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Role of a novel dual flavin reductase (NR1) and an associated histidine triad protein (DCS-1) in menadione-induced cytotoxicity ☆

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Abstract

Microsomal cytochrome P450 reductase catalyzes the one-electron transfer from NADPH via FAD and FMN to various electron acceptors, such as cytochrome P450s or to some anti-cancer quinone drugs. This results in generation of free radicals and toxic oxygen metabolites, which can contribute to the cytotoxicity of these compounds. Recently, a cytosolic NADPH-dependent flavin reductase, NR1, has been described which is highly homologous to the microsomal cytochrome P450 reductase. In this study, we show that over-expression of NR1 in human embryonic kidney cells enhances the cytotoxic action of the model quinone, menadione. Furthermore, we show that a novel human histidine triad protein DCS-1, which is expressed together with NR1 in many tissues, can significantly reduce menadione-induced cytotoxicity in these cells. We also show that DCS-1 binds NF1 and directly modulates its activity. These results suggest that NR1 may play a role in carcinogenicity and cell death associated with one-electron reductions.

Keywords: NADPH-dependent flavin reductase; Microsomal cytochrome P450 reductase; Flavoproteins

Flavoproteins are components of the electron transport pathways in living systems involved in such important processes as respiration, photosynthesis, signal transduction, detoxification, and metabolism. NADPH-cytochrome P450 reductase (CPR) is a member of the flavoprotein family present in plants, yeast, and animals where it is associated with the endoplasmic reticulum membrane [1,2]. CPR works together with other membrane-associated enzymes to metabolize a variety of substrates. Its main function is the transfer of electrons from NADPH via FAD and FMN cofactors to cytochrome P450 isoenzymes [3,4] as well as to other heme-containing enzymes such as heme oxygenase [5], cytochrome *c*, and cytochrome *b*5 [6]. It also plays critical roles in the bioactivation of one-electron acceptors such as the therapeutically important anticancer agents mitomycin c or tirapazamine [7–11]. Nitric oxide synthase (NOS) appears to have evolved from the fusion of an NADPH-reductase domain with a heme-containing oxidase [12]. NOS can also catalyze the activation of redox cycling and bioreductive anticancer agents including menadione [13–15], and this requires only the soluble reductase domain of NOS [16]. NR1 is a recently described cytosolic NADPH-dependent flavin reductase, which shows a high homology to CPR and the reductase domain of NOS [17,18] (Fig. 1). NR1 is expressed in a variety of cancer cell lines and human tissues, including kidney, placenta, and brain [17,19]. Like CPR, NR1 can catalyze the activation of the one-electron acceptors doxorubicin, potassium ferricyanide, and menadione [17].

In *Caenorhabditis elegans*, the NR1 homologue, FRE-1, is encoded in a two-gene operon together with a novel histidine triad protein, DCS-1 [19]. Since proteins encoded polycistronically often functionally interact, we identified a human DCS-1 and show that it is coordinately expressed with NR1 in both worm and human tissues [19]. These

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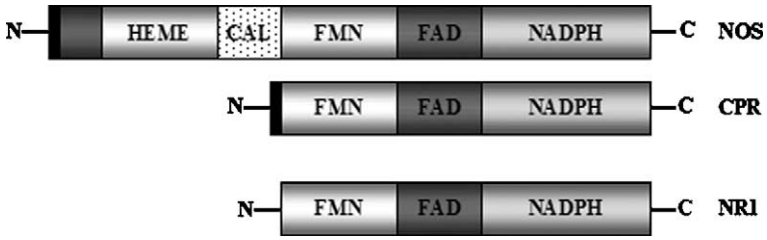

Fig. 1. Schematic representation of the primary structure of NOS (nitric oxide synthase), CPR (microsomal P450 reductase), and NR1 (NADPH-dependent flavin reductase). Consensus binding sites for NADPH, FAD, and FMN are conserved between all three proteins.

observations indicate that NR1 and DCS-1 are highly conserved proteins, which may play a role in cellular stress.

