Rapid oxidation of dichlorodihydrofluorescin with heme and hemoproteins: formation of the fluorescein is independent of the generation of reactive oxygen species

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Abstract Oxidative stress and the generation of reactive oxygen species (ROS) have been implicated in the pathogenesis of cellular damage. These events have usually been reported in terms of oxidation of a reporter molecule such as 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA). Treatment of HeLa cells with hemin or metalloporphyrins resulted in a rapid oxidation of DCFH in a time- and dose-dependent manner. This oxidation was inhibited by treatment of the cells with a large amount of superoxide dismutase and catalase, which is different from observations that these enzymes had no effect on the induction of heme oxygenase-1, a stress-induced protein, in hemin-treated cells. To examine the possibility that the oxidation of DCFH is independent of the generation of ROS, the oxidation was measured using hemoglobin-synthesizing erythroleukemia K562 cells. When K562 cells were treated with δ-aminolevulinic acid, a precursor of heme, oxidation of DCFH increased depending on the heme content in cells. Then DCFH-DA was oxidized directly with heme, hemoglobin, myoglobin and cytochrome *c*. These results suggest that oxidation of DCFH is not always related to the generation of ROS but may be related to heme content in cells.

Key words: Heme; Reactive oxygen species; 2′,7′-Dichlorodihydrofluorescin diacetate; Hemoprotein; Heme oxygenase-1

1. Introduction

2′,7′-Dichlorodihydrofluorescin (DCFH) is widely used to measure oxidative stress in cells. The diacetate ester form of DCFH, 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA), is relatively resistant to oxidation, but when taken up by cells, is de-acetylated to form DCFH, which then forms a two-electron oxidation product, the highly fluorescent compound dichlorofluorescin (DCF), in a reaction with the oxidizing species liberated [1,2]. The oxidant involved is H_2O_2 [1,2], but in the absence of cellular peroxidase the actual oxidant is more likely to be a Fenton-type species rather than the peroxide [3]. Other oxidizing species include nitric oxide [4], peroxynitrite [5], hypothiocyanite [6] and peroxyl radical [7]. Thus, formed reactive oxygen species (ROS) are important regulators of apoptosis [8].

Heme is the cofactor of proteins involved in a variety of biological processes such as oxygen transport, oxygenation and electron transfer. Free heme is a major source of iron that contributes to the generation of hydroxyl radicals by the Fenton reaction [9], damaging lipids and proteins, events which potentiate oxidative damage. Moreover, the generation of ROS as well as the radical reaction occurs upon injury to various tissues and red blood cells and the production of hydroxyl radical from hydrogen peroxide by releasing heme of hemoglobin and myoglobin follows cell membrane damage [10]. Since both heme and iron can be involved in the generation of potentially harmful ROS, the breakdown of heme and subsequent sequestration of heme iron in ferritin may be a mechanism of protection of cells from oxidative stress [11].

Heme oxygenase (HO) is the rate-limiting enzyme in the cellular catabolism of heme, in which heme is degraded to biliverdin, carbon monoxide, and ferric ions. Biliverdin is rapidly converted to bilirubin by biliverdin reductase. Three isoforms of HO have been identified, the inducible isoform HO-1, the constitutive isoform HO-2, and a recently characterized isoform HO-3 with low enzyme activity [10,11]. Expression of HO-1, a protein also known as heat shock protein 32, is induced by its substrate heme, but also following exposure to a variety of stress stimuli, including UV irradiation, H_2O_2, NO, heavy metals, phorbol ester, endotoxin and organic chemicals [11,12]. Interestingly, most of the known HO-1 inducers stimulate the production of ROS or lead to depletion of glutathione levels, suggesting the involvement of HO-1 activity in cellular protection against oxidative stress. However, any effect of HO-1 on hemin-induced oxidative stress has not been defined.

