Gene Transfection of H25A Mutant Heme Oxygenase-1 Protects Cells against Hydroperoxide-induced Cytotoxicity

Heme oxygenase (HO)-1 is a stress-inducible enzyme protecting cells against oxidative stress, and mechanisms have been considered to depend exclusively on its enzyme activity. This study aimed to examine if the protein lacking catalytic activities could also display such resistance against oxidative stress. Stable transfectants of rat wild type HO-1 cDNA (HO-1-U937) and those of its H25A mutant gene (mHO-1-U937) were established using human monoblastic lymphoma cell U937. HO-1-U937 and mHO-1-U937 used in the study exhibited similar levels of the protein expression, while only the former increased enzyme activities. HO-1- and mHO-1-U937 cells became more and less sensitive to H2O2 than mock transfectants, respectively; such distinct susceptibility between the cells was ascribable to differences in the capacity to scavenge H2O2 through catalase and to execute iron-mediated oxidant propagation. On the other hand, both cell lines exhibited greater resistance to tert-buty1 hydroperoxide than mock transfectants. The resistance of HO-1-U937 to hydroperoxides appeared to result from antioxidant properties of bilirubin, an HO-derived product, while that of mHO-1-U937 was ascribable to increased contents of catalase and glutathione. These results provided evidence that gene transfection of the activity-lacking mutant HO-1 protects cells against oxidative stress through multiple mechanisms involving up-regulation of catalase and glutathione contents.

Cells and tissues have the ability to protect themselves against oxidative stress through up-regulation of a wide range of antioxidative gene products. Among such products, heme oxygenase (HO)-1 has attracted great interest as a modifier of oxidative susceptibility; pretreatment with the HO-1 induction or its gene transfection markedly protects cells and tissues against oxidative damages in vitro and in vivo (1–9). Such an HO-1-mediated protection has been thought to result from multiple mechanisms. This stress-inducible enzyme degrades protoheme-IX, the substrate of HO that derives from hemoglobin or myoglobin and serves as a catalyst for enhancement of peroxide toxicity (10). Second, the HO reaction generates biliverdin, CO, and divalent iron as products, each of which is biologically active and ameliorates the oxidative susceptibility of cells through distinct mechanisms. Biliverdin serves as a substrate for biliverdin reductase, which generates bilirubin, a potent radical scavenger that eliminates lipid peroxide radicals (11). Furthermore, CO could inhibit excessive NO generation and suppress the catalytic activity of cytochrome P450 monooxygenases (12, 13); these enzyme systems account for endogenous generator of reactive oxygen species. Another cytotoxic outcome of the HO-1 induction could be ascribable to an increase in bioavailability of divalent iron that causes in turn activation of iron-responsive proteins such as aconitate and subsequent up-regulation of ferritin (14). Up-regulated protein synthesis of ferritin causes overall reduction of free iron in cells and thereby attenuate oxidative susceptibility (15). However, since an excess of divalent iron leads to Fenton reaction through reducing hydrogen peroxide (H2O2), overexpression of HO-1 through excess expression of the gene could, in contrast, aggravate oxidative cell susceptibility under circumstances where effects of the iron-mediated oxidant propagation overwhelms antioxidative capacity of the HO-1 reaction (16, 17). Thus, the mechanisms by which the HO-1 induction alter oxidative susceptibility and their link to the catalytic activities have not fully been addressed yet.

If fundamental mechanisms for the aforementioned protection of cells by the HO-1 induction are ascribable to attenuation of oxidative insults mediated by heme and nonheme irons, the protection by HO-1 overexpression should be observed irrespective of the choice of pro-oxidant substances such as protoheme-IX, hydrogen peroxide, and organic hydroperoxides. This study aimed to examine if overexpression of the HO-1 protein leads to resistance against oxidative insults only through its catalytic action. To address this question, we have established the wild type and the activity-lacking mutant HO-1-overexpressing cells and characterized their differences in antioxidative properties against varied pro-oxidant reagents such as H2O2, tert-butyl hydroperoxide (t-BuOOH), and protoheme-IX. The current study showed that the mutant HO-1 gene transfection...
turned out to render cells more resistant to H$_2$O$_2$ and the organic hydroperoxide through mechanisms involving up-regulation of catalase expression and cellular contents of reduced glutathione while causing greater susceptibility to overloading of proteome-IX. At the same time, these results shed light on novel aspects for multiplicity of the HO-1-mediated mechanisms against oxidative stress.

