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The Antioxidant Role of a Reagent, 2′,7′-Dichlorodihydrofluorescin Diacetate, Detecting Reactive-Oxygen Species and Blocking the Induction of Heme Oxygenase-1 and Preventing Cytotoxicity

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Heme oxygenase-1 (HO-1) degrades heme into biliverdin, iron and CO. The enzyme participates in adaptive and protective responses to oxidative stress and various inflammatory stimuli, and is induced in response to reactive oxygen species (ROS). 2′,7′-Dichlorodihydrofluorescin diacetate (DCFH-DA) is a common reagent used to detect ROS by the oxidation of 2′,7′-dichlorodihydrofluorescin (DCFH) to fluorescent dichlorodihydrofluorescein. We previously found that rapid oxidation of DCFH occurred with heme-compounds as well as ROS [Ohashi, T. *et al.* (2002) *FEBS Lett.* 511, 21–27], and then examined the effect of DCFH-DA on the induction of HO-1 expression by arsenite, cadmium and hemin, which induce oxidative stress and cytotoxicity. We found suppression of the arsenite-, cadmium- and hemin-dependent induction of HO-1 with DCFH-DA. The suppression occurred at the transcriptional level since the promoter activity of the Maf-recognition site of the HO-1 gene decreased with the DCFH-DA treatment. DCFH abolished the phosphorylation of extracellular signal–regulated kinase, the nuclear translocation of a transcriptional activator Nrf2, and cell death. An antioxidant, *N*-acetylcysteine (NAC), also suppressed the induction by arsenite and cadmium, but not that by hemin, indicating that DCFH blocked a different site in the stress signal pathway from NAC. Considering that the oxidation of DCFH diminishes ROS generated by various stressors, our findings provide a potential strategy for protection of cells from toxic insults using DCFH-like molecules.

Key words: cytotoxicity, 2′,7′-dichlorodihydrofluorescin, heme oxygenase-1, Nrf2, ROS.

Abbreviations: HO, heme oxygenase; ROS, reactive oxygen species; DCFH-DA, 2′,7′-dichlorodihydrofluorescin diacetate; DCF, 2′,7′-dichlorodihydrofluorescein; FCS, fetal calf serum; SDS, sodium dodecylsulfate; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; ERK, extracellular signal–regulated kinase: SAPK/JNK, stress-activated protein kinase/c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; PVDF, poly(vinylidene difluoride); ARE, anti-oxidant responsive element; NAC, *N*-acetylcysteine.

HO catalyzes the initial catabolism of heme to biliverdin, carbon monoxide and free iron. Biliverdin is rapidly converted to bilirubin by biliverdin reductase (*1*). Two isoforms of HO have been identified, inducible isoform HO-1 and constitutive isoform HO-2 (*2–4*). HO-1 is an important cytoprotective enzyme and a widely known marker of oxidative stress (*1*, *2*). The expression of HO-1 is induced by its substrate, heme (hemin), but also following exposure to a variety of stress stimuli, including UV irradiation, H_2O_2, NO, heavy metals, cytokines and electrophilic chemicals (*2*, *5*, *6*). Interestingly, most known HO-1 inducers stimulate the production of ROS or lead to depletion of glutathione (*7*, *8*), suggesting the involvement of HO-1 activity in cellular protection against oxidative stress. On the other hand, a HO-1 deficiency in mice and humans increases the susceptibility to inflammation (*9*, *10*). Although several studies have revealed that carbon monoxide plays a key role in the cytoprotective effect preventing cellular stress, the mechanisms underlying the cytoprotection by HO-1 expression in response to ROS have not been well demonstrated.

