

## Ferritin and the response to oxidative stress

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Iron is required for normal cell growth and proliferation. However, excess iron is potentially harmful, as it can catalyse the formation of toxic reactive oxygen species (ROS) via Fenton chemistry. For this reason, cells have evolved highly regulated mechanisms for controlling intracellular iron levels. Chief among these is the sequestration of iron in ferritin. Ferritin is a 24 subunit protein composed of two subunit types, termed H and L. The ferritin H subunit has a potent ferroxidase activity that catalyses the oxidation of ferrous iron, whereas ferritin L plays a role in iron nucleation and protein stability. In the present study we report that increased synthesis of both subunits of ferritin occurs in HeLa cells exposed to oxidative stress. An increase in the activity of iron responsive element binding proteins

in response to oxidative stress was also observed. However, this activation was transient, allowing ferritin protein induction to subsequently proceed. To assess whether ferritin induction reduced the accumulation of ROS, and to test the relative contribution of ferritin H and L subunits in this process, we prepared stable transfectants that overexpressed either ferritin H or ferritin L cDNA under control of a tetracycline-responsive promoter. We observed that overexpression of either ferritin H or ferritin L reduced the accumulation of ROS in response to oxidant challenge.

**Key words:** hydrogen peroxide, iron, iron storage proteins, reactive oxygen species, transfection.

### INTRODUCTION

Iron is an essential element for mammalian cell growth. It is a required constituent of numerous enzymes, including iron-sulphur and haem proteins of the respiratory chain, as well as ribonucleotide reductase, which catalyses the rate-limiting step in DNA synthesis [1]. However, 'free' iron has the capacity to participate in oxygen free radical formation via Fenton chemistry [2]. Balancing the deleterious and beneficial effects of iron thus emerges as an essential aspect of cell survival.

Ferritin plays a central role in the maintenance of this delicate intracellular iron balance [3–5]. This protein has the capacity to sequester up to 4500 atoms of iron in a ferrihydrite mineral core, and functions to store iron not required for immediate metabolic needs. Ferritin is a 24 subunit protein composed of two subunit types, termed H and L, which perform complementary functions in the protein. The H subunit is thought to play a role in the rapid detoxification of iron [it contains the majority of the ferroxidase activity that oxidizes iron to the Fe(III) form for deposition within the core], whereas the L subunit facilitates iron nucleation, mineralization and long-term iron storage [3,6]. Ferritin is highly conserved in evolution [4], and murine and human ferritin H subunits appear to play analogous roles [6]. Thus murine ferritin H, like human ferritin H, possesses a ferroxidase activity not found in human or mouse ferritin L [6]. These functions of ferritin suggest that it might serve as a cytoprotective protein, minimizing oxygen free radical formation by sequestering intracellular iron.

Several results support a role of ferritin as a protectant against oxygen free radical-mediated damage. Exposure of endothelial

cells to haemin was observed to induce ferritin synthesis and concordantly reduce the cytotoxic response of these cells to toxic doses of H<sub>2</sub>O<sub>2</sub> [7]. A 6-fold induction of ferritin synthesis was observed in liver slices from rats treated with phorone, a glutathione-depleting drug that increases intracellular levels of reactive oxygen species (ROS) [8]. UV irradiation, which produces oxygen free radicals and damages DNA, has been shown to induce ferritin H mRNA [9], and ferritin protein [10,11].

In contrast with these studies, which suggest that induction of ferritin may accompany oxidant stress and, by inference, protect against it, observations that superoxide can mobilize iron from ferritin led to the suggestion that exposure to oxygen radicals may actually increase the pool of reactive (reduced) iron and exacerbate oxidant injury [12]. In addition, there are reports [13] that H<sub>2</sub>O<sub>2</sub> activates iron regulatory protein (IRP)-1, possibly through direct disassembly of the 4Fe–4S cubane cluster [14], or by activating a signal transduction pathway [15]. Activated IRP-1 and IRP-2 are proteins that function in the regulation of ferritin and other iron-responsive mRNA species (reviewed in [3,16–18]), repressing ferritin synthesis at a translational level [19,20]. Thus activation of IRPs would reduce, not increase, synthesis of both ferritin H and L in response to oxidative stress. However, other reports have suggested that IRP is inactivated in rat livers in an *in vivo* model of ischaemia/reperfusion, an injury associated with the production of ROS [21]. IRP was also inactivated by superoxide anions and H<sub>2</sub>O<sub>2</sub> in a cell-free system [22]. Thus the relationship between oxidant stress and ferritin synthesis exhibits considerable complexity.