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Abbreviations: ROS, reactive oxygen species; DCFH-DA, 2′,7′-dichlorodihydrofluorescin diacetate; DCFH, dichlorodihydrofluorescin; DCF, dichlorofluorescin; SOD, superoxide dismutase; HO, heme oxygenase; ALA, δ-aminolevulinic acid; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; FCS, fetal calf serum; HRP, horseradish peroxidase

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During a study of which heme regulates the transcription of genes such as the HO-1 and *c-fos* genes [13,14], we examined the relationship between the generation of ROS and the expression of HO-1 induced by hemin and found that the oxidation of DCFH occurred on treatment of the cells with hemin. In addition, we found that the fluorescence did not reflect the generation of ROS but that the probe reacted directly with heme and hemoproteins. Obstacles to evaluating the generation of ROS with these chemicals are discussed.

2. Materials and methods

2.1. Materials

[α-^{32}P]dCTP (3000 Ci/mmol) was obtained from NEN. Zinc-, cobalt-, and tin-protoporphyrins were gifts from Dr. S. Sassa of The Rockefeller University. DCFH-DA was from Molecular Probes Co. Dihydrorhodamine 123, δ-aminolevulinic acid (ALA), *N*-acetylcysteine, cytochrome *c* (bovine heart) and hemoglobin (bovine) were products of Sigma Chemicals Co. Catalase (bovine liver, 6500 U/mg) and superoxide dismutase (SOD; bovine erythrocytes, 5000 U/mg) were purchased from Wako Pure Chemicals Co. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and RPMI 1640 medium were from Gibco–Invitrogen Co. Hemin and metalloporphyrins were dissolved in dimethyl sulfoxide at a concentration of 20 mM, then diluted 20-fold with phosphate-buffered saline (PBS) containing 10 mg/ml bovine serum albumin (BSA). All other chemicals used were of analytical grade.

2.2. Cell culture

HeLa cells were maintained in DMEM supplemented with 10% FCS, 50 U/ml penicillin, and 50 mg/ml streptomycin. To expose them to hemin, the cells were washed twice with PBS, placed in DMEM containing 1 mg/ml BSA, and then incubated in the presence of 10 μM hemin at 37°C for a specific period. The cells were incubated with SOD or catalase at 37°C for 1 h, followed by hemin. They were washed twice with PBS after collected. Human erythroleukemia K562 cells were grown in RPMI 1640 medium supplemented with 10% FCS, 50 U/ml penicillin, and 50 mg/ml streptomycin. For differentiation, the cells were incubated with 100 μM ALA or 25 μM hemin for 48 h. The heme content of the cells was determined by fluorescence spectrophotometry [15].

2.3. Northern blots

Total RNA from the cells was prepared by the guanidium isothiocyanate method [14]. The RNA preparations were electrophoresed in a 1% agarose gel under denaturing conditions, and then transferred onto a nylon filter. Conditions for hybridization with a radiolabeled fragment of HO-1 cDNA and washing were as described [13,14].

