.2% SDS and 0.2 mg/ml proteinase K) was added to the cell pellet (2×10^5 cells) and incubated at 37°C overnight. DNA was obtained by ethanol precipitation, separated in a 0.8% agarose gel and visualized under UV light.

Cellular fractionation

To analyse the sub-cellular distribution of cytochrome c and apoptosis inducing factor (AIF), cells were fractionated into cytosolic and membrane-bound fractions using low concentrations of digitonin, which selectively permeabilizes the plasma membrane to release the cytosol. Cells were extracted with digitonin in sucrose buffer (250 mM sucrose, 75 mM NaCl, 1 mM NaH_2PO_4, 8 mM $NaHPO_4$, 1 mM PMSF, 5 μg/ml leupeptin, 21 μg/ml aporotinin, 12.5 μg/ml digitonin) to collect the cytosol. Cells were centrifuged at $10000 \times g$ for 5 min and the supernatants were immunoblotted with antibodies against cytochrome c and AIF.

Caspase activity assay

Cells were washed three times with chilled PBS, then incubated with 75 μl of lysis buffer (50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 10 mM EGTA, 10 μM digitonin, 0.5 mM PMSF) for 30 min at 37°C. Thereafter, the contents from three wells were pooled and centrifuged at $20\,000 \times g$ for 20 min at 4°C. The supernatant was mixed (1:1) with reaction buffer (100 mM HEPES, 1 mM EDTA, 10 mM DTT, 0.5 mM PMSF, 10% glycerol). The reaction began with the addition of 5 μl (5 mg/ml) of the colorimetric agent Ac-DEVD-pNA (caspase-3 substrate) and caspase activity was measured as the absorbance at 405 nM for 1 h after incubation of the mixture at 37°C. Caspase activity was calculated as (absorbance/mg of protein in treated sample)/(absorbance/mg of protein in control sample).

Quantitation of relative fluorescence

The averages of fluorescence intensity from fluorescence images were calculated as described [26].

Statistical analysis

The difference between two mean values was analysed by Student's *t*-test and was considered to be statistically significant when $p < 0.05$.

Replicates

Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

Results

We have used *in vitro*-transcribed siRNAs specific for the mRNA of IDPc for the transient transfection of HeLa cells. Both IDPc mRNA level measured by RT-PCR and IDPc protein level measured by Western blotting were significantly decreased in IDPc siRNA-transfected cells compared to scrambled siRNA-transfected control cells (Figure 1A and B). Neither the transfection procedure itself, nor the transfection of HeLa cells with a control of unspecific siRNA (scrambled siRNA), had an effect on IDPc levels. The IDPc siRNA–transfected HeLa cells exhibited ~80% less IDPc activity when compared with that of scrambled siRNA-transfected control cells. However, the activity of IDPm was not affected by the transfection of IDPc siRNA (Figure 1C). When cultured HeLa cells were treated with 200 nM staurosporine, a time-dependent decrease in cell viability was observed. However, HeLa cells transfected with IDPc siRNA were more sensitive than control cells transfected with scrambled siRNA (Figure 1D).

The effects of transfection of the IDPc siRNA on the cellular features of apoptosis were studied to determine whether these could correlate with changes in the apoptotic pathways. To observe the morphologic characteristics of apoptosis, cells were stained with Hoechst 33258 after HeLa cells were exposed to 200 nM staurosporine for 3 h and detected by fluorescence microscopy. Control cells showed even distribution of the stain and round homogeneous nuclei feature. Apoptotic cells increased in IDPc siRNA-transfected cells exposed to staurosporine and displayed typical changes, including reduction of cellular volume, staining bright and condensed or fragmented nucleus (Figure 2A). Staurosporine-triggered apoptosis in HeLa cells was also determined by the measurement of DNA fragmentation using agarose gel electrophoresis. As shown in Figure 2B, DNA fragmentation was more apparent in IDPc siRNA-transfected cells compared to control cells on the exposure to staurosporine (Figure 2B). As shown in Figure 2C, cleavage of caspase-3 and caspase-9 induced by staurosporine was more pronounced in IDPc siRNA-transfected cells. Induction of the formation of fragments which represents proteolytic cleavage of PARP and lamin B indicates an oncoming apoptotic process. The cleaved products of PARP and lamin B increased markedly in IDPc siRNA-transfected cells compared to control cells upon exposure to staurosporine. The survival signal elicited by Akt proceeds through several mechanisms including inactivation of caspase-9 and stimulation of NFκB [27]. Phosphorylated Akt (pAkt) decreased markedly in IDPc siRNA-transfected cells compared to control cells upon exposure to staurosporine. Caspase-3 activation in HeLa cells was also assessed by caspase colorimetric assay. Caspase-3 activity was significantly increased in IDPc siRNA-transfected cells as compared to control cells (Figure 2D). Taken together, staurosporine-induced the cleavage of procaspase-3 into the active form of caspase-3 and that caspase-3 induces degradation of PARP or lamin B.