CPR is an important enzyme for the cellular metabolism of quinone anticancer drugs [20]. One-electron reductive activation of quinones by enzymes such as CPR generates semiquinones that can produce harmful oxyradicals [21]. The use of heterologously expressed reductases has important implications for studies of drug metabolism and drug development [22]. The heterologous expression of CPR in cell lines increases their sensitivity to quinone-type cytotoxins such as menadione [23]. In this study, we developed human embryonic kidney cells which over-express NR1 and examined their response to the model quinone, menadione. Our results show that a treatment of HEK cells stably expressing NR1 with menadione leads to increased cytotoxicity and cell death. Furthermore, we show that coexpression of DCS-1 can significantly protect NR1 expressing cells against the cytotoxicity of menadione. NR1 is expressed in a variety of cancer cell lines and may yield a useful target for the development of bioreductive anticancer drugs.

Materials and methods

Cell culture and cell lines. HEK 7X cell lines were obtained from the American Tissue Culture Collection and were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) with 5% FBS and in DMEM containing 800 μg/ml geniticin (G418, Life Technologies) as a selection drug used for the stable line. Stable and transient cell lines were used. Cells were transfected with FLAG-tagged NR1 and 6-His-tagged DCS-1 constructs in pCEP4 vector [19] using the LipofectAMINE reagent (Life Technologies) and incubated at 37 °C humidified incubator with 5% CO_2. As a control for transfection efficiency cells were transfected with β-galactosidase reporter gene in pCEP4 vector. First, HEK 7X cells were transfected with NR1 and a stable population of cells was established after approximately 4 weeks.

Cytotoxicity assays. We performed three types of cell transfections in 10 mm dishes: (1) stable cells over-expressing NR1 transfected with the β-galactosidase reporter gene, (2) stable cells over-expressing NR1 transfected with DCS-1 constructs, and (3) cells expressing endogenous levels of NR1 transfected with the β-galactosidase as a control. Cells were allowed 24 h for protein expression and then were split into 24-well plates to obtain equal transfection efficiency in each well. The transfection efficiency was 80–90% in every trial transfection as determined using β-galactosidase staining and immunodetection analysis against anti-His monoclonal primary antibodies for DCS-1 detection. All cells were incubated in MEM without the G418 for 48 h prior to incubation with menadione. Cells were cultured on coverslips for 6, 18, and 24 h with medium containing various concentrations of menadione (MD) (0, 25, 50, 75, and 100 μM). After various incubation periods (6–24 h), cells were fixed in 4% paraformaldehyde, pH 7.3, for 1 h, washed several times with PBS, and stained with Hoechst 33258 dye (Molecular Probes) for 10 min at 4 °C. SlowFade Antifade reagent (Molecular Probes) was applied and coverslips were sealed on a microscope slide. 1000 cells were scored blindly per slide under fluorescence at 356 nm using a 40× Plan-NEOFLUAR lens on a Zeiss Axiophot microscope. Cells were scored as apoptotic only if they display a globular, brightly fluorescent nucleus, typical of chromatin condensation.

To measure the cytotoxicity in response to menadione, we used the Cytotoxicity Detection Kit (Roche Molecular Biochemicals). Release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was measured in cell culture supernatant. We used DMEM (as described above) without phenol red to prevent interference with the assay. The three cell transfections chosen for this experiment are described above. First, 10 mm plates were transfected and then split after 24 h into 24-well plates on coverslips at 5×10^4 cells/well. The efficiency of transfections was tested by β-galactosidase and immunocytochemistry staining using specific antibodies. We allowed 12 h for the cells to recover from splitting and then treated them with various concentrations of menadione (0, 25, 50, 75, and 100 μM). Toxic concentrations were determined before this experiment using a wide range of menadione concentrations. After various incubation periods (6–24 h), cell-free culture supernatant was collected and the amount of LDH released from damaged cells was measured (Cytotoxicity Detection Kit, Roche) in microtiter plate reader (Labsystems Multiskan Ascent) with a 492 nm absorbance filter. The cells remaining on coverslips were fixed in 4% paraformaldehyde, pH 7.3, for 1 h and stained as described above.