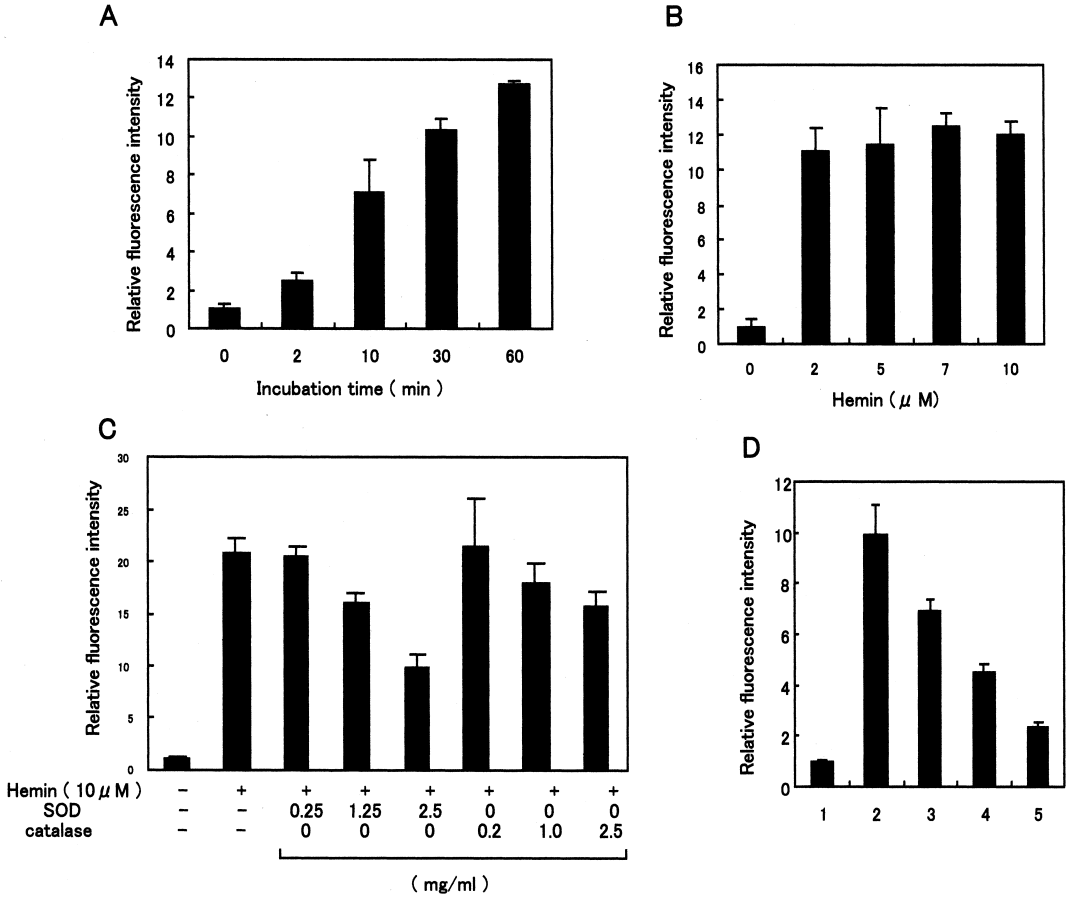

Fig. 1. Oxidation of DCFH in hemin- and metalloporphyrin-treated HeLa cells. A: HeLa cells (1×10^5 cells) were incubated with 10 μM hemin for the periods indicated, followed by 10 μM DCFH-DA for 10 min. The cells were washed and lysed, and the fluorescence of homogenates was determined. B: The cells were treated with the indicated concentrations of hemin for 60 min. The DCF fluorescence in the cells was measured as above. C: The cells were pretreated with the indicated amounts of catalase or SOD for 30 min, then incubated with 10 μM hemin for 60 min. After a 10 min incubation with DCFH-DA, cells were collected and washed, and the fluorescence in the cells was measured. D: The cells were left untreated (lane 1) or treated with 10 μM hemin (lane 2), 10 μM cobalt-protoporphyrin (lane 3), 10 μM tin-protoporphyrin (lane 4) or 10 μM zinc-protoporphyrin (lane 5) for 60 min. The oxidation of DCFH was measured as above. Data are expressed as the mean ± standard deviation of triplicate experiments.