To investigate whether the difference in apoptotic cell death of control and IDPc siRNA-transfected cells upon exposure to staurosporine is associated with ROS formation, the levels of intracellular peroxides in HeLa cells were evaluated by confocal microscopy with the oxidant-sensitive probe DCFH-DA. As shown in Figure 3A, an increase in DCF fluorescence

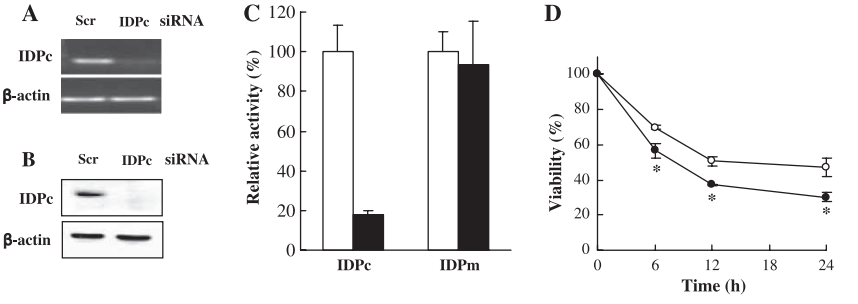

Figure 1. Knockdown of IDPc by siRNA in HeLa cells. HeLa cells were transfected with scrambled siRNA (Scr) or IDPc siRNA. After 48 h, the transfected cells were disrupted by sonication and then mRNA levels (A), protein levels (B) and the activity (C) of ICDH were determined. Open and solid bars represent HeLa cells transfected with scrambled siRNA and IDPc siRNA, respectively. Data are presented as means ± SD of three separate experiments. (D) Viability of transfectant cancer cell exposed to staurosporine. After IDPc siRNA- (*closed circles*) or control scrambled siRNA-transfected (*open circles*) HeLa cells were exposed to 200 nM staurosporine for various lengths of time at 37°C, viability of cells was determined by MTS assay. Data are presented as means ± SD of three separate experiments. * $p < 0.01$ vs control scrambled siRNA-transfected cells exposed to staurosporine.