2′,7′-Dichlorodihydrofluorescin (DCFH) is used to evaluate oxidative stress in cells. Dichlorodihydrofluorescin diacetate (DCFH-DA) is relatively resistant to oxidation, but when taken up by cells, it is de-acetylated into DCFH, which then forms a 2-electron oxidation product, the highly fluorescent compound DCF, in a reaction with the oxidizing species liberated (*11*). We previously examined the relationship between the generation of ROS and the expression of HO-1, and found that rapid oxidation of DCFH occurred not only with the generation of ROS but also in the presence of hemin (*11*, *12*). We also found that HO-1 was heterogeneously expressed in individual cells under unstressed conditions in parallel with the DCFH reaction (*13*). We further tried to determine the cytoprotective effect

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of the ROS-dependent oxidation of DCFH-DA. In this study, we found that the treatment of HeLa cells with DCFH-DA resulted in abolition of the induction of HO-1 by hemin, arsenite and cadmium. DCFH-DA decreased the stressor-dependent induction of HO-1/ARE reporter gene activity. DCFH-DA blocked the nuclear translocation of a transcription factor, Nrf2. These treatments decreased not only the HO-1 induction but also the decrease in cytotoxicity caused by hemin, arsenite and cadmium. The mechanisms of oxidants such as DCFH leading to a loss of ROS in cells will be discussed.

MATERIALS AND METHODS

Materials—DCFH-DA was purchased from Molecular Probes (Eugene, OR). DMEM and FCS were from GIBCO-Invitrogen Co. (San Jose, CA). Plasmids pRBGP3 and pHO-1-Luc (*14*) carried the rabbit β-globin TATA box and a 161-base pair fragment of the AB1 enhancer of the mouse *ho-1* gene placed upstream of the rabbit β-globin TATA box, respectively, and were kind gifts from Dr. K. Itoh, Tsukuba University. Antibodies for actin, HSP70, and Nrf2 were products of Santa Crutz Co. (Santa Crutz, CA). All other chemicals used were of analytical grade.

Cell Culture—HeLa cells were maintained in DMEM supplemented with 7% FCS, 50 U/ml of penicillin, and 50 μg/ml of streptomycin (*13*). To expose the cells to chemicals, the culture medium was changed to fresh medium-containing 10 μM sodium arsenite without or with 10–40 μM DCFH-DA, and the cells were then incubated at 37°C for the indicated period. The cells were also treated with cadmium chloride (1 μM) or hemin (10 μM) at 37°C for 5 h in the presence of 10–40 μM DCFH-DA. They were washed twice with PBS after being collected.

To measure the oxidation of DCFH with the cells, the cells were incubated with the indicated concentration of a stressor in the presence of 20 μM DCFH-DA for 15 min at 37°C. The cells were washed twice and lysed. The DCF fluorescence was measured with a spectrofluorometer with excitation at 490 nm and scanning of emission from 500 to 550 nm (*11*, *12*).

Antibodies—Antibodies against human HO-2 were prepared by injecting a rabbit with 0.5 mg of the maltose-binding protein (New England Biolabs Inc., Boston, MA)–HO-2 (*15*) fusion protein in Freund's complete adjuvant. After three subsequent injections at two-week intervals, antiserum was collected. The resulting antiserum was affinity-purified and used for immunoblotting. Antibodies against HO-1 and actin were as described previously (*13*).

Immunoblotting and RNA Blotting—The cellular proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Bio-Rad Labolatories, Herculus, CA). The conditions for immunoblotting for HO-1 and other antigens, and the detection of cross-reacted antigens were as described previously (*13*). Isolation of the nuclear and cytoplasmic fractions was carried out as described previously (*16*). RNA blots of HO-1 mRNA were performed as described previously (*13*, *16*).

Immunofluorescence Microscopy—Cells were washed with PBS (+) (PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$), fixed with 4% paraformaldehyde for 20 min, and then permeabilized in PBS (+) containing 0.1% Triton X-100 for 1 h. After blocking with 2% FCS in PBS (+), incubation with anti–HO-1 as the primary antibody was carried out, followed by incubation with Cy2-conjugated goat anti-rabbit immunoglobulin (Amersham Biosciences Co., Buckinghamshire, UK) (*13*).

Luciferase Assay—Cells were transfected with reporter plasmids pRBGP3, pHO-1-Luc and pRL-SV40 (Promega Co., Madison, WI) using Lipofectamine reagent, according to the manufacturer's recommendation. The cells were treated with hemin, sodium arsenite or cadmium chloride for 16h after the transfection and then washed twice with PBS. They were then lysed in a Reporter lysis buffer (Promega Co.), and the lysates were centrifuged and the supernatants were assayed for luciferase. The *Photinus* and *Renilla* luciferase assays were performed according to the protocol for the Dual Luciferase Assay System (Promega Co.). Transfection efficiency was normalized on the basis of *Renilla* luciferase activity.