To calculate the percent of cytotoxicity, the following controls were performed in each experiment setup: (1) background control, containing the assay medium, provides information about LDH activity in the medium. (2) Low control, containing untreated cells, and (3) high control, cells treated with 2% Triton X-100 solution in assay medium for maximum release of LDH. Additional controls included: (1) different concentrations of menadione were added to the assay medium without cells; there was no change in absorbance among the wells, (2) to test for possible interference by detergent, and to determine if an equal number of cells were distributed among each well (5×10^4 cells/well), 2% Triton X-100 was added to each concentrations of menadione used in the assay; the absorbance was equal in each well, which indicates that the cells were in fact plated out equally, (3) we performed the assay using various concentration of menadione with 5% and 2.5% FBS with no cells present, and noticed no change in absorbance among the wells. We concluded that neither the menadione, Triton X-100 nor FBS interfered with the assay.

The assay was performed in three trials (from three separate transfections). In each trial, the average absorbance of at least three measurements from each well was taken and the percentage cytotoxicity was determined by using the following equation:

$$\text{Cytotoxicity } (\%) = [(\text{exp. value} - \text{low control})/(\text{high control} - \text{low control})] \times 100.$$

The average percentage cytotoxicity from all three trials was plotted.

β-Galactosidase staining. HEK 7X cells were fixed for 5 min, washed in PBS, and incubated in a solution of PBS containing 0.1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase), 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, and $K_4Fe(CN)_6$. Cells were incubated at 37 °C for 15 min.

Statistical analyses. Where applicable, data are presented as means ± the standard error of mean of the trials. Statistical analysis was performed between stable line and control groups ($n \geqslant 2$ for both control group and stable line). The data were subjected to one-way analysis of variance (ANOVA) followed by Tukey test to determine which means were significantly different from each other with $p < 0.05$.

Immunoprecipitation. Cells transfected with FLAG-tagged NR1 were lysed by the addition of 500 μl cold 1× lysis buffer (50 mM Hepes, pH 7.2, 250 mM NaCl, 10% glycerol, 2 mM EDTA, 0.1% NP-40, and 0.25 μM phenylmethylsulfonyl fluoride) containing 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 mM PMSF, pH 7.4, and 1% NP-40 for 20 min at 4 °C with agitation. Supernatant was collected after 15 min centrifugation at high speed at 4 °C. Recovered supernatants were incubated with 10 μg of anti-DCS-1 pre-bound to 80 μl of protein A–Sepharose beads slurry and incubated with agitation overnight at 4 °C. Recovered immune complexes were washed twice with lysis buffer containing 1% NP-40 and twice with lysis buffer without NP-40 and boiled in 40 μl SDS sample buffer to extract immunoprecipitated proteins. Beads were collected between washes by centrifugation at 1200 rpm for 2 min at 4 °C.

Western blot analysis. Forty micrograms of protein was loaded in each lane, separated on SDS–PAGE gel, and transferred to nitrocellulose membranes Hybond-C extra (Amersham Pharmacia). Then membranes were washed twice with TBS (25 mM Tris, 0.8% NaCl, and 0.02% KCl, pH 7.6). Non-specific bindings were blocked in blocking solution (5% non-fat milk in TBS containing 0.05% Tween 20) for 2 h. Membranes were rinsed three times in TBS, followed by six time washes in TBS-T, 10 min each. Membranes were incubated overnight at 4 °C with primary mouse anti-Flag antibodies, rabbit anti-DCS-1 (Affinity Bioreagents), and polyclonal rabbit anti-rat HO-1 antibodies (SantaCruz Biotechnology). Then, the membranes were incubated with species specific secondary antibodies (linked to horseradish peroxidase) (1:5000 dilution) in 2% non-fat milk in TBS-T for 1 h at room temperature. Membranes were washed six times in TBS-T incubated with ECL reagents (Amersham Pharmacia) and exposed to X-ray films (Kodak) to visualize protein bands.