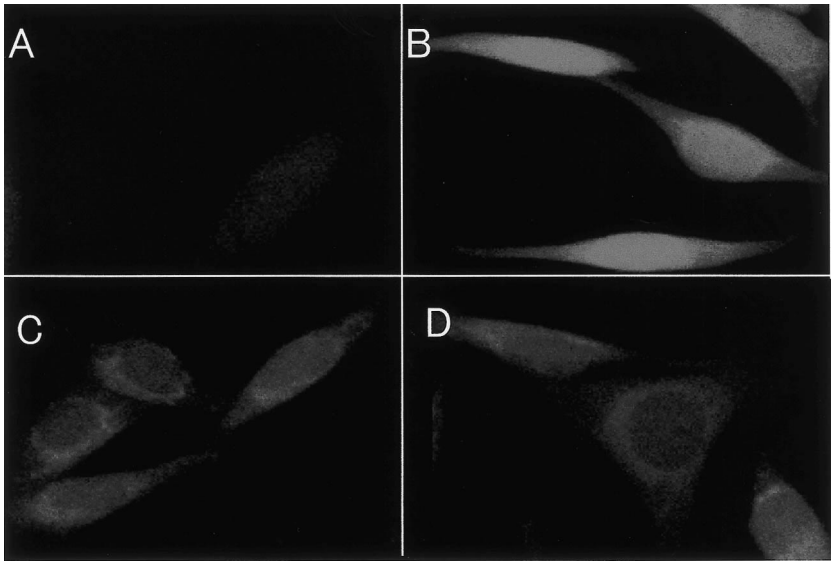

Fig. 2. Microscopic observation of DCF fluorescence in hemin- and cobalt-protoporphyrin-treated HeLa cells. Cells were incubated alone (A) or with hemin (B) and cobalt-protoporphyrin (C), followed by DCFH-DA, under conditions similar to those outlined in the legend of Fig. 1D. Cells were also incubated with 10 μM hemin plus 2.5 mg/ml SOD (D), as above, washed twice with PBS, and then visualized by confocal microscopy.

2.4. Fluorescence spectrophotometry

Cells were incubated with DCFH-DA (10 μM) at 37°C for 10 min. They were then washed twice with PBS, and lysed with 10 mM Tris–HCl buffer, pH 7.4, containing 0.5% Tween 20. The homogenates were centrifuged at $10\,000\times g$ for 10 min to remove cell debris. DCF fluorescence in the supernatant was measured using a spectrofluorometer with excitation of 500 nm and emission by scanning from 500 to 550 nm [11]. Oxidation of dihydrorhodamine 123 was measured with excitation at 500 nm and emission at 536 nm. Data were normalized to values obtained from untreated controls.

2.5. Microscopy

The localization of the fluorescence in cells treated with DCFH-DA was examined, using a Carl Zeiss LSM 510 confocal microscope. Cells incubated with DCFH-DA were washed twice with PBS and living cells were observed.

3. Results

3.1. Oxidation of DCFH-DA and dihydrorhodamine 123 in hemin-treated HeLa cells

To explore the possibility that treatment with hemin results in the generation of ROS, HeLa cells were incubated with 10 μM hemin for a given period, and the generation of ROS was evaluated on addition of DCFH-DA at 37°C for 10 min. The fluorescence with emission peaked at about 525 nm rapidly appeared in a time- and dose-dependent manner (Fig. 1A,B). To clarify the mechanisms by which exogenously added hemin generates superoxide or hydrogen peroxide, which is able to promote the oxidation of DCFH, cells were pretreated with ROS-metabolizing enzymes including catalase and SOD, followed by incubation with hemin. The pretreatment of the cells with a high concentration of SOD decreased the fluorescence, and catalase partly inhibited this effect (Fig. 1C). Antioxidants *N*-acetylcysteine (2 mM), pyrrolidine dithiocarbamate (100 μM) and bilirubin (10 μM) were without effect. When cobalt-protoporphyrin (10 μM), which is a potent inducer of HO-1 [10,14], was incubated with the cells, the oxidation of DCFH also occurred, but to a lesser extent (Fig. 1D). Even tin-protoporphyrin (10 μM) and zinc-protoporphyrin (10 μM), inhibitors of HO-1 [10,16,17], caused the oxidation. We further examined the localization of the fluorescence in cells by confocal microscopy. Intense DCFH-derived fluorescence was found in whole cells treated with 10 μM hemin or 10 μM cobalt-protoporphyrin (Fig. 2B,D), while pretreatment of the cells with SOD led to a diminishment of the fluorescence (Fig. 2C). We then examined the oxidation of dihydrorhodamine 123, another marker of ROS generation, in hemin-treated HeLa cells (Fig. 3). The fluorescence in the cells also markedly increased on treatment with hemin, similar to the case with DCFH.