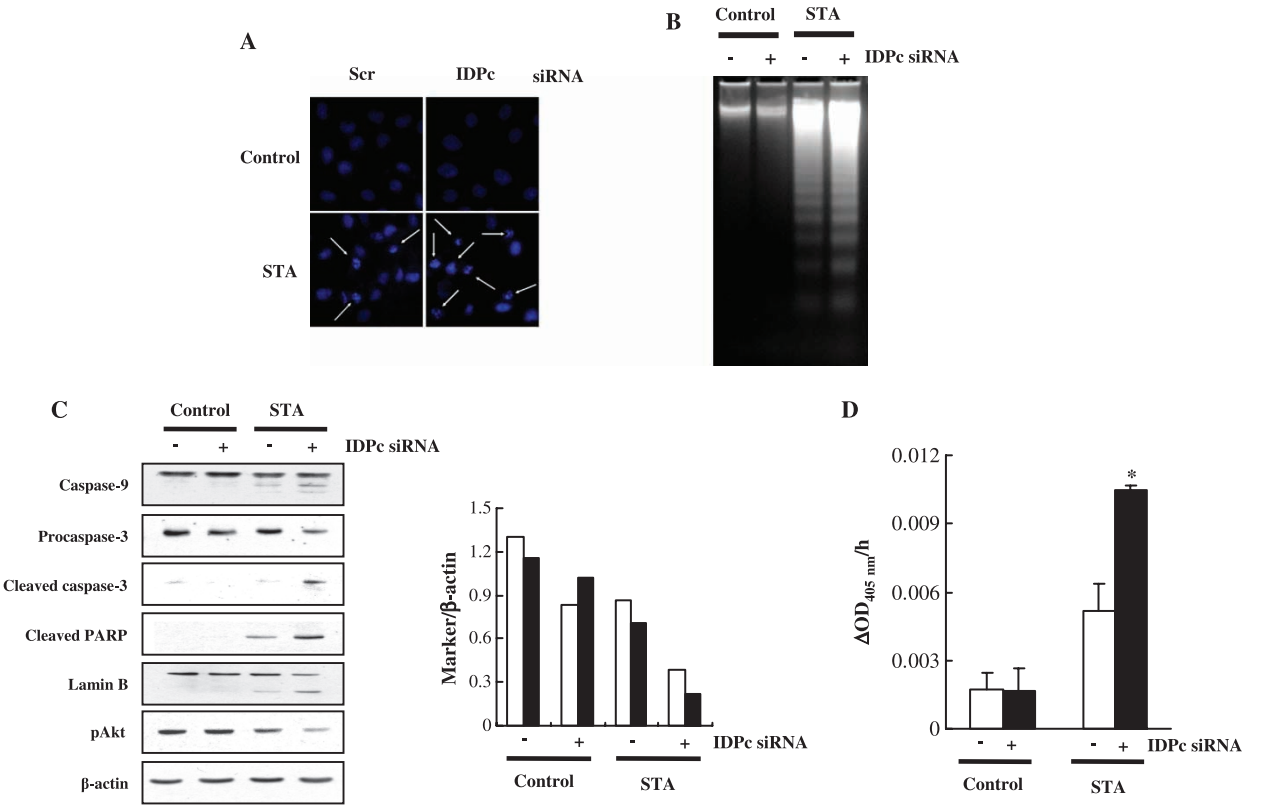

Figure 2. Staurosporine-induced apoptosis in IDPc siRNA transfectant HeLa cells. (A) HeLa cells were treated with 200 nm staurosporine for 3 h. Staurosporine-mediated morphologic changes were examined by Hoechst 33258 staining and observed under a fluorescence microscope. (B) Agarose gel electrophoresis of nuclear DNA fragments of HeLa transfectant cells exposed to staurosporine for 3 h. (C) Immunoblot analysis of various apoptosis-related proteins in HeLa transfectant cells unexposed or exposed to 200 nm staurosporine for 3 h. Cell extracts were subjected to 10–12.5% SDS-PAGE and immunoblotted with antibodies against cleaved caspase-3, caspase-9, procaspase-3, cleaved PARP, lamin B and pAkt. β-Actin was run as an internal control. Bands were analysed by densitometry and normalized to β-actin. Open and shaded bars represent procaspase-3 and pAkt, respectively. (D) Activation of caspase-3 in HeLa transfectant cells exposed to staurosporine for 3 h. HeLa cells were lysed and centrifuged. The supernatant was then added to Ac-DEVD-*p*NA and subjected to caspase colorimetric activity. Protease activity of caspase-3 was calculated by monitoring the absorbance at 405 nm. Data are presented as means $\pm$ SD of three separate experiments. * $p < 0.01$ vs control scrambled siRNA-transfected cells exposed to staurosporine.

was observed in HeLa cells when they were exposed to staurosporine and the increase of fluorescence was significantly enhanced in IDPc siRNA-transfected cells. These data strengthen the conclusion that IDPc provided protection from the staurosporine-induced apoptosis by decreasing the steady-state level of intracellular oxidants. GSH is one of the most abundant intracellular antioxidants and determination of changes in its concentration provides an alternative method of monitoring oxidative stress within cells. Cellular GSH levels determined with the GSH-sensitive fluorescent dye CMAC in U937 cells exposed to staurosporine were significantly decreased in IDPc siRNA-transfected cells as compared to that of control cells (Figure 3B). 8-OH-dG, the most abundant and most studied lesion in DNA generated by intracellular ROS, has been used as an indicator of oxidative DNA damage *in vivo* and *in vitro* [28]. Recently, it has been shown that 8-OH-dG level is specifically measured by a fluorescent binding assay using avidin-conjugated TRITC [25]. The fluorescent intensity which reflects the endogenous levels of 8-OH-dG in DNA was significantly increased in IDPc siRNA-transfected cells as compared to that of control cells exposed to staurosporine (Figure 3C). The significance of redox status in staurosporine-induced apoptosis was further confirmed by the blocking effect of NAC, a scavenger of ROS on the modulation of apoptotic marker proteins (Figure 3D).