**EXPERIMENTAL PROCEDURE**

**Reagents and Cell Culture**—All reagents used in the present study were purchased from Sigma unless otherwise mentioned. Human monoblastic lymphoma cell line U937 was used for experiments. Epstein-Barr virus-transformed lymphoblastoid cell line (LCL) were prepared by transforming lymphocytes from normal controls and the patient with HO-1 deficiency using supernatant from B95-8. LCL cells were further cloned by a limiting dilution method (18). LCL cells were prepared by transforming lymphocytes from the patient, designated as YK-14. These cells were cultured at 37°C in the presence of 5% CO$_2$ in plastic culture flasks that were filled with RPMI 1640 medium containing penicillin/streptomycin and 10% fetal bovine serum (Invitrogen).

**Establishment of Rat HO-1 and Mutant HO-1 Transfectants**—We prepared pEFneo-H25A mutant HO-1 cDNA construct using the PCR-based site-directed mutagenesis according to previous reports (19). In brief, PCR was performed in pEFneo cDNA in pEFneo using primers that mutate His$^{25}$ of rat HO-1 protein to Ala; the imidazole residue of the His serves as the proximal ligand of the substrate proteome-IX, and its replacement with Ala interferes with binding of molecular oxygen at the distal side, causing loss of the enzyme activity (20). The H25A mutant of HO-1 in pEFneo was synthesized and amplified with the primers bearing 5'-GGC CAC CAA GGA GGT GCC CAT CCG TGC GGA AAG TTC TG-3' and 5'-CAG AAT TCT CTT CAC GGA TGG CCA CCT TGG TGG CC-3', followed by the digestion with DpnI to remove the template vector. The wild type and H25A mutant gene constructs were transfected into U937 cells in culture, and their stable transfectants were cloned as described elsewhere (19). Amounts of the transfected protein in these cells were examined in their cellular lysates by Western blot and fluorescence-activated cell sorting analyses using GTS-1, the MoAb against HO-1, as described previously (19, 21). Several different clones of the wild type and H25A mutant HO-1 transfectants were obtained through these procedures.

**HO Activities in the Transformants—**HO activities in the established cell lines were determined by measuring the formation of bilirubin while causing greater susceptibility to overloading of proteome-IX. As seen in Fig. 2, these cell lines exhibited distinct susceptibilities to the effects of HO-1 and HO activities in the parent cell line U937. The cells in the first group were treated with the culture medium alone, while those in the second and third groups were treated with 400 μM H$_2$O$_2$, 400 μM t-BuOOH for 2 h. The cells in the next seven groups were treated with the same concentration of H$_2$O$_2$ or t-BuOOH-induced injury of U937 cells. The cells in the first group were treated with the culture medium alone, while those in the second and third groups were treated with 400 μM H$_2$O$_2$, 400 μM t-BuOOH for 2 h. The cells in the next seven groups were treated with the same concentration of H$_2$O$_2$ or t-BuOOH-induced injury of U937 cells. The cells in the first group were treated with the culture medium alone, while those in the second and third groups were treated with 400 μM H$_2$O$_2$, 400 μM t-BuOOH for 2 h. The cells in the next seven groups were treated with the same concentration of H$_2$O$_2$ or t-BuOOH-induced injury of U937 cells. The cells in the first group were treated with the culture medium alone, while those in the second and third groups were treated with 400 μM H$_2$O$_2$, 400 μM t-BuOOH for 2 h.

**Analysis of Cell Viability—**Cell viability of U937 cells and LCL cells were determined by the methylthiazole tetrazolium assay as described previously (Cell Count Kit 8; Dojindo, Kumamoto, Japan). To this end, 100 μl of the cell suspension (2 × 10$^5$ cells/ml) were plated per well on 24-well plates and cultured in RPMI 1640 containing 10% fetal calf serum. After cells underwent exposure to reagents described above, they were washed with phosphate-buffered saline, resuspended in the same medium and plated in a 96-well plate for 1 h. Ten μl of the reaction mixture containing 3-4,5-dimethythiazolyl-2-yl-2,5-diphenyl tetrazoilo bromide was added in each well. After a 2 h incubation under 95% N$_2$, 5% CO$_2$ at 37°C, the solubilized formazan product was quantified spectrophotometrically using a microplate reader. Absorbance was measured at 450 nm.