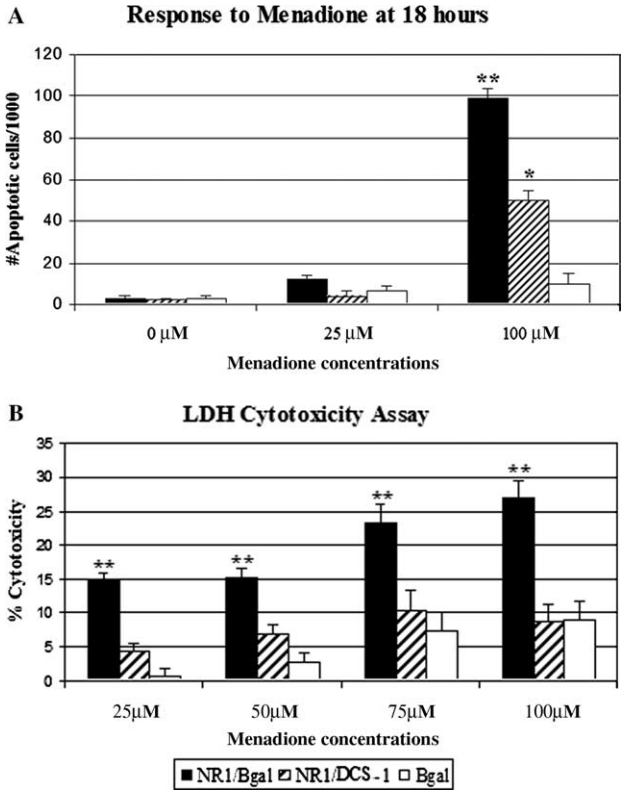

Fig. 2. Determination of cell death in HEK 7X cells expressing NR1. HEK 7X cells were seeded at a concentration 5×10^4 cells/well and incubated at 37 °C in 5% CO_2, 90% humidity and incubated with various concentrations of menadione (MD) for 18 h. (A) Apoptotic nuclei were visualized with Hoechst 33342. Cells (1000) per slide were scored blind, and a significantly higher number of apoptotic nuclei were observed after 18 h incubation with 100 μM MD, compared with control cells not expressing NR1. (B) Colorimetric assay for the quantification of cell death and cell lysis, based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant. An increased cytotoxicity was observed in the stable line over-expressing NR1 and statistically significant suppression of that cytotoxicity was observed in cells co-expressed with DCS-1. Cells used: (1) NR1/β-Gal., stable cells over-expressing NR1 transfected with the β-galactosidase reporter gene (2) NR1/DcpS, stable cells over-expressing NR1 transfected with DCS-1 constructs, (3) β-Gal., HEK 7X cells expressing endogenous levels of NR1 transfected with the β-galactosidase as a control ($n = 3$ different transfections, $p < 0.05$).

Results

Cooperative action of NR1 and DCS-1

In this study, we used menadione as a model quinone and examined its cytotoxicity in stable HEK cell lines over-expressing NR1. The cytotoxicity of menadione was compared between HEK cells stably expressing NR1 transfected with: (1) the β-galactosidase reporter gene or (2) DCS-1. Additionally, HEK cells expressing endogenous levels of NR1 were transfected with β-galactosidase as a control. Our results show that menadione induced cell death in cells expressing NR1, as indicated by an increased toxicity to 100 μM concentration of MD after 18 h of exposure, compared to controls (Fig. 2A). A high number of condensed and fragmented nuclei were observed in stable line expressing NR1 exposed to 100 μM MD, whereas the same stable line co-transfected with the DCS-1 showed a statistically significant decrease in the number of apoptotic nuclei. 10 ± 5 of control cells showed apoptotic nuclei, while 100 ± 5 of the NR1 expressing cells, and 50 ± 5 of NR1 and DCS-1 expressing cells showed picnotic nuclei. This was also apparent by microscopic examination of cells. Cell detachment from the dishes and overall change of cell phenotype were observed in the stable cells expressing only NR1 after exposure to menadione, whereas the same cells transfected with DCS-1 were healthy and differentiated, similar to control cells (Fig. 3).