Cell Viability—Cell viability was monitored by measuring the activity of lactate dehydrogenase released into the medium, using an LDH-cytotoxicity Assay Kit (BioVision Co., Mountain View, CA) and the trypan blue dye exclusion test. Apoptotic cells were evaluated by measurement of caspase-3 activity (Promega Co.).

RESULTS

Suppression of the Induction of HO-1 in HeLa Cells Treated with DCFH-DA—A variety of stressors including heavy metal ions and hemin induce the expression of HO-1, *via* the generation of ROS (*2*, *5*, *17*). DCFH detects ROS and is oxidized by diminishing ROS generated. To investigate the relationship of the oxidation of DCFH with the induction of HO-1 by ROS, HeLa cells were treated with sodium arsenite (10 μM), cadmium chloride (1 μM), or hemin (10 μM) in the absence or presence of DCFH-DA. As shown in Fig. 1A, the level of HO-1 increased in arsenite-, cadmium- and hemin-treated cells, and the increases were prevented by the treatment with 40 μM DCFH-DA. The level of HO-2 remained unchanged with these treatments. The induction of a heat shock protein, HSP70, was observed on treatment with arsenite or cadmium, but no decrease in the HSP70 level was observed with DCFH-DA. When the concentration of DCFH-DA was increased, the hemin-dependent induction of HO-1 was suppressed in a dose-dependent manner (Fig. 1B). The dose-dependent decrease with DCFH-DA was also observed in the cases of sodium arsenite and cadmium chloride (data not shown). The oxidation of DCFH was 2.2-, 1.8- and 10-fold increased in arsenite- and cadmium- and hemin-treated cells, respectively (Fig. 1C). Immunofluorescence observation revealed that HO-1 in arsenite-, cadmium- and hemin-treated HeLa cells was strongly stained with anti–HO-1, and that the staining was abolished on treatment with DCFH-DA (Fig. 1D). In a separate experiment, cells were treated with an antioxidant, NAC, to compare the effect of DCFH-DA on the expression of HO-1. The treatment of the cells with 5 mM NAC resulted in decreases in arsenite- and cadmium-dependent induction of HO-1, but not in hemin-dependent induction (Fig. 2). These results indicated that the HO-1 induction was suppressed by DCFH-DA and that the mechanism underlying