Since treatment with SOD resulted in a decrease of the hemin-dependent oxidation of DCFH, we next examined the effect of SOD on the induction of HO-1 mRNA in hemin-treated HeLa cells. As shown in Fig. 4, treatment of cells with 10 μM hemin for 2 h resulted in the induction of HO-1

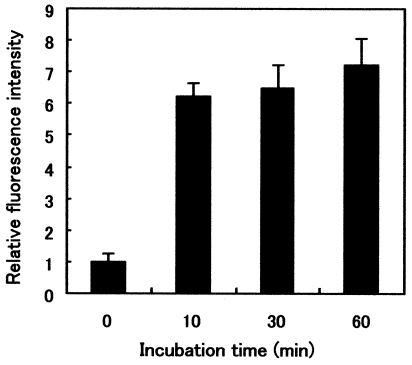

Fig. 3. Oxidation of dihydrorhodamine in hemin-treated HeLa cells. Cells were incubated with 10 μM hemin for the period indicated, and then with 10 μM dihydrorhodamine for 10 min. They were then collected, washed twice with PBS, and lysed. The fluorescence in the cells was measured as described in Section 2. Bars represent standard deviation ($n = 3$).

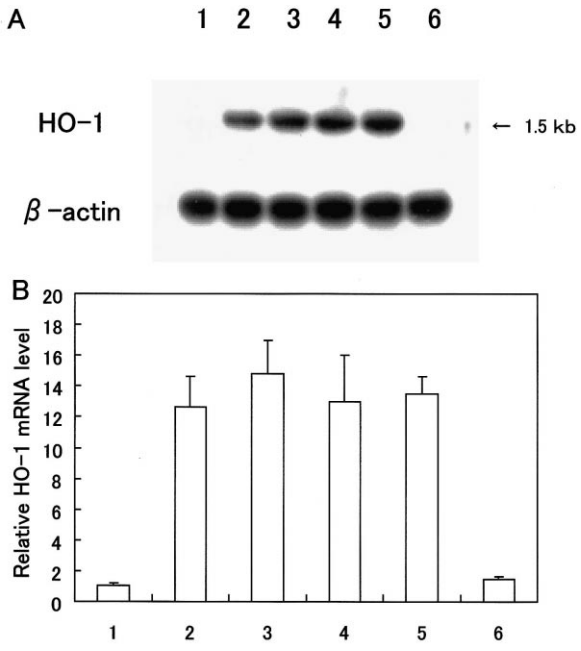

Fig. 4. RNA blots of HO-1 in hemin-treated HeLa cells. A: Total RNA (10 μg) from cells left untreated (lane 1) or treated with 10 μM hemin (lane 2), 10 μM hemin+2.5 mg/ml SOD (lane 3), 10 μM hemin+2.5 mg/ml catalase (lane 4), 10 μM hemin+2.5 mg/ml SOD+2.5 mg/ml catalase (lane 5) or 2.5 mg/ml SOD+2.5 mg/ml catalase (lane 6) for 2 h was electrophoresed, transferred onto filters, and hybridized with cDNA fragments of human HO-1 (upper panel) and β-actin (lower panel). An arrow shows the position of HO-1 mRNA. B: Densitometric quantitation of HO-1 mRNA. The lanes shown are the same as above. Values are expressed as the mean ± standard deviation of triplicate experiments.

mRNA, and the hemin-dependent induction did not change on treatment with SOD or catalase. These results indicated that the oxidation of DCFH is not paralleled by the expression of HO-1 mRNA induced by hemin.

3.2. *Oxidation of DCFH is not reflected by ROS generation but associated with heme content in hemoglobin-synthesizing K562 cells*

It is possible that heme and not ROS causes the direct oxidation of DCFH. We then examined the oxidation of DCFH in human erythroleukemia K562 cells which are capable of the biosynthesis of hemoglobin. When K562 cells were cultured in the presence of 50 μM ALA, a precursor of heme, for 48 h, the heme content of cells increased 20-fold compared to the control (Fig. 5). Then, using the ALA-treated cells, the oxidation of DCFH was compared with that in control cells. The DCF fluorescence in ALA-treated cells was five-fold that in the control. On incubation with 100 μM ALA, the fluorescence was higher than that in 50 μM ALA-treated cells. Incubation of K562 cells with hemin for 48 h led to an increase in the DCF fluorescence, and the extent of the fluorescence depended on the content of the intracellular heme (Fig. 5). Strong fluorescence was observed in hemoglobin-synthesizing K562 cells, and the intensity of the DCF fluorescence in hemin-treated cells was similar to that in ALA-treated cells. These results suggest that the formation of DCF fluorescence does not always depend on the generation of ROS but can be attributed to the heme content of the cells.