**Characterization of the Activity of Catalase and Glutathione Content—**Activities of catalase and glutathione peroxidase (GPx) were examined in cell lines established in the current study. Briefly, catalase activity was determined by monitoring the rate of decomposition of H$_2$O$_2$ into oxygen through an oxygen-sensing microelectrode as described previously (23). GPx activity was measured by a method described elsewhere (24). The 100-μl assay mixture contained 1.85 μM reduced OSH, 0.09 units of yeast glutathione reductase, 185 μM NADPH, and 100 μl of the sonicated cell suspension in 10 μM TE buffer, pH 8.0. The reaction was initiated by the addition of 750 μl t-BuOOH of a 10-μl volume to a mixture of the other ingredients in 100 μl. The GPx activity was quantitated from the decrease in NADPH absorbance at 340 nm. Total cellular contents of glutathione and those of the reduced form alone (GSH) were measured as described previously (25).

**Isolation of RNA and RT-PCR—**Total cellular RNA was isolated from U-937 cells using TRIZol reagent (Invitrogen). A total of 4 μg of total RNA were reverse-transcribed in 50 μl of reaction mixture containing the RT buffer (Invitrogen), 10 μM dithiothreitol (Invitrogen), 1 μM each deoxynucleotide triphosphate (dNTP) (Applied Biosystems), 1 unit/μl RNase inhibitor (Promega, Madison, WI), 0.01 μM/μl pd(N)$_6$ random primer (Amersham Biosciences) and 10 units/μl Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCRs were performed in a final volume of 25 μl of the PCR buffer (Applied Biosystems) containing 0.2 μM concentration of each dNTP, a 0.5 μM concentration of each primer (Invitrogen), and 0.25 units/μl Taq polymerase (Applied Biosystems). The amount of cDNAs added to the reaction mixture was normalized by the intensity of the glyceraldehyde-3-phosphate dehydrogenase amplicon. Aliquots were electrophoresed in 2% agarose gels and stained with ethidium bromide (WAKO, Osaka, Japan). Primers used in this study were as follows: catalase sense, 5'-GGT TTG GCC TCA CAA GGA CTA C-3'; antisense, 5'-TCA TTG GCA GTG TTG AAT CTC C-3'; GPx-1 sense, 5'-GGGA GAA CCC CAA GGA AG-3'; antisense, 5'-CAG AAT TCT CTT GAG AGC GGC-3'; GPx-4 sense, 5'-TGT CCG CCT ACT GAA GCC-3'; antisense, 5'-TGC GTT CCT GCC TCA CTG G-3'.

**Statistical Analyses—**Data in the present study are expressed as means ± S.E. of measurements. Differences in the mean values among the groups were analyzed by one-way analysis of variance combined with Fisher’s multiple comparison test. p values of <0.05 were considered statistically significant.

**RESULTS**

**Characterization of HO-1-cDNA-transfected U937 Cells and Susceptibility to H$_2$O$_2$**—Fig. 1 illustrates characterization of the expression of HO-1 protein in U937 cells. As seen in A, parent and mock transfectant cells did not show any notable expression of the HO-1 protein. On the other hand, the cells transfected with the wild type and mutant HO-1 cDNA displayed a marked expression of the protein at 32 kDa. Several different cell clones of the wild type (H1A, H1B, and H1C) and H25A mutant transfectants (mH1A, mH1B, and mH1C) that expressed comparable amounts of the protein became available for the current analyses. These cell clones obtained in the current study displayed a single peak population and expressed comparable amounts of the HO-1 protein (Fig. 1B). Under these circumstances, the wild type transfectant produced the 5-fold greater amount of the catalytic substrates versus the mock transfectant, while the H25A mutant transfectant did not show any notable elevation of the activities as compared with the parent and mock transfectant cell lines (Fig. 1C).