The relative roles of the H and L subunits of ferritin in the response to oxidative stress are uncertain. We have previously

Abbreviations used: CAT, chloramphenicol acetyltransferase; DCF, 2',7'-dichlorofluorescein; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FBS, fetal bovine serum; H<sub>2</sub>DCF-DA, 2',7'-dichlorofluorescein-diacetate; IRE, iron responsive element; IRP, iron regulatory protein; ROS, reactive oxygen species.

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observed that treatment of cells with tumour necrosis factor, a cytokine that can induce oxidative stress [23], results in the selective induction of the H subunit of ferritin [24]. Oxidants, however, induced both ferritin H and ferritin L [25]. Using a tetracycline-inducible system similar to that described in the present study, it was recently reported that overexpression of ferritin H, but not a mutant of ferritin H with impaired ferroxidase activity, increases resistance of HeLa cells to the toxicity of H<sub>2</sub>O<sub>2</sub> [26]. Epsztejn et al. [27] have also shown that overexpression of ferritin H in clonal isolates of murine erythroleukemia cells reduces ROS formation and protects cells against cell death induced by exposure to H<sub>2</sub>O<sub>2</sub>. The experiments described in the present study utilized transfection of HeLa cells with ferritin H and L to assess the role of ferritin and its constituent subunits in the response to oxidative stress. We observed that overexpression of either ferritin H or ferritin L reduces the accumulation of ROS in oxidatively challenged cells.

## MATERIALS AND METHODS

### Cell culture

HeLa cells were a gift from Dr C. Morrow (Department of Biochemistry, Wake Forest University School of Medicine). Cells were cultured at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4.5 g/l) and supplemented with 10% (v/v) fetal bovine serum (FBS). The cells were passaged every 3–4 days.

### RNA analysis

Total RNA was prepared from 100 mm dishes of HeLa cells. Cells were removed from the plate by trypsin treatment, spun down, and RNA was isolated using TRIzol (Gibco BRL) as indicated in the manufacturer's instructions. Northern-blot analysis was performed as described previously [28]. Hybridization and signal detection were performed using the Genius system (Boehringer Mannheim) or using [<sup>32</sup>P]dCTP cDNA probes labelled using the random primer method, as previously described [24]. Mouse ferritin H cDNA (mFH) [24] and  $\beta$ -actin cDNA [29] were used as probes.  $\beta$ -Actin was generally used as an RNA loading control; similar results were seen using ribosomal RNA to control for loading. Films were scanned with a Pharmacia densitometer or PhosphorImager (Molecular Dynamics).

### Metabolic labelling and immunoprecipitation

HeLa cells in 35 mm dishes were washed twice with PBS and incubated for 15–30 min in 1 ml of methionine-free minimum essential medium. Tran<sup>35</sup>S-label (ICN; 100  $\mu$ Ci) was added to the medium and incubation continued for 2 h. Whole-cell extracts were prepared as previously described [28]. Whole-cell extract (1  $\times$  10<sup>7</sup> c.p.m.) was incubated with 6  $\mu$ l of anti-(human ferritin) antibody (Dako) and 20  $\mu$ l of Protein A-agarose (Calbiochem), and the slurry was rocked overnight at 4 °C. The immunoprecipitate was pelleted, washed with cold PBS, boiled for 5 min in 2  $\times$  loading buffer [125 mM Tris/HCl (pH 6.8), 4% (w/v) SDS, 80 mM dithiothreitol (DTT), 20% (v/v) glycerol and 0.01% Bromophenol Blue] and separated by SDS/PAGE.