3.3. *Heme, hemoglobin, myoglobin and cytochrome c oxidize DCFH-DA in vitro*

Finally, we examined if heme in the reduced or oxidized form can directly oxidize DCFH-DA, since the oxidation of DCFH occurred in frozen K562 cells after thawing when incubated with hemin–albumin. Then, we tried to perform experiments without cells. The hemin–albumin complex was treated with sodium hydrosulfite to reduce heme. A reduced form of the complex was obtained by gel filtration, and immediately incubated with DCFH-DA at 37°C for a specific period. As shown in Fig. 6, the oxidation of DCFH occurred with reduced heme in a time-dependent manner. Reduced forms of hemoglobin, myoglobin and cytochrome *c* also caused a rapid oxidation of DCFH. Oxidized [Fe(III)] forms of heme (hemin), hemoglobin and myoglobin oxidized DCFH as well, but to much lesser extent than the reduced hemoproteins. On incubation of the oxidized form of cytochrome *c* with DCFH-DA, no fluorescence was observed. Thus, the oxidation of DCFH occurs directly with heme and hemoproteins.

4. Discussion

The present study demonstrates that DCFH, a probe used to measure the generation of ROS, is directly oxidized to DCF by heme and hemoproteins. Another chemical, dihydrorhodamine, also reacts with heme. Treatment of cells with metalloporphyrins including cobalt-, tin-, and zinc-protoporphyrin caused the oxidation of DCFH. Of the compounds tested, only SOD and catalase inhibited the heme-dependent oxidation of DCFH. These observations were similar to those by Rota et al. [18] who found that the oxidation of DCFH to DCF occurred with horseradish peroxidase (HRP) in the presence or absence of the substrate H_2O_2. The level of HRP-dependent DCF fluorescence was higher with than that without H_2O_2. Reduction of HRP–heme by H_2O_2 [18] supports our in vitro observation that reduced hemoproteins oxidize DCFH much better than do oxidized hemoproteins. Similarly, Burkitt and Wardman [19] reported that cytochrome *c* is a potent catalyst of DCFH oxidation and claimed that DCF fluorescence in pre-apoptotic and apoptotic cells is a measure of cytosolic cytochrome *c* rather than superoxide and H_2O_2.

Since the extent of oxidation of DCFH with reduced heme was unexpectedly much higher than that with oxidized heme, the reaction mechanisms in the two cases may be different. As DCFH is a highly reactive reductant, hemin and the oxidized form of hemoglobin or myoglobin were reduced with a concomitant oxidation of DCFH-DA. On the other hand, the reduced heme reacts with DCFH in the presence of oxygen to cause a more effective oxidation of DCFH, although the mechanism involved such as the formation of a ternary complex of heme, oxygen and DCFH remains unexplored.

There are several controversies in studies concerning the effect of the addition of SOD on oxidative stress in cells as monitored with DCFH. Some studies reported an inhibitory effect by SOD [20–22], whereas almost the same number of studies reported that SOD had no effect [23–25]. The finding made here, that SOD inhibited the oxidation of DCFH, could provide an explanation for discrepancies regarding the effect of SOD on the DCFH test for oxidative stress. Namely, it is possible that the amount of ROS was overestimated using heme or hemoproteins in cells in some cases.