As seen in Fig. 2, these cell lines exhibited distinct suscep-

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tibility to exposure to H$_2$O$_2$. Namely, cells transfected with the wild type or H25A mutant HO-1 cDNA and their susceptibility to H$_2$O$_2$. A, Western blot analysis of the HO-1 protein by anti-rat MoAb GTS-1. H1A or mH1A denotes a wild type or an H25A HO-1 mutant transfected cell, respectively. B, representative fluorescence-activated cell sorting analyses of the protein expression in the wild type (H1A, H1B, and H1C) and mutant HO-1-transfected cells (mH1A, mH1B, and mH1C). Cells were fixed and penetrated with saponin to allow access of the anti-HO-1 MoAb GTS-1. C, differences in HO activities among cells. Data indicate mean ± S.E. of 3–7 separate experiments. *, p < 0.05 as compared with parent, mock transfectant, and mHO-1 cells.

FIG. 1. Characterization of human monoblastic lymphoma U937 cells transfected with rat wild type or H25A mutant HO-1 cDNA and their susceptibility to H$_2$O$_2$. A, Western blot analysis of the HO-1 protein by anti-rat MoAb GTS-1. H1A or mH1A denotes a wild type or an H25A HO-1 mutant transfected cell, respectively. B, representative fluorescence-activated cell sorting analyses of the protein expression in the wild type (H1A, H1B, and H1C) and mutant HO-1-transfected cells (mH1A, mH1B, and mH1C). Cells were fixed and penetrated with saponin to allow access of the anti-HO-1 MoAb GTS-1. C, differences in HO activities among cells. Data indicate mean ± S.E. of 3–7 separate experiments. *, p < 0.05 as compared with parent, mock transfectant, and mHO-1 cells.

FIG. 2. Differences in susceptibility to H$_2$O$_2$ among parent, mock, wild type (H1A, H1B, H1C), and H25A mutant (mH1A, mH1B, mH1C) transfectant cells. Cells were exposed to 400 μM H$_2$O$_2$ for 2 h. The value of 100% indicates the viable cell number in the vehicle-treated cells for each group. Data represent mean ± S.E. of more than 13 separate experiments for the left six groups and five separate experiments for the mH1B and mH1C groups. *, p < 0.05; significantly lower than the values of the H$_2$O$_2$-treated mock group. †, p < 0.05; significantly greater than the values in the H$_2$O$_2$-treated mock group.

Catalytic Activity of HO-1 Is a Determinant of Oxidative Susceptibility—Observation that the HO-1 gene transfection significantly altered oxidative susceptibility of U937 cells led us to examine if these events occurred dependently on the catalytic activities of the enzyme. To address this question, effects of ZnPP, a competitive inhibitor of the HO reaction, were examined in the mock transfectant, the wild type (H1A), and H25A mutant HO-1-overexpressing (mH1A) U937 cells. As shown in Fig. 3, the application of ZnPP at 5 μM decreased the H$_2$O$_2$-induced cytotoxicity in these cell lines. On the other hand, the same concentration of copper protoporphyrin-IX, a metalloprotoporphyrin that does not inhibit the HO activity, did not suppress the cytotoxic effect of H$_2$O$_2$ (data not shown).

These results suggested the catalytic activity of HO could be a determinant of the H$_2$O$_2$-elicited cytotoxicity and further encouraged us to investigate roles of divalent iron, an end
product of the heme degradation as a catalyst of \( \text{H}_2\text{O}_2 \)-induced oxyradical propagation. Treatment of the cells with the iron chelator DFO at 1 mM significantly attenuated the \( \text{H}_2\text{O}_2 \)-induced cell death in all of these cell lines. On the other hand, the iron-saturated DFO (Fe-DFO) did not exhibit such an inhibitory action on the \( \text{H}_2\text{O}_2 \)-induced cell damages, suggesting that effects of DFO result from its action to chelate free iron. Furthermore, DPD, another iron chelator with greater lipophilicity than DFO, attenuated the cell injury almost completely at 1 mM. These results showed that deprivation of iron reduces differences in \( \text{H}_2\text{O}_2 \) susceptibility among the cells. Considering the results shown in Fig. 1, these results collectively suggest that divalent iron generated through the HO reaction is a determinant of the cell susceptibility to the \( \text{H}_2\text{O}_2 \) exposure.