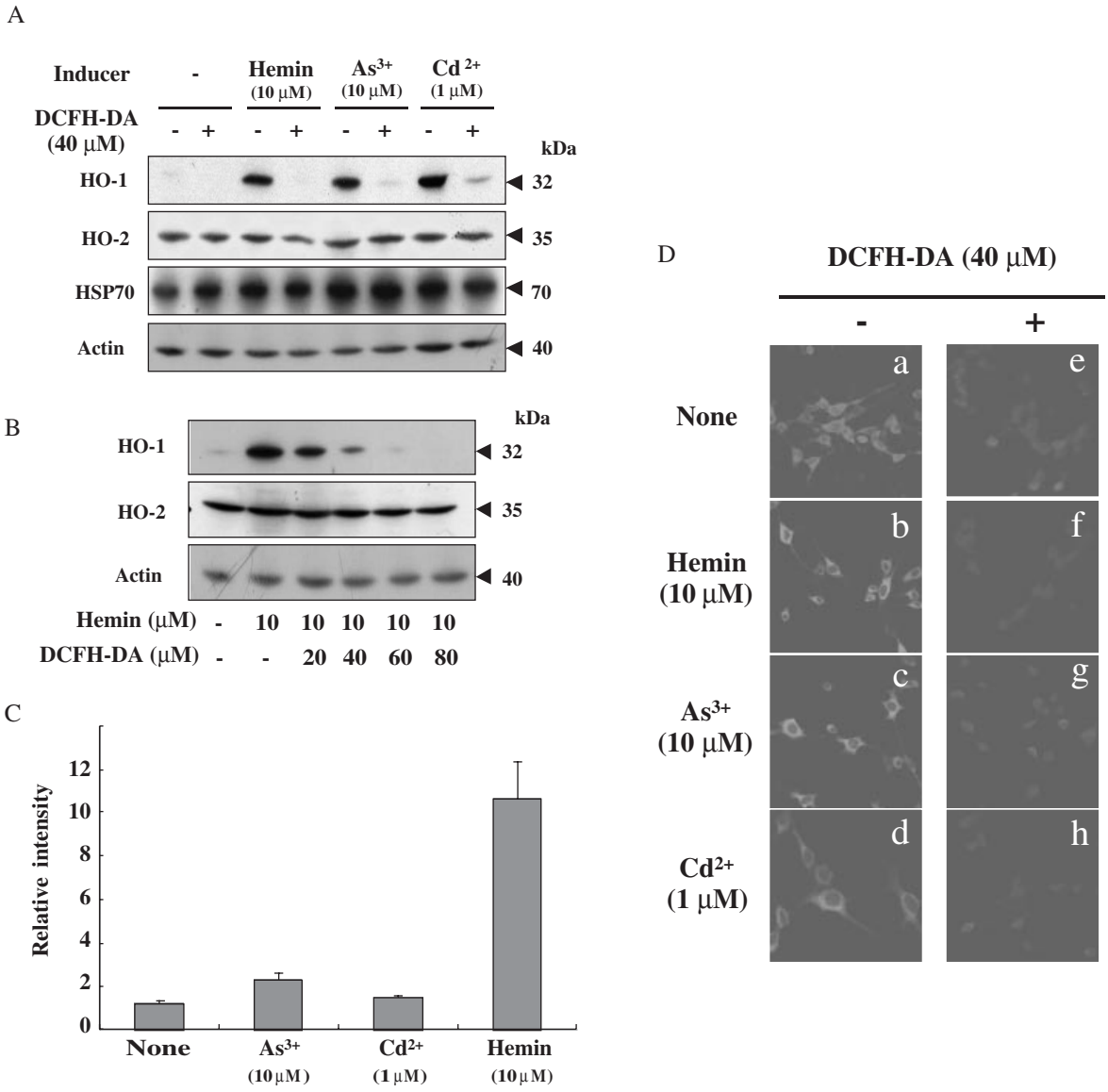

Fig. 1. **Effect of DCFH-DA on the induction of HO-1 in hemin-, arsenite- and cadmium-treated HeLa cells.** (A) Cells were incubated without (lanes 1 and 2) or with 10 μM hemin (lanes 3 and 4), 10 μM sodium arsenite (lanes 5 and 6) or 1 μM cadmium chloride plus 40 μM DCFH-DA (lanes 2, 4, 6 and 8) for 5 h. Cellular proteins were analyzed by SDS-PAGE and electroblotted onto a PVDF membrane. Immunoblotting was carried out using anti–HO-1, anti–HO-2, anti-HSP70, and anti-actin. (B) Effect of the DCFH-DA concentration on the HO-1 induction in hemin-treated cells. Cells were incubated with 10 μM hemin plus the indicated concentration of DCFH-DA for 5 h. (C) The oxidation of DCFH. The treatment of cells with the indicated reagents for 15 min was performed as above, except for the addition of 20 μM DCFH-DA. The cells were washed and lysed. The DCF fluorescence was quantitated with a spectrofluorometer. The data are the means of triplicate experiments ± SD. (D) Immunofluorescence staining of HO-1 in HeLa cells. Permeabilized cells treated without (a) or with 10 μM hemin (b), 10 μM sodium arsenite (c), or 1 μM cadmium chloride (d), or the inducers plus 40 μM DCFH (e–h) were labeled with anti–HO-1.

the suppression by DCFH was different from that in the case of NAC.