The immunofluorescent analysis of apoptosis was confirmed using a quantitative cytotoxicity analysis, determined by measurement of lactate dehydrogenase (LDH) activity released from damaged cells. An increase in the amount of dead or plasma membrane-damaged cells results in an increase in the amount of the LDH enzyme activity in the culture supernatant. The results show that menadione was highly toxic to NR1 expressing cells and this increased with increasing concentrations of the drug (Fig. 2B). At 100 μM concentration, for example, the cytotoxicity reached $27\% \pm 3$ as compared to the controls at $8.6\% \pm 3$. A significant decrease in measured LDH level

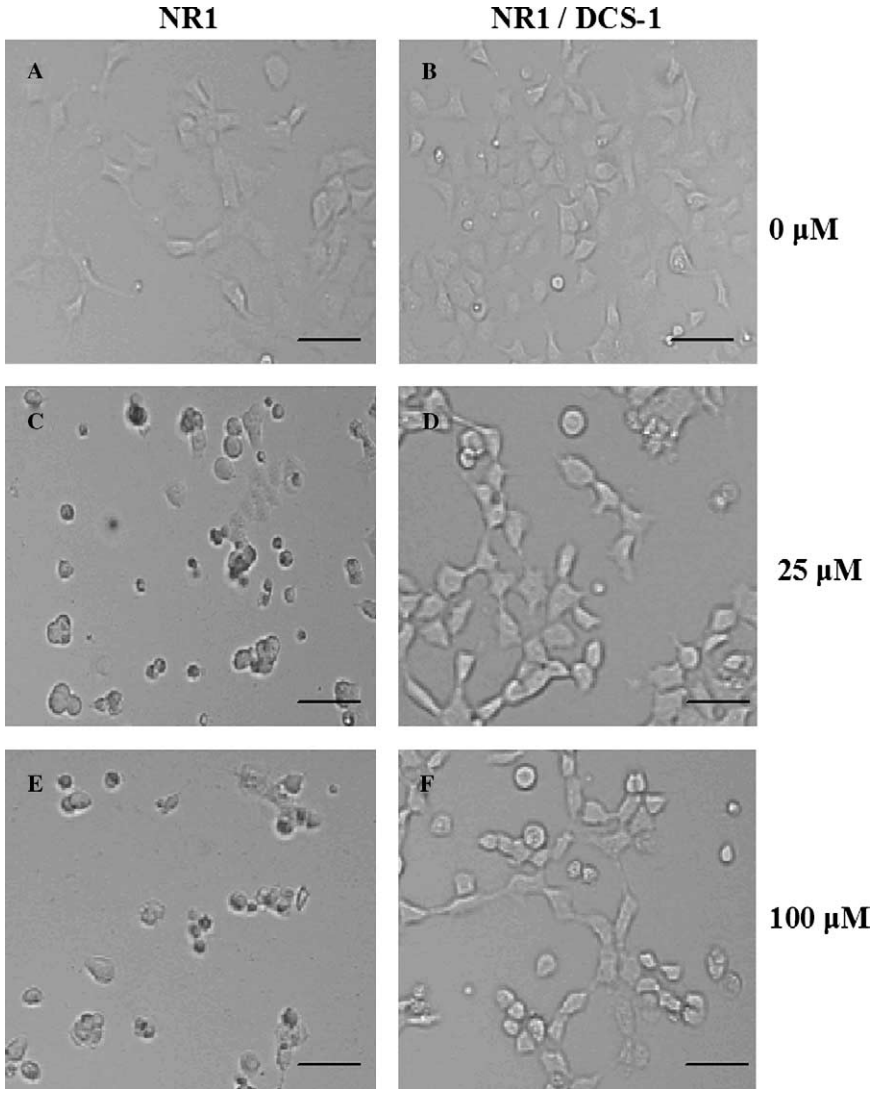

Fig. 3. Effect of various concentrations of menadione on phenotype of HEK 7X cells stably expressing NR1. Stable cells were transfected with β-galactosidase gene in pCEP4 (left panel A,C,E) and with DCS-1 cDNA in pCEP4 (right panel B,D,F) using LipofectAMINE method. Cells were incubated in DMEM for approximately 48 h at 37 °C and 5% CO_2 for the protein expression. Cells were exposed to various concentrations of menadione for 18 h. Cells expressing NR1 also show a higher number of shrivelled and rounded cells, whereas cells expressing both NR1 and DCS-1 were more abundant in number and more differentiated. Scale bar is 50 μm.