Alterations in mitochondrial integrity and function may play an important role in the apoptotic cascade. MPT, associated with the opening of large pores in the mitochondrial membranes, is a very important event in apoptosis, and ROS is one of the major stimuli that change MPT [29]. To answer whether IDPc modulates the MPT upon exposure to staurosporine, we determined the change in MPT by intensity of fluorescence emitting from a lipophilic cation dye, Rh 123. High fluorescence represents high $\Delta\psi$m, which correlates with healthy mitochondria. Significantly less Rh 123 dye was taken up by the mitochondria of IDPc siRNA-transfected cells, compared with

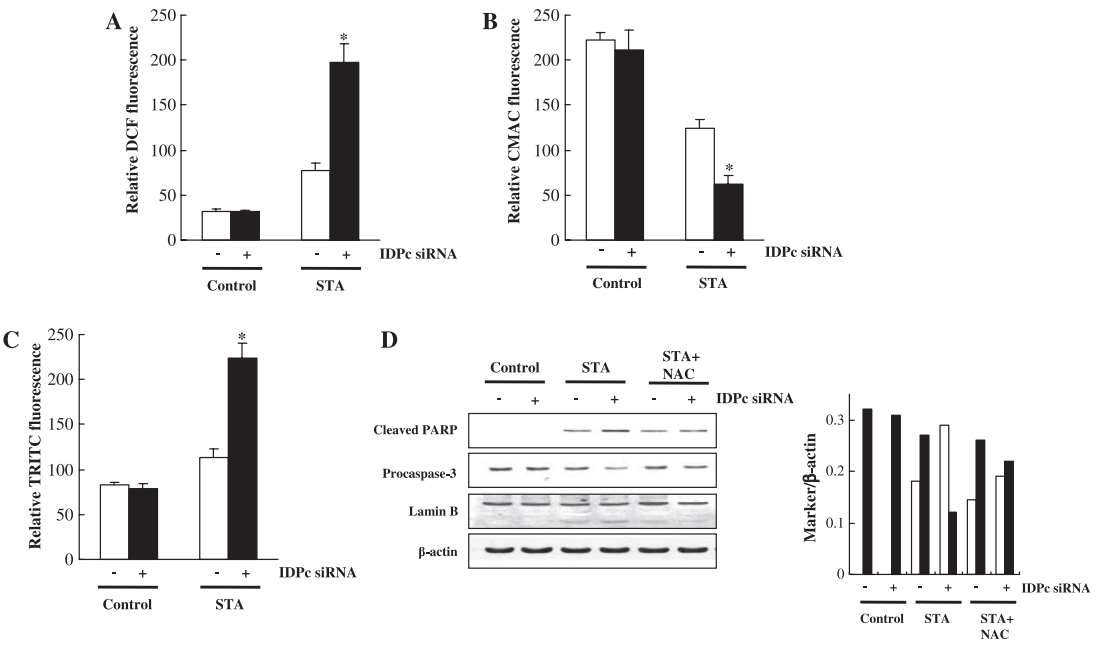

Figure 3. Cellular redox status and oxidative DNA damage in IDPc siRNA transfectant HeLa cells. (A) Measurement of *in vivo* molecular oxidation. DCF fluorescence was measured in HeLa transfectant cells exposed to staurosporine by confocal microscopy. (B) Effect of IDPc siRNA transfection on GSH levels. Fluorescence image of CMAC-loaded cells was obtained under microscopy. (C) 8-OH-dG levels reflected by the binding of avidin-TRITC were visualized by a fluorescence microscope with 488 nm excitation and 580 nm emission. (A–C) The averages of fluorescence intensity were calculated as described [27]. Data are presented as means ± SD of three separate experiments. * $p < 0.01$ vs control scrambled siRNA-transfected cells exposed to staurosporine. (D) IDPc siRNA transfected HeLa cells pre-incubated for 2 h in the presence or absence of 10 mm NAC were induced to undergo apoptosis by treatment with staurosporine. Cell extracts were subjected to 10–12.5% SDS-PAGE and immunoblotted with antibodies against procaspase-3, cleaved PARP and lamin B. β-Actin was run as an internal control. Bands were analysed by densitometry and normalized to β-actin. Open and shaded bars represent cleaved PARP and procaspase-3, respectively.