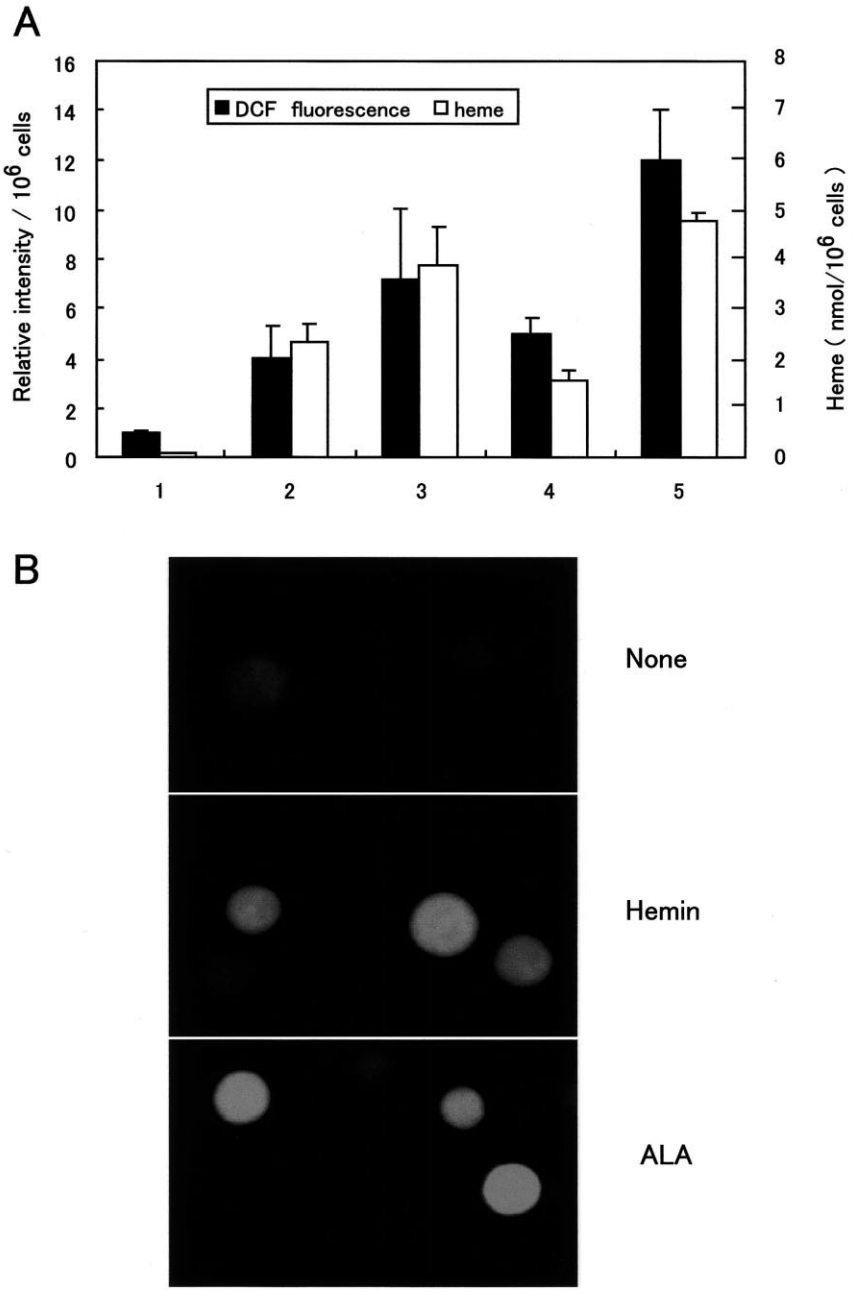

Fig. 5. Oxidation of DCFH in human erythroleukemia K562 cells induced to differentiate by treatment with ALA and hemin. A: K562 cells were left untreated (lane 1) or treated with 25 μM (lane 2) or 50 μM hemin (lane 3), and incubated with 50 μM (lane 4) or 100 μM ALA (lane 5) for 48 h. The amount of DCF fluorescence and heme in the cells was determined by fluorescence spectrophotometry. Bars represent the standard deviation ($n = 3$). B: Confocal microscopic observations. Untreated K562 cells (top); 50 μM hemin-treated K562 cells (center) and; 100 μM ALA-treated K562 cells (bottom).

Metalloporphyrins are highly reactive molecules and effectively mediate destruction of macromolecules through the generation of ROS, leading to apoptosis [10]. The finding that treatment of cells with metalloporphyrins including cobalt-, zinc- and tin-protoporphyrin caused oxidation of DCFH can be related to the generation of ROS in cells, but this possibility was ruled out by observations that these metalloporphyrins also oxidize DCFH in vitro. Furthermore, the oxidation of DCFH is not related to the induction of HO-1 by metalloporphyrins since cobalt-protoporphyrin is an potent inducer of HO-1, but tin- and zinc-protoporphyrin are inhibitors. Metalloporphyrins are capable of binding to oxygen [26], suggesting that metalloporphyrins are potent catalysts of the oxidation of reactive DCFH. It is unclear, however, whether the mechanisms involved in the oxidation of DCFH by metalloporphyrins and by hemin are the same.