was observed when NR1 expressing cells were compared with cells expressing both NR1 and DCS-1 after exposure to increased doses of menadione. The cytotoxicity in cells expressing both proteins was $9\% \pm 3$. In summary, these results show that one-electron reduction of menadione by NR1 elicits cell death in a dose and time dependent manner and that co-expression of DCS-1 significantly suppresses this process.

Increase of NF1 and DCS-1 cytosolic levels after exposure to menadione

Western blot analysis was used to determine cytosolic levels of NR1 and DCS-1 after exposure to various concentrations of menadione. Untransfected HEK cells were subjected to various concentrations of the drug for 3 and 6 h, and cytosolic fractions were used for the Western blot. Membranes were probed with anti-NR1 and anti-DCS-1 affinity purified antibodies. In addition, heme oxygenase 1 (HO-1) antibodies were used as a control of oxidative stress and anti-actin antibodies to control for equal amounts of protein loaded in each well. The results showed no change in NR1 and DCS-1 levels and no detection of HO-1 after 3 h of exposure to menadione (data not shown). We observed a dose dependent increase in NR1, DCS-1, and HO-1 level after 6 h of exposure to the drug (Fig. 4). The above results show that (i) menadione can induce NR1 and DCS-1 level in HEK cells in a time and dose dependent manner, (ii) the reduction of menadione causes cell death in NR1 expressing HEK cells, and that (iii) DCS-1 might have a protective role against cytotoxic effects of the drug.