**Alterations in \( \text{H}_2\text{O}_2 \)-scavenging Enzyme Systems by HO-1 Gene Transfection**—Results showing distinct susceptibility to \( \text{H}_2\text{O}_2 \) led us to hypothesize that the HO-1 gene transfection could alter the capacity to scavenge the peroxide. To address this hypothesis, differences in two major enzyme systems for elimination of \( \text{H}_2\text{O}_2 \) such as catalase and glutathione peroxidase (GPx-1 and -4), and total glutathione contents among the cells. As seen in Fig. 5A, the cells undergoing the HO-1 gene transfection exhibited a 25% reduction of the total GPx activity. This reduction by the mutant transfection appeared to result from decreased expression of GPx-4 rather than that of GPx-1, so far as judged by the corresponding RT-PCR analyses in Fig. 5B.

Considering that the abundance of this enzyme system to eliminate \( \text{H}_2\text{O}_2 \) is thought to depend on the content of glutathione rather than that of the enzyme (26), we next examined total glutathione contents in the cells. As seen in Fig. 5C (open bars), the total glutathione contents displayed a 1.8-fold elevation in the mutant transfectant versus the mock or wild type transfectants. This elevation appeared to result from an increase in the reduced form of glutathione (GSH; closed bars), a compound responsible for amelioration of oxidative stress. These results showed that the wild type and mutant HO-1 transfectants exhibit distinct responses of the peroxide-scavenging systems. Namely, the wild type transfectant reduces the catalase activity but does not exhibit any notable changes in the glutathione peroxidase system, whereas the H25A mutant transfectant
increased both the activity of catalase and cellular glutathione contents simultaneously.

To examine whether the decreased sensitivity of H25A mutant HO-1 cells to H2O2 could depend on the activity of catalase and/or cellular glutathione contents, the effects of ATZ, a potent inhibitor of catalase, or BSO, a reagent depleting glutathione, were examined. As seen in Fig. 6, the H2O2-induced cytotoxicity was significantly enhanced by pretreatment with either ATZ or BSO. The enhancement of cell susceptibility after the pretreatment was comparable among different cell types (i.e., mock, wild type (H1A), and mutant (mH1A) transfectants). Collectively, H25A mutant HO-1 gene transfection turned out to render cells more resistant to H2O2 through mechanisms involving up-regulation of catalase expression and cellular contents of reduced glutathione, suggesting a novel antioxidative action of the activity-lacking mutant protein.

Alterations in Cell Susceptibility to tert-Butyl Hydroperoxide by HO-1 Gene Transfection—Results in Figs. 1 and 2 showing that the wild type HO-1 gene transfection aggravates H2O2 cytotoxicity led us to hypothesize that the HO-1 transfectant could also increase its susceptibility to other types of hydroperoxide. To address this hypothesis, differences in susceptibility to t-BuOOH were examined among the cells (Fig. 7). Application of the peroxide at 400 μM markedly reduced the number of viable cells in all of these groups, whereas both the wild type (H1A) and mutant HO-1 (mH1A) transfectants exhibited greater cell survival than the mock transfectant. Namely, the wild type HO-1 gene transfectant displayed quite different pictures of the susceptibility between H2O2 and t-BuOOH. We inquired whether iron could be involved in the mechanisms for t-BuOOH-induced injury as was observed in the H2O2 injury system. As seen in the right columns of Fig. 7, pretreatment with DFO or DPD attenuated the hydroperoxide-induced cell injury almost completely in these cell lines. Most importantly, the treatment of these chelators canceled out different susceptibility to the peroxide among the cells. Open, solid, and hatched bars denote data collected from mock-, wild type (H1A)-, and mutant HO-1-transfected cells (mH1A), respectively. Data represent mean ± S.E. of three or four separate experiments. *, p < 0.05 as compared with the t-BuOOH-treated mock transfectants. †, p < 0.05 as compared with the t-BuOOH-treated cells.

wild type HO-1 gene transfectant displayed quite different pictures of the susceptibility between H2O2 and t-BuOOH. We inquired whether iron could be involved in the mechanisms for t-BuOOH-induced injury as was observed in the H2O2 injury system. As seen in the right columns of Fig. 7, pretreatment with DFO or DPD attenuated the hydroperoxide-induced cell injury almost completely in these cell lines. Most importantly, the treatment of these chelators canceled out different susceptibility to the peroxide among the mock-, wild type (H1A)-, and H25A mutant (mH1A)-transfected cells.