Heme is an oxidant in several model systems [11,27]. The present study showed that hemin-dependent induction of HO-1 mRNA expression was not affected by treatment with ROS-modifying enzymes such as SOD and catalase which detoxify the mediators of oxidative stress (Fig. 4). The binding sites of many transcriptional factors have been identified in the promoter region of the HO-1 gene, and Nrf2 is found to be a major factor in the response to heavy metals and oxidative

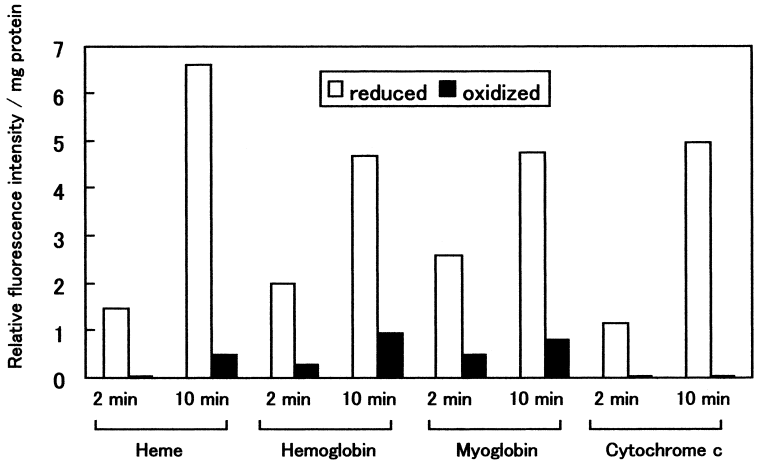

Fig. 6. Oxidation of DCFH with reduced and oxidized forms of heme, hemoglobin, myoglobin and cytochrome *c*. Reduced forms of heme–albumin complex and hemoproteins were obtained by passing them through a small Sephadex G-50 column (0.5×2.0 cm) after treatment with sodium hydrosulfite. Then, DCFH-DA (10 μM) was incubated with reduced or oxidized hemoproteins (0.2 mg of protein) at 37°C for the period indicated. Aliquots of each sample were withdrawn and the DCF fluorescence was measured. Data are the mean of duplicate experiments.

stress [28]. In response to hemin treatment, an increase in the binding of a number of transcriptional factors has been demonstrated, most significantly, AP-2 and NF-κB [11]. We previously showed that a rapid and transient activation of the AP-1 transcriptional complex occurred in hemin-treated HeLa cells, without translocation and activation of NF-κB [14]. The mechanisms involved in the induction of HO-1 mRNA expression by hemin treatment have yet to be established.

K562 cells undergo erythroid differentiation on treatment with hemin and anti-tumor drugs including aclarubicin and doxorubicin [29]. The treatment of K562 cells with hemin induced stress-related cellular modifications such as an increase in heat shock protein and thioredoxin expression [30]. During these treatments, the cells generate ROS which regulate cell growth, indicating that oxygen stress can be essential for the differentiation process [31]. In addition, human myeloleukemic HL60 cells were used to study NADPH oxidase-generating superoxide or chemically induced apoptosis [32,33]. Neutrophils were also employed as a model of superoxide production by NADPH oxidase [34]. In these studies, DCFH has proved a good marker for measuring ROS. However, these cells contain a large amount of hemoproteins including myeloperoxidase and hemoglobin [29,35,36]. Based on our observation that heme oxidizes DCFH directly, it is possible that changes in the level of cellular hemoproteins lead to an inaccurate estimation of the amount of ROS in cells by measuring oxidation of DCFH. Alternatively, measuring the oxidation of DCFH is a sensitive method to estimate the heme content of cells.

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