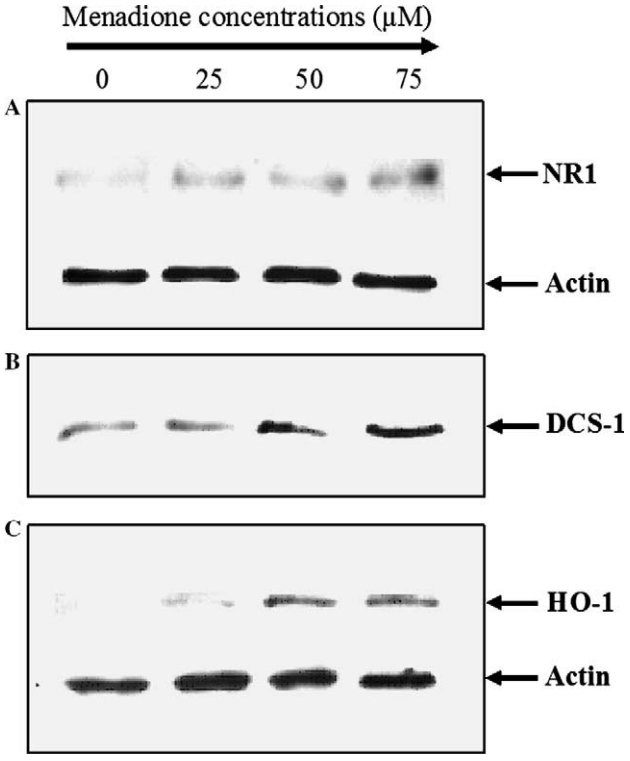

Fig. 4. Western blot analysis of NR1 and DCS-1 in cytosolic fractions. Expression of NR1 and DCS-1 was compared in cytosolic fractions from untransfected HEK 7X cells after incubation with various concentrations of menadione (MD) for 6 h. The cells were lysed and separated by 8.5% SDS–polyacrylamide gel electrophoresis, and immunoblotted using antibodies as indicated. Each well contains 40 μg of protein. Expression of both NR1 and DCS-1 increased with increased dose of MD. Blots were first probed with anti-NR1 IgG and rabbit anti-actin IgG (A) and then reprobed with rabbit anti-DCS-1 (B). Rabbit anti-HO-1 IgG was also used on the same samples as a control of the oxidative stress (C). Expression of NR1 and DCS-1 was normalized on actin expression in the same lane as indicated. The same results were obtained in at least three separate treatments.

Interaction between NR1 and DCS-1

A potential interaction between NF1 and DCS-1 was analyzed using immunoprecipitation assay. HEK cells were transiently transfected with Flag-NR1 and DCS-1 constructs, and cell extracts were immunoprecipitated with affinity purified anti-DCS-1 antibodies (Fig. 5). DCS-1-immunoreactive band was detected in cells transfected with Flag-NR1 plus DCS-1 or β-galactosidase control. In cells transfected with Flag-NR1 plus DCS-1, a band corresponding to NR1 (see crude lysates) was detected with the anti-Flag antibody following immunoprecipitation with the DCS-1 antibody. This band was not seen in immunoprecipitates from cells transfected with the β-galactosidase control construct. These results demonstrate that DCS-1 can physically associate with NR1 and potentially modulate its activity.

Discussion

We have isolated a novel human NADPH-dependent flavin reductase, NR1, from a variety of human tissues including kidney, placenta, and brain. This is a human ortholog of *C. elegans fre-1* [19]. Previous study shows that NR1 is also expressed in various cancer cell lines [17]. NR1 belongs to a family of flavin-containing proteins and shows strong sequence conservation in the regions shown to be involved in FMN, FAD, and NADPH cofactor binding. The N-terminus of human NADPH-cytochrome P450 reductase comprises a hydrophobic domain, which targets the enzyme to the membrane of the endoplasmic reticulum [24]. In contrast, the N-terminus of the NR1 flavin reductase lacks this hydrophobic domain, consistent with a cytosolic localization of this enzyme. NR1 would thus be unlikely to utilize the hydrophobic cytochrome P450s as substrates.

In *C. elegans,* the NR1 homolog is expressed from a two-gene operon together with a novel histidine triad protein, DCS-1 [19]. Human DCS-1 is a 336 amino acid protein with a predicted molecular mass of 37 kDa. It belongs to the histidine triad (HIT) superfamily named for the HIT motif, containing seven conserved amino acids with three His residues, His-ϕ-His-ϕ-His-ϕ-ϕ (ϕ is a hydrophobic amino acid) [25,26]. This HIT family of proteins is highly conserved in nature, but their physiological role remains puzzling. Recently, DCS-1 has been identified as the scavenger decapping enzyme which plays an important role in RNA processing [19,27]. DCS-1 is co-expressed together with NR1 in a variety of human tissues [19]. Our study performed on HEK cells further suggests a functional cooperation between DCS-1 and NR1.