DCFH Regulates HO-1 Expression at the Transcription Level—To determine whether suppression of HO-1 expression occurs at the transcriptional level, Northern blots of HO-1 were carried out. The increased levels of HO-1 mRNA in arsenite-, cadmium- and hemin-treated cells decreased on treatment with DCFH-DA (Fig. 3A). It has been reported that the AB1 and SX2 enhancers of the *ho-1* gene (*18*) contain three and two copies, respectively, of the Maf recognition element (MARE), which overlap with ARE (*19*). To clarify whether or not the DCFH-dependent decrease in HO-1 mRNA is regulated at the transcriptional level, we investigated the effect of DCFH-DA on arsenite-, cadmium- and hemin-dependent induction of a *ho-1* AB1 enhancer-luciferase (pHO-Luc) reporter in HeLa cells. The reporter activity of enhancer-less pRBGP3 was weak and no change in the activity was observed on treatment with any compounds for 15 h (Fig. 3B). The reporter activity was high in pHO-Luc–transfected HeLa cells and increased on treatment with arsenite, cadmium and hemin. The increases in the activities of arsenite- and hemin-treated

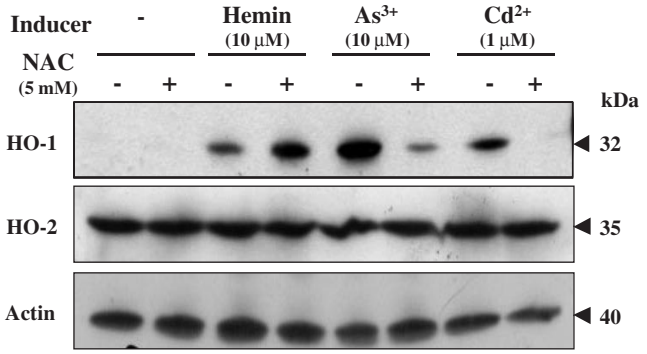

Fig. 2. **Effect of NAC on the expression of HO-1 in HeLa cells cultured with stressors.** Cells were incubated without (lanes 1 and 2) or with 10 μM hemin (lanes 3 and 4), 10 μM sodium arsenite (lanes 5 and 6) or 1 μM cadmium chloride plus 5 mM NAC (lanes 2, 4, 6 and 8) for 5 h. Analysis of cellular proteins and immunoblotting, using anti–HO-1, anti–HO-2, and anti-actin, were carried out as described in the legend to Fig. 1.

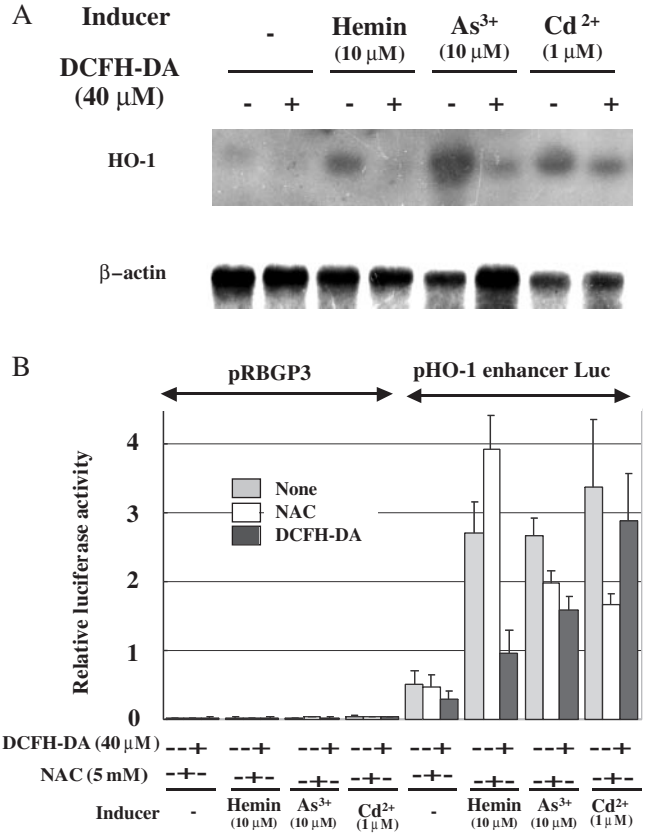

Fig. 3. **Regulation of the HO-1 gene enhancer in hemin-, arsenite-, and cadmium-treated HeLa cells by DCFH-DA or NAC.** (A) Decrease of HO-1 mRNA in hemin-, arsenite- and cadmium-treated cells by DCFH-DA. RNA from cells treated under the same conditions as those in the legend in Fig. 1 was separated, transferred to nylon membranes, and then hybridized with the fragments of human HO-1 (upper panel) and β-actin (lower panel) cRNAs. (B) Cells were transfected with the pRBGP3 or pHO-1-Luc plasmid and then cultured in the presence of the indicated compounds for 15 h. The concentrations of reagents added were the same as described in Figs. 1 and 2. Luciferase activity was measured and normalized as to *Renilla* luciferase activity. Data are the means of four experiments ± SD.