control cells (Figure 4A). To determine if changes in MPT were accompanied by changes in ROS, the levels of peroxides in the mitochondria of HeLa cells were evaluated by confocal microscopy with the oxidant-sensitive probe DHR 123. As shown in Figure 4B, the intensity of fluorescence was significantly lower in control cells compared to that in the mitochondria of IDPc siRNA-transfected cells when HeLa cells were exposed to staurosporine. In order to verify that IDPc siRNA-induced enhancing effects on mitochondrial dysfunction was due to increase in mitochondria-derived ROS we employed MnSOD mimetic, MnTMPyP. As shown in Figure 4A and B, MnTMPyP efficiently suppressed changes in the MPT and the level of ROS in mitochondria. These results indicate that staurosporine most likely leads to increased mitochondrial injury while IDPc protects mitochondria from oxidative damage. We examined whether IDPc siRNA induces cell death by modulating expression of the Bcl-2 family, which ultimately determine the cellular response to apoptotic stimuli. As shown in Figure 4C, the abundance of pro-apoptotic Bax in mitochondria of HeLa cells was significantly increased in IDPc siRNA-transfected cells as compared to that of control cells when exposed to staurosporine. It has been reported that Bid, a death agonist member of the Bcl-2/BclX_L family, is a specific proximal substrate of caspase-8 in the Fas signalling pathway [30]. When cells were exposed to staurosporine, the cleavege of Bid were enhanced in IDPc siRNA-transfected cells compared with control cells. The role of the mitochondrial pathway of apoptosis in the staurosporine-induced death of HeLa cells was evaluated by immunoblot analysis of released cytochrome c. Mitochondria play an important role in the regulation of apoptosis by releasing apoptogenic molecules that include cytochrome c [31]. The abundance of cytochrome c in cytosol of HeLa cells was significantly increased in IDPc siRNA-transfected cells as compared to that of control cells when exposed to staurosporine. The release of AIF from mitochondria to nucleus was also significantly increased in IDPc siRNA-transfected cells.

Discussion

When cells are grown in air, NADPH must be used to maintain a level of GSH as well as reduced thioredoxin to combat oxidative damage. Glutathione reductase converts GSSG to GSH in the cell using NADPH as a reductant [12]. The oxidized form of thioredoxin, with a disulphide bridge between the half-cystines, is reduced by NADPH in the presence of a flavoprotein, thioredoxin reductase [32]. Reduced thioredoxin may provide reducing equivalents