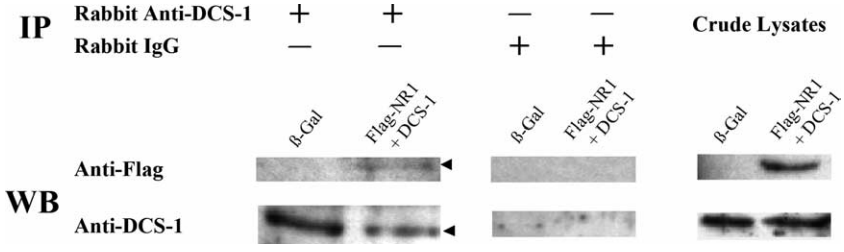

Fig. 5. Co-immunoprecipitation of DCS-1 and NR1. DCS-1 immunoprecipitates with Flag-NR1. HEK cells were transfected with β-galactosidase control gene and Flag-NR1 with DCS-1 cDNAs in pCEP4 mammalian expression vector, and immunoprecipitated (IP) with affinity purified anti-DCS-1 IgG. Proteins were separated by SDS–PAGE, blotted and probed with rabbit anti-Flag and rabbit anti-DCS-1 (WB). Arrows indicate NR1 and DCS-1 immunoreactive bands (left panel). No immunoprecipitation of NR1 was detected when immunoprecipitation was performed with a control rabbit IgG antibody (middle panel). The right panel illustrates that high levels of both NR1 and DCS-1 are present in crude lysates.

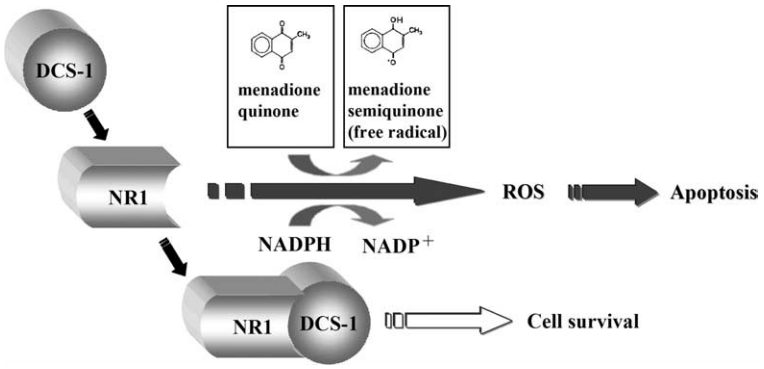

Fig. 6. Proposed model of NF1 and DCS-1 cooperative regulation. NF1 transfers electrons provided by NADPH via FAD and FMN to menadione. One-electron reduction of menadione results in the formation of free radicals (semiquinone) and reactive oxygen species (ROS) leading to cell death. DCS-1 association with NF1 prevents the formation of ROS and promotes cell survival.

In this study, we use an artificial electron acceptor menadione, a model quinone. The data presented here show that menadione induced NR1 and DCS-1 level in a time and dose dependent manner in HEK cells over-expressing NR1 and consequently induced cell death, as indicated by an increased toxicity to 100 μM concentration of menadione. Cell detachment from the dishes and overall change of cell phenotype were observed in these cells. Cells over-expressing both NR1 and DCS-1 showed a statistically significant decrease in the number of apoptotic nuclei compared with cells expressing NR1 and β-galactosidase. Quantitative measurement of LDH activity, which is released from damaged cells, showed high levels in NR1 expressing cells exposed to a variety of menadione concentrations. A significant decrease in measured LDH level was observed in the presence of DCS-1. This suggests that DCS-1 negatively regulates NR1 activity.

Furthermore, our immunoprecipitation analysis showed that DCS-1 physically binds NR1. The interaction between NR1 and DCS-1 is interesting in the light of recent reports of similar HIT proteins modulating functions of other proteins by a direct association. It has been shown that the yeast trehalase-associated protein YLR270Wp, which is a HIT protein homologous to DCS-1, can regulate neutral trehalase activity [28]. Hint, another member of HIT family, interacts with cyclin-dependent kinase 7 [29]. Protein kinase C-interacting protein 1 (PKCI), also a member of the HIT protein family, associates with and negatively regulates the microphthalmia transcription factor *mi* that plays a major role in cell proliferation and differentiation [30]. PKCI also interacts and co-localizes at the nucleus with ATDC (ataxia telangiectasia group D-complementing) gene product [31]. Our study shows that the HIT protein DCS-1 can physically bind and directly modulate NR1 activity (Fig. 6). This interaction may be important in regulating drug metabolism and activation of environmental chemicals. NR1 is expressed in a variety of cancer cell lines; therefore, the potential of these proteins as therapeutic drug targets should be addressed.

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