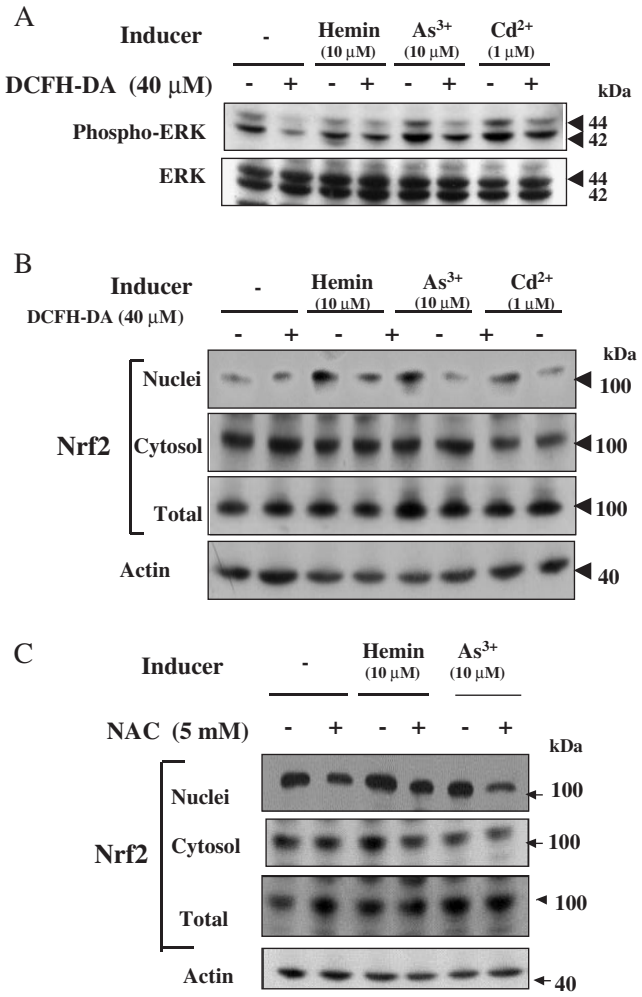

Fig. 4. **DCFH-DA suppressed the phosphorylation of ERK and the translocation of Nrf2 to nuclei.** HeLa cells were treated without or with the indicated concentrations of inducers in the absence or presence of 40 μM DCFH-DA for 1 h. (A) Immunoblotting was performed with antibodies for phospho-ERK and ERK. (B) Whole cell extracts, cytoplasmic and nuclear extracts were prepared from HeLa cells treated with the indicated compounds for 1 h. Eact extract was separated, blotted, and subjected to Western analysis using antibodies against Nrf2 and actin. (C) The translocation of Nrf2 was also examined in cells incubated under the same conditions as above, without or with 5 mM NAC.

cells were abolished by the addition of 40 μM DCFH-DA, and that of cadmium-treated cells was partially decreased by DCFH. We next examined the effect of an antioxidant, NAC, on the activity of the MARE site of the HO-1 gene. NAC decreased the activity induced by arsenite and cadmium, but not that by hemin. These results were compatible with the observation of a decrease in the expression of HO-1 protein caused by DCFH.