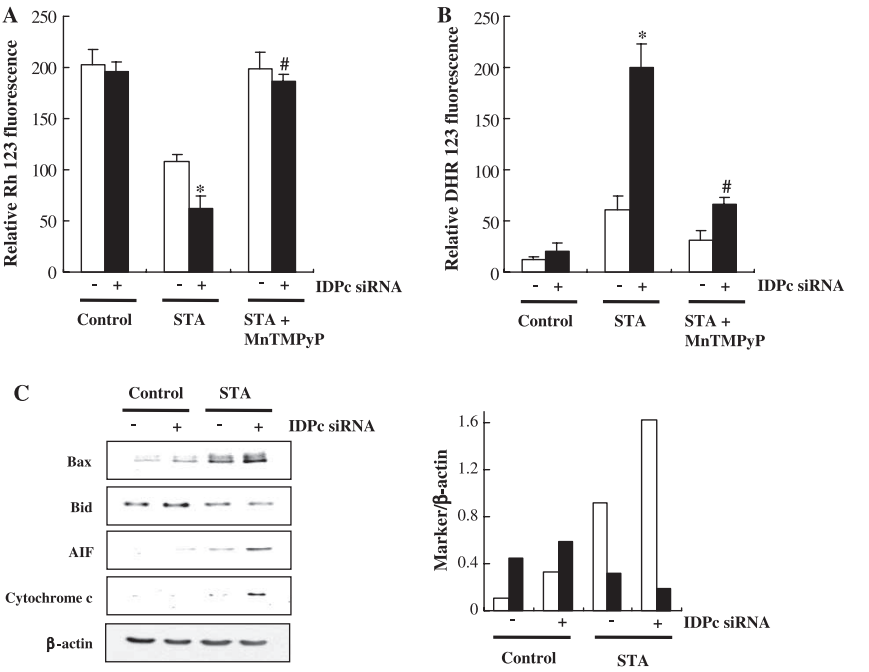

Figure 4. Effects of IDPc siRNA on mitochondrial dysfunction and mitochondrial redox status of HeLa transfectant cells exposed to staurosporine. (A) Effect of IDPc siRNA on MPT. MPT of HeLa transfectant cells was measured by the incorporation of Rh 123 dye into the mitochondria. (B) Effect of IDPc on mitochondrial ROS generation. DHR 123 was employed to detect mitochondrial ROS. Fluorescence was measured by confocal microscopy. (A–B) IDPc siRNA transfected HeLa cells pre-incubated for 2 h in the presence or absence of 5 μM MnTMPyP were treated with 200 nm staurosporine for 3 h. The averages of fluorescence intensity were calculated as described [27]. Data are presented as means ± SD of three separate experiments. * $p < 0.01$ vs control scrambled siRNA-transfected cells exposed to staurosporine. # $p < 0.01$ vs IDPc siRNA-transfected cells exposed to staurosporine in the absence of MnTMPyP. (C) Immunoblot analysis of Bax, Bid, AIF and cytochrome c in HeLa transfectant cells unexposed or exposed to 200 nm staurosporine for 3 h. β-Actin was run as an internal control. Bands were analysed by densitometry and normalized to β-actin. Open and shaded bars represent Bax and Bid, respectively.

to several enzymes including thioredoxin peroxidases and methionine sulphoxide reductase, presumably involving the defense against oxidative stress. Recently, a family of antioxidant proteins from eucaryotes and procaryotes are purified and these proteins remove hydrogen peroxide using hydrogen provided by the NADPH-dependent thioredoxin system and thus are called thioredoxin peroxidases [12,33]. Reduced thioredoxin may also provide reducing power to methionine sulphoxide reductase which can reactivate proteins damaged by previous oxidation of their methionine residues [34]. Glucose 6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme of the pentose phosphate pathway, has long been regarded as the major enzyme to generate NADPH. In fact, the role of G6PD in the cell response to oxidative stress is well established in yeast, in human erythrocytes and in the mouse embryonic stem cells [35–37]. However, two other $NADP^+$-linked dehydrogenases, malic enzyme and IDPc, are also responsible for the generation of cytosolic NADPH [38]. Earlier study indicated that IDPc in the rat liver was 16- and 18-fold more active in producing NADPH than G6PD and malic enzyme, respectively [39], suggesting an important role of IDPc in the production of NADPH and eventually for the cellular defense against oxidative stress. Recently, IDPc that is preferentially expressed in bovine corneal epithelium has been identified. The role of this enzyme in contributing to corneal transparency is likely attributed to its protective effect against UV radiation [40]. We also demonstrated that the control of cytosolic redox balance and oxidative damage is one of the primary functions of IDPc [14].

The aim of the present work was to evaluate the role of IDPc siRNA in sensitizing HeLa cells from various anti-cancer agents in regards to apoptotic cell death, cellular redox status, mitochondrial dysfunction and oxidative damage to cells. In the present study, events reflecting staurosporine-induced apoptosis, such as the elevation of ROS level, MPT alteration, caspase-3 activation and DNA fragmentation, were observed. The suppression of IDPc expression by siRNA significantly increased ROS level and enhanced the whole apoptotic pathway.

The involvement of mitochondria in apoptosis has been extensively discussed [31]. It is well established that mitochondrial dysfunction is directly and indirectly involved in cell death including apoptosis and a variety of pathological states [31]. All the changes caused by staurosporine are compatible with mitochondrial failure, generation of ROS and accumulation of Rh 123 which reflect mitochondrial swelling or changes in the mitochondrial inner membrane.

A clear enhancement of such damages in IDPc siRNA-transfected cells compared to control cells exacerbates a deterioration of bioenergetic state.

Chemotherapy plays a major role in the control of malignant tumours. However, resistance of many tumours to consecutive administration of anti-cancer drugs constitutes a major problem in the cancer therapy. Improvements in the therapeutic index depend on enhancement of tumour response to anti-cancer drugs. This study indicates that IDPc siRNA enhances the susceptibility of anti-cancer drug-induced apoptosis through inhibiting IDPc activity and subsequently modulating the mitochondrial redox status and mitochondrial function. The observed effects of IDPc siRNA in HeLa cells offer the possibility of developing a modifier of chemotherapy involving a variety of anti-cancer drugs to elicit ROS formation.

In conclusion, the present study demonstrates that IDPc abrogates the anti-cancer agent-induced early production of ROS, leading to protection against apoptotic cell death. In this regard, IDPc siRNA could play a role as a sensitizer in tumour chemotherapy.

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