Increased Susceptibility to Heme Overloading by H25A Mutant Gene Transfection—Results showing increased resistance of H25A mutant transfectant cells against hydroperoxides led us to further inquire whether the same cells could also exhibit a comparable resistance against hemin, the substrate of the HO reaction that is known to enhance oxidative stress as a pro-oxidant. Fig. 8 illustrated the effects of 12-h exposure to 400 μM hemin on viability of different U937 cell lines. When transfected with the wild type HO-1 cDNA, U937 cells did not
reduce their viability upon the hemin exposure, and one of the clones that displayed the greatest activity of HO (H1A; Fig. 1C) exhibited a significant resistance against the stimulus. On the other hand, the mutant HO-1-transfected cells (mH1A, mH1B, and mH1C) exhibited greater susceptibility to hemin than the mock transfected, indicating distinct susceptibility to this particular pro-oxidant as compared with H2O2 and t-BuOOH. These results tempted us to hypothesize whether the cell-overexpressing the activity-lacking mutant HO-1 might display greater cell susceptibility to hemin through cancellation of intrinsic HO activities. In order to address this hypothesis, we compared oxidative susceptibility of the HO-1-null LCL (YK-14) that was established from the first human case of the gene deficiency. As seen in Fig. 9A, the YK-14 cells displayed greater sensitivity to hemin than two different LCL cells expressing HO-1 normally. On the other hand, when susceptibility of these cell lines to H2O2 was compared, there were no significant differences between YK-14 and two different control LCL cells (Fig. 9B). These results suggest that the H25A mutant transfecants exhibit greater susceptibility to hemin that is analogous to the HO-1-deficient cells.

**DISCUSSION**

The present study first provided evidence that gene transfection of the activity-lacking mutant HO-1 cDNA leads to antioxidative properties to cells. HO-1 is a stress-inducible protein that modulates cell susceptibility against oxidative stress, and mechanisms for such alterations in the susceptibility have been thought to depend exclusively on its catalytic activities to reduce pro-oxidants such as protoheme-IX and to generate biliverdin and/or bilirubin as antioxidants. Since the same reaction also provides divalent iron simultaneously, the overexpression of HO-1 was shown to cause an increased cell susceptibility to H2O2 under certain circumstances, particularly where the iron-mediated oxidant propagation overwhelms antioxidant properties of the HO reaction. Overexpression of the mutant protein was therefore expected to interfere with the binding of naturally occurring HO-1 to NADPH-cytochrome P450 reductase, the electron transfer system necessary to execute the catalytic reaction of HO, and thus suppress the intrinsic enzyme activities, displaying a phenotype analogous to dominant-negative effects. Such a hypothesis was plausible, so far as both the mutant-overexpressed U937 cells and the YK-14 cell established from the HO-1-deficient patient comparably displayed greater susceptibility to hemin, the substrate of the HO reaction, than their control cells. However, considering results showing the cell susceptibility to other pro-oxidants such as H2O2 and t-BuOOH, overexpression of H25A mutant HO-1 appears to alter the sensitivity to these oxidative insults through different mechanisms.

Current results on the mutant HO-1-mediated alterations in cellular peroxide-scavenging systems such as the glutathione-glutathione peroxide pathway and catalase raise several important questions against previously proposed mechanisms by which the wild type HO-1 overexpression leads to alterations in oxidative susceptibility. Previous studies suggested that overexpression of HO-1 causes increased susceptibility of cells to H2O2 through an elevation of divalent iron available for Fenton reaction (16, 17). If the increase in the HO-derived iron available as a result of the gene transfection simply dictates the oxidative susceptibility, the same cell should exhibit a similar susceptibility to t-BuOOH, since the cytotoxicity of this peroxide is also aggravated by iron-mediated oxidant propagation. However, such a scenario is obviously inconsistent with the current result showing that the same HO-1 gene transfectant displayed decreased susceptibility to t-BuOOH. To understand such a discrepancy of the oxidative susceptibility between different peroxides, several different possibilities should be taken into account. First, a recent investigation by Wilks and Ortiz de Montellano (27) is of biological importance, showing that H2O2 but not other organic hydroperoxides, such as t-BuOOH, could serve as a substrate of the HO reaction; besides molecular oxygen, the former helps HO-dependent oxidative cleavage of heme into verdoheine even in the absence of NADPH, while the latter does not undergo the reaction. Accordingly, H2O2 could easily drive the delivery of divalent iron in the wild type HO-1 transfectant and increase the susceptibility to greater extents than t-BuOOH. Second, as seen in the current study, the expression of catalase at both transcriptional and post-transcriptional levels is down-regulated in the wild type HO-1 transfectant as compared with that in the mock transfecant and parent cells. Since blockade of catalase by ATZ canceled out the difference in the susceptibility among the cells, the reduction in its activity by the gene transfection appears to result in the increased susceptibility to H2O2. Furthermore, this observa-