### Construction of expression plasmids

Expression plasmids were constructed using the tetracycline-inducible vector system [30]. The pUHD15-1neo (tet transactivator) and the pUHG10-3 (tetO) plasmids were gifts from Dr

H. Bujard (ZMBH, Universitat Heidelberg, Germany). To prepare pUHGmFH (where mFH corresponds to mouse ferritin H cDNA) mouse ferritin H cDNA [24] was digested with *Hpa*II and treated with the Klenow fragment of DNA polymerase I. The pUHG10-3 vector was digested with *Bam*HI, treated with Klenow and calf intestinal phosphatase, and ligated to the  $\sim$ 780 nt *Hpa*II fragment of mouse ferritin H cDNA. This produced a mouse ferritin H cDNA lacking the iron responsive element (IRE), but containing an intact coding sequence. Restriction digestion was used to identify transformants carrying the mouse ferritin H cDNA insert in the sense orientation. pUHGmFL was constructed similarly. Mouse ferritin L was excised from pMLF27 (a generous gift from Dr C. Beaumont, INSERM Unite 409, Faculte de Medecine Xavier Bichat, Paris, France) using *Sma*I/*Hind*III and filled in with Klenow to produce a fragment lacking the IRE, but containing an intact coding sequence. pUHG10-3 was digested with *Bam*HI/*Xba*I, filled in with Klenow and treated with calf intestinal phosphatase. The vector and fragment were blunt-end ligated to yield pUHGmFL. pUHG-CAT (where CAT corresponds to chloramphenicol acetyltransferase), containing the CAT gene driven by the tetO promoter, was a gift from Dr J. Ninomiya-Tsuji (Department of Molecular Biology, Graduate School of Science, Nagoya University, Nagoya, Japan).

### Transfection

HeLa cells were electroporated essentially as previously described [31], using 250 mV. For transient transfections, three plasmids were electroporated in each sample: 25  $\mu$ g of pUHD15-1neo, the transactivator-containing plasmid; 5  $\mu$ g of the desired expression vector (pUHG10-3, pUHGmFH or pUHGmFL); and 5  $\mu$ g of pUHG-CAT, an electroporation efficiency control. For isolation of stable transfectants, HeLa tet-off cells (ClonTech Laboratories), in which gene expression is repressed in the presence of doxycycline, were cotransfected with either pUHGmFH or pUHGmFL and pHygSRalpha [32] using LIPOFECTAMINE<sup>®</sup> (Gibco BRL), and selected in medium containing 200  $\mu$ g/ml hygromycin and 5 ng/ml doxycycline.

### Measurement of ROS

HeLa cells stably transfected with ferritin H (clones H46 and H66) or ferritin L (clones L6 and L46) were thawed and grown in DMEM containing 10% (v/v) FBS and 5 ng/ml doxycycline. Cells were then trypsin-treated and plated at a density of 5000 cells/well into 96 well dishes in medium either containing doxycycline or without doxycycline. Cells were allowed to grow for 48 h. 2',7'-Dichlorofluorescein-diacetate (H<sub>2</sub>DCF-DA; Molecular Probes, Eugene, OR, U.S.A.) was then added directly into the growth medium at a final concentration of 4  $\mu$ M for 1 h at 37 °C. H<sub>2</sub>DCF-DA is a non-fluorescent permeant molecule, which is acted upon by intracellular esterases to produce H<sub>2</sub>DCF [33]. In the presence of intracellular ROS, H<sub>2</sub>DCF is rapidly oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF) [34]. Cells were washed twice with 150 mM NaCl/10 mM Hepes/Tris (pH 7.3) (HBS) buffer, and were then treated with HBS containing graded concentrations of freshly prepared H<sub>2</sub>O<sub>2</sub>. Plates were placed in a fluorescent microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) at 37 °C. Fluorescence was continuously monitored for 30 min, using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. In each experiment, fluorescence increase was measured in six replicate cultures for each concentration of H<sub>2</sub>O<sub>2</sub>.

### *In vitro* transcription for preparation of $^{32}\text{P}$ -labelled IRE RNA

IRE band shifts were performed using the 3' untranslated region of human transferrin receptor cDNA. A 1.3 kb *Xba*I–*Hind*III DNA fragment was excised from pCD-TR1 and ligated to an *Xba*I linker, followed by digestion with *Xba*I and *Bal*I. The *Bal*I–*Xba*I fragment containing human transferrin receptor IREs was inserted into the *Eco*RV–*Xba*I site of the pcDNA3.1 (+) plasmid in the sense orientation relative to the promoter for T7 RNA polymerase. The resulting plasmid was designated pIRE700.  $^{32}\text{P}$ -labelled RNA was *in vitro* transcribed from 1  $\mu\text{g}$  of the linearized (*Xba*I-digested) pIRE700 with T7 RNA polymerase in the presence of [ $\alpha$ - $^{32}\text{P}$ ]CTP (Amersham).

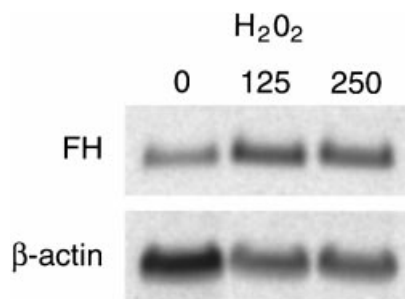
### Preparation of cytosolic extracts and RNA–protein binding assay

Preparation of cytosolic extracts and the IRP binding assay were performed using the method of Hanson and Leibold [35], with some modification. For cytosolic extract preparation, cells were incubated in 150  $\mu\text{l}$  of extraction buffer [10 mM Hepes/HCl (pH 7.6), 3 mM  $\text{MgCl}_2$ , 40 mM KCl, 5% (v/v) glycerol, 1 mM DTT and 0.2% Nonidet P40] for 5 min. Cell debris was removed by centrifugation at 15000  $g$  for 30 min at 4  $^\circ\text{C}$ , and protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad). Cytosolic extract (12  $\mu\text{g}$ ) was incubated with a  $^{32}\text{P}$ -labelled IRE RNA (40000 c.p.m.) for 30 min at 25  $^\circ\text{C}$ . Unbound RNA was digested with RNase T1 (10 units) for 10 min. Heparin was added to give a final concentration of 5 mg/ml for another 10 min. RNA–protein complexes were separated on 5% non-denaturing polyacrylamide gels. For quantification of RNA binding activity, intensity of shifted bands was analysed using a Molecular Dynamics Phosphor-Imager. To measure total IRP binding activity, cytosolic extracts were preincubated with 2% 2-mercaptoethanol prior to addition of the RNA probe.

## RESULTS

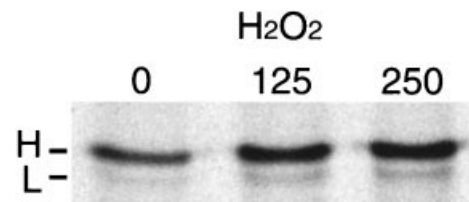
### $\text{H}_2\text{O}_2$ induces ferritin in HeLa cells

In order to test whether ferritin is induced in response to oxidants, we treated cells directly with  $\text{H}_2\text{O}_2$  and assessed the effects on ferritin mRNA and protein. As seen in Figure 1, treatment of HeLa cells with 125 or 250  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 8 h led to an increase in ferritin mRNA. Increased synthesis of both the H and L subunits of the ferritin protein was also seen, as shown in Figure 2. Thus in these cells, an increase in ferritin protein is observed following sustained (8 h) exposure to oxidants.



**Figure 1**  $\text{H}_2\text{O}_2$  increases ferritin mRNA in HeLa cells

Cells were treated for 8 h with 125 or 250  $\mu\text{M}$   $\text{H}_2\text{O}_2$  in growth medium. RNA was isolated, and Northern blots were prepared and probed with cDNA for ferritin H (FH) and  $\beta$ -actin. Ferritin induction was seen in three independent experiments.

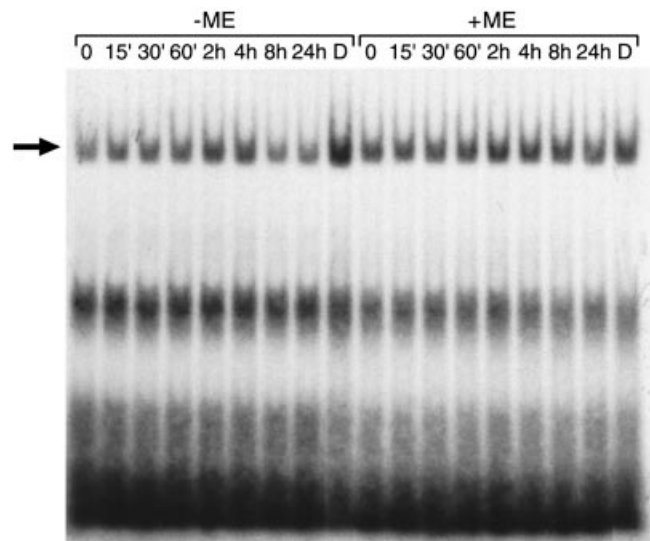


**Figure 2**  $\text{H}_2\text{O}_2$  increases ferritin protein synthesis in HeLa cells

Cells were treated for 8 h with 125 or 250  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and then labelled for 2 h with  $\text{Tran}^{35}\text{S}$ -label. Ferritin was isolated by immunoprecipitation and analysed by SDS/PAGE [12.5% (w/v) polyacrylamide]. The H and L subunits of ferritin are indicated. The 1.8-fold induction seen in this figure was confirmed in three independent experiments.

### Oxidative stress has a transient effect on IRP in HeLa cells

Ferritin translation is regulated by IRPs (see [3] for review). These translational regulators function to repress ferritin synthesis when intracellular iron levels are low; when iron is abundant, repression is relieved and ferritin translation occurs. IRP-1 and IRP-2 share sequence similarity, but are differentially regulated in response to iron: IRP-1 is regulated by assembly/disassembly of its iron–sulphur cluster, whereas IRP-2 is regulated by degradation [16,17]. Activation of the repressor function of IRP-1 has been reported under conditions of oxidative stress [13]. Such a response would reduce newly synthesized ferritin protein under pro-oxidant conditions. In order to assess whether activation of total IRP (IRP-1 and IRP-2) occurred in HeLa cells exposed to  $\text{H}_2\text{O}_2$ , cell extracts were prepared after various time intervals following treatment with  $\text{H}_2\text{O}_2$  and total IRP activity was measured. Cells were also treated with desferrioxamine, a known activator of IRP activity [36], as a positive control. As shown in Figure 3, there was a transient increase in IRP activity



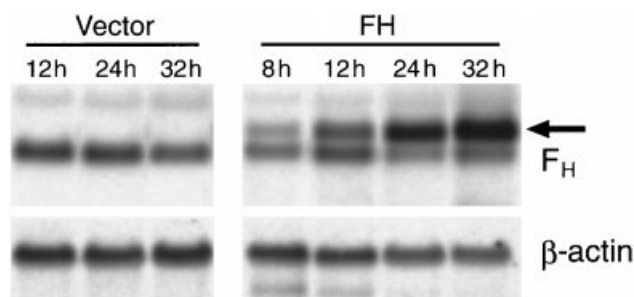
**Figure 3**  $\text{H}_2\text{O}_2$  has a transient effect on IRP activation in HeLa cells

Cells were treated with 125  $\mu\text{M}$   $\text{H}_2\text{O}_2$  in growth medium for the indicated times. Cytosolic extracts were prepared and IRP activity was assayed in the presence and absence of 2-mercaptoethanol (ME), as described in the Materials and methods section. 'D' indicates cells treated with 150  $\mu\text{M}$  desferrioxamine for 24 h. Active IRPs were measured in the absence of 2-mercaptoethanol; total IRP was measured in its presence. IRPs are indicated by the arrow.

**Table 1** Hydrogen peroxide transiently activates IRP-1

HeLa cells were treated with 125  $\mu$ M H<sub>2</sub>O<sub>2</sub> for the indicated times. Results are presented as means  $\pm$  S.E.M., *n* = three independent experiments.

Time	Active IRP/total IRP
0	0.82 $\pm$ 0.06
15 min	1.02 $\pm$ 0.05
30 min	0.97 $\pm$ 0.07
60 min	1.03 $\pm$ 0.02
2 h	1.03 $\pm$ 0.03
4 h	0.96 $\pm$ 0.05
8 h	0.67 $\pm$ 0.01
24 h	0.83 $\pm$ 0.04

**Figure 4** Transient overexpression of ferritin H mRNA in HeLa cells

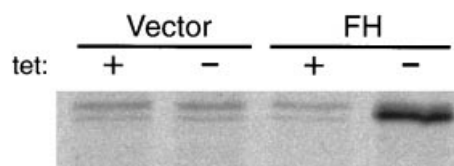
Cells were co-transfected with pUHD15-1neo and pUHGMFH (FH) or pUHD15-1neo and pUHG (Vector), and RNA was isolated after the indicated time intervals. RNA (10  $\mu$ g) was used to prepare Northern blots, which were hybridized sequentially with mouse ferritin H cDNA (F<sub>H</sub>) and  $\beta$ -actin cDNA probes. The arrow indicates the transfected gene (which produces a larger transcript than the endogenous human ferritin H mRNA, probably through utilization of an alternative polyadenylation site present in the vector).

following treatment with H<sub>2</sub>O<sub>2</sub> in these cells. Quantification of three independent experiments showed a 24% increase in the ratio of active to total IRP after 15 min (Table 1). This ratio remained constant for approximately 4 h, then gradually declined, returning to baseline after 24 h. The extent of activation at 8 and 24 h was identical at 125 and 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> (results not shown). Thus although rapid IRP activation occurred in response to oxidant challenge, it was not sustained beyond 4 h.

#### Overexpression of ferritin H in transfected HeLa cells

In order to directly address whether ferritin reduces oxidative stress, and to assess the contributions of the H and L subunits to this effect, we used transfection to independently augment levels of ferritin L or ferritin H. We then assessed the response of transfected cells to a model oxidant, H<sub>2</sub>O<sub>2</sub>.

To selectively modulate expression of ferritin, we first used transient transfection. HeLa cells were transfected with ferritin H cloned in a tetracycline-regulated expression plasmid that allows expression of ferritin H when tetracycline is withdrawn [30]. HeLa cells were electroporated with pUHD15-1neo/pUHG-CAT or pUHD15-1neo/pUHGMFH/pUHG-CAT, and plated into medium in the absence of tetracycline for RNA and protein analysis. Total RNA was harvested at the times indicated and 10  $\mu$ g was used for Northern-blot analysis. As seen in Figure 4, ferritin H mRNA was efficiently expressed under these conditions.

**Figure 5** Transient overexpression of ferritin H protein using the tet vector system

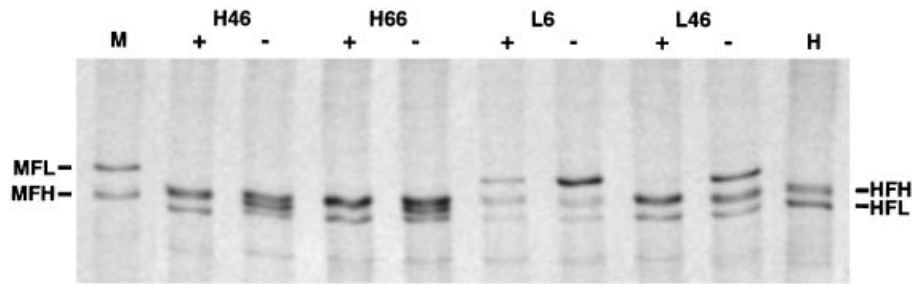
HeLa cells were electroporated with pUHD15-1neo and pUHG10-3 (Vector) or with pUHD15-1neo and pUHGMFH (FH) and plated into growth medium containing no tetracycline (–) or 2  $\mu$ g/ml tetracycline (tet; +). After 16 h, cells were labelled and ferritin was immunoprecipitated.

In order to test whether ferritin protein was also expressed in transiently transfected cells, HeLa cells were electroporated with pUHD15-1neo/pUHG10-3/pUHG-CAT or pUHD15-1neo/pUHGMFH/pUHG-CAT, plated into medium in the presence and absence of tetracycline, and ferritin protein synthesis assessed 16 h later. As seen in Figure 5, ferritin protein is overexpressed in transfected HeLa cells. Scanning densitometry of two independent experiments revealed that ferritin protein synthesis was increased by 5.2–5.3-fold in transfected cells. Tetracycline regulation also functioned under these conditions, as no expression of the transfected gene product was seen in the presence of tetracycline. Time course studies revealed that RNA accumulation and protein synthesis peaked between 16–32 h (results not shown).

#### Overexpression of either ferritin H or ferritin L reduces ROS formation in response to oxidant challenge