Blocking of the Activation of ERK and Nrf2 Involved in the Expression of HO-1 Caused by DCFH-DA—Since a transcription factor, Nrf2, can regulate the inducible expression of HO-1 mediated by the MARE site of the *ho-1* gene (*14*, *19*), we next tried to define the signal transduction pathway involved in the regulation of the Nrf2 activity, followed by that of HO-1 expression. It was found that marked phosphorylation of ERK occurred on

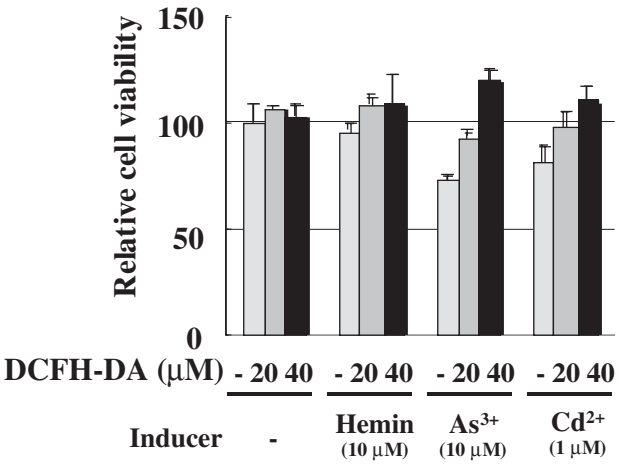

Fig. 5. **The protection of HeLa cells from the cell toxicity of hemin, arsenite and cadmium by treatment with DCFH-DA.** After treatment of the cells without or with the indicated compounds for 24 h, cell viability was estimated by measuring the lactate dehydrogenase activity released into the medium. Data are the means of four experiments ± SD.

treatment with 10 μM hemin, 10 μM sodium arsenite and 1 μM cadmium chloride (Fig. 4A). This phosphorylation was decreased by 40 μM DCFH-DA. To address whether DCFH affects the stressor-dependent activation of Nrf2, the translocation of Nrf2 to the nucleus was measured using DCFH-treated HeLa cells. As shown in Fig. 4B, Nrf2 was translocated to the nuclei of hemin-, arsenite- and cadmium-treated HeLa cells, but the translocation was inhibited by treatment with DCFH-DA. The nuclear translocation of Nrf2 in arsenite-, but not hemin-, treated cells was blocked by NAC (Fig. 4C). These results indicated that DCFH blocked the activation of ERK and Nrf2, followed by a decrease in the hemin-, arsenite- and cadmium-dependent induction of HO-1.

Protection from Hemin-, Arsenite- and Cadmium-Induced Cytotoxicity by Treatment with DCFH-DA—To address whether treatment with DCFH-DA affects cell viability, we incubated HeLa cells with 10 μM sodium arsenite, 1 μM cadmium chloride and 10 μM hemin in the absence or presence of 20–40 μM DCFH-DA for 24 h, and then measured cell viability by means of the cytotoxicity assay. As shown in Fig. 5, cell viability decreased on treatment with hemin, arsenite and cadmium, but the presence of DCFH-DA led to an increase in the viability, indicating that DCFH seems to contribute significantly to cellular defense mechanisms against toxic insults.

DISCUSSION

The present study demonstrated that the induction of HO-1 on treatment of HeLa cells with hemin, arsenite and cadmium was suppressed by DFCH-DA. The suppression by DCFH was dose-dependent, through coupling with the oxidation of DCFH. The induction and suppression of HO-1 expression were transcriptionally regulated *via* Nrf2. The mechanism involved in the suppression of the HO-1 induction by DCFH was different from that in the case of an antioxidant NAC since NAC abolished arsenite- and cadmium-, but not hemin-, dependent induction of HO-1. We (*20*) previously found that an increase in the ERK1/2 activity occurred in arsenite- and cadmium-treated HeLa cells. DCFH abolished the phosphorylation of these kinases (Fig. 4A). The responses of ERK were tightly associated with the nuclear translocation of Nrf2 (*21*). Judging from the fact that the oxidation of DCFH to fluorescent DCF is coupled with the destruction of ROS (*22*, *23*), the induction of HO-1 by inducers could be totally dependent on the generation of ROS.

The oxidation of DCFH with hemin was much higher than that with arsenite or cadmium (Fig. 1C). We previously reported that heme and hemoproteins directly oxidized DCFH, which was free from the reaction of ROS (*11*, *12*). Thus, the level of DCF fluorescence does not seem to be reflected by the generated ROS in hemin-treated cells. On the other hand, heme is an oxidant in several model systems (*24*, *25*). Several studies (*26–28*) have demonstrated the ability of hemin to trigger lipid peroxidation, protein degradation and DNA damage. To prevent these oxidant-mediated injuries, HO-1 may act as an anti-oxidant enzyme. The binding sites for many transcriptional factors identified in the promoter region of the HO-1 gene have been found to induce the enzyme, and Nrf2 was found to be a major factor as to the response to heavy metals and oxidative stress (*14*). In response to hemin treatment, an increase in the binding of Nrf2 as well as NF-κB and AP-2 has been demonstrated (*6*). We previously revealed that rapid and transient activation of the AP-1 transcriptional complex occurred in hemin-treated HeLa cells, without activation of NF-κB (*20*). The present study showed an increase in the nuclear translocation of Nrf2 in hemin-treated HeLa cells. DCFH decreased the translocation of Nrf2 into nuclei, followed by suppression of the induction of HO-1. Considering that the oxidation of DCFH is coupled with the reduction of hemin, DCFH contributes to prevent the disturbance of the redox state in cells by exogenously added hemin.

In addition to a variety of growth factors, cytokines and mitogens, ERKs are activated by cytotoxic insults (*29*, *30*). Cellular ERK activation either inhibits or enhances apoptosis in some cells (*30*, *31*). Even in the same cell, the role of ERK activation is likely to differ depending on the cellular insult (*30*). In HeLa cells, cadmium-, arsenite- and hemin-induced cell death was inhibited by treatment with DCFH, with concomitant suppression of HO-1 induction (Figs. 1 and 5). An antioxidant, NAC, also inhibited the arsenite- and cadmium-induced HO-1 enhancer activities, but not the hemin-induced one, indicating that the mechanism underlying the inhibition of the HO-1 induction by DCFH was different from that in the case of NAC. The findings also suggest that different cytotoxic insults are likely to be linked to different signaling molecules.

Several studies (*32–35*) have shown that the activation of p38 MAPK and SAPK/JNK occurred with various stressors generating ROS. We (*13*, *20*) have also reported that ERK1/2 and SAPK/JNK were activated in stressors-treated HeLa cells. However, the inhibition of ERK, SAPK/JNK or p38 MAPK activation individually was insufficient to completely abolish the induction of HO-1 in HeLa cells in response to these stressors (*36*). The inhibition of these MAPK pathways in combination resulted in a significant decrease in the HO-1 induction (*37*), suggesting that these kinases act

in concert to regulate the HO-1 induction. The present data show that DCFH totally abolished the phosphorylation of ERK as well as the activation of Nrf2 in hemin-, arsenite- and cadmium-treated cells, by quenching ROS.

The metal ions generate ROS, followed by hydrogen peroxide, and a free radical scavenger or antioxidants including NAC and sulforaphane clearly reduced the metal-induced toxicity (*37*, *38*), NAC almost completely abolished cadmium-induced activation of SAPK/JNK and c-Jun as well as apoptosis (*37*). The present study demonstrated that DCFH abolished HO-1 induction and cytotoxicity by metal ions and hemin, although the HO-1 gene activation by stressors occurred through different regulatory mechanisms. Based on the observation that DCFH decreased the phosphorylation of ERK and the translocation of Nrf2 to the nucleus, the DCFH-sensitive signal pathway upstream of ERK mainly contributes to the control of the HO-1 gene activation by hemin, arsenite and cadmium.

It has been reported that the AP-1 family of transcription factors is involved in the activation of the HO-1 gene by various oxidative stress agents, which in many cases is mediated by activation of ERK (*30*, *31*). It has also been shown that the nuclear translocation of Nrf2 could be dependent on ERK phosphorylation (*21*, *30*). In addition, the phosphorylation of SAPK/JNK, occurring upstream of c-Jun activation, was observed in cells expressing the HO-1 protein (*37*). Thus, the activation of different factors by stress agents occurred via multiple MAPK signals. DCFH can totally abolish the stress-activated signal pathways, and application of DCFH-like reagents facilitates the detoxication of ROS generated by various stressors.

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