![Fig. 8. Differences in sensitivity of hemin-induced cytotoxicity among different U937 cells. Cells were treated with 400 μM of hemin for 12 h. Open, solid, and hatched bars denote data collected from mock, wild type (H1A, H1B, H1C)-, and H25A mutant HO-1 (mH1A, mH1B, mH1C)-transfected cells, respectively. Data represent means ± S.E. of 15 separate experiments for the mock, H1A, and mH1A groups and those of three separate experiments for H1B, H1C mH1B, and mH1C groups. *, p < 0.05; significantly elevated as compared with hemin-treated mock transfecants. †, p < 0.05; significantly decreased versus the hemin-treated mock transfecants.](image1)

![Fig. 9. Susceptibility of LCL established from a patient with HO-1 deficiency to oxidative stress. A, differences in the hemin susceptibility between the LCL cells established from two healthy subjects (control 1 and control 2) and those from a patient with HO-1 deficiency, designated as YK-14. Open circles and open squares denote data from control 1 and control 2, respectively, whereas closed circles indicate those from YK-14. Data represent mean ± S.E. of five separate experiments for individual groups. Hemin was applied at 100–400 μM for 12 h. *, p < 0.05 as compared with control 1; †, p < 0.05 as compared with both control cells. B, differences in the H2O2 susceptibility between two control cells and YK-14. Open circles and open squares denote data from control 1 and control 2, respectively, whereas closed circles indicate those from YK-14. H2O2 was applied at 100–400 μM for 2 h.](image2)
tion simultaneously explains the absence of alterations in the susceptibility to t-BuOOH, an oxidant that is not degraded by catalase. At present, the mechanisms by which the HO-1 gene transfection down-regulates the expression of catalase are unknown. Moreover, since the transfection with the mutant protein conversely up-regulated catalase, further investigation should be necessary to address whether the expression of this \( \text{H}_2\text{O}_2 \)-scavenging enzyme is elevated in the mutant transfectant as a result of the aforementioned dominant negative effects on the cells. However, the present results first suggest that the HO-1 gene transfection could enhance the \( \text{H}_2\text{O}_2 \) cytotoxicity not only through enhancement of iron-mediated oxyradical propagation but also through down-regulation of catalase expression.

Distinct from the wild type transfectant, the activity-lacking H25A mutant HO-1 transfectant not only up-regulated the catalase activity but also increased the cellular contents of glutathione and thereby acquired resistance to both organic and inorganic hydroperoxides. These results suggest that cells and/or tissues overexpressing the H25A mutant HO-1 protein become less sensitive to oxidative stress caused by these hydroperoxides. Since HO-1-deficient patients with a function-directed point mutation have never been reported, the biological and physiologic significance of such a mutant protein remains largely unknown. However, the current study sheds light on a possibility that patients with such a mutation, if any, could hardly be discovered clinically because of antioxidative properties of the mutant protein. Different from the first human case of the HO-1 deficiency, who totally lacked the protein expression of HO-1 through a heterogeneous homozygote of the corresponding gene, such a point-mutated subject exposed to stressors might induce the mutant HO-1 protein and thereby increase the catalase expression and cellular glutathione contents. Under these circumstances, enhancement of oxidative susceptibility of cells and tissues that is evident generally in the HO-1 knockout mice or its human homologue could hardly be observed as phenotypic changes. Considering a marked susceptibility of the mutant transfectant to hemin \textit{in vitro}, human cases with such a functionally critical mutation of HO-1 might be predictable if the gene is analyzed among patients who undergo acute overloading of free heme and resultant tissue injury without displaying notable hyperbilirubinemia under varied clinical conditions (e.g., hemolysis, sepsis).

Novel biological actions of the activity-lacking mutant HO-1 protein suggested in the current study deserve further studies provided that the knock-in mice display any notable resistance against oxidative stress \textit{in vivo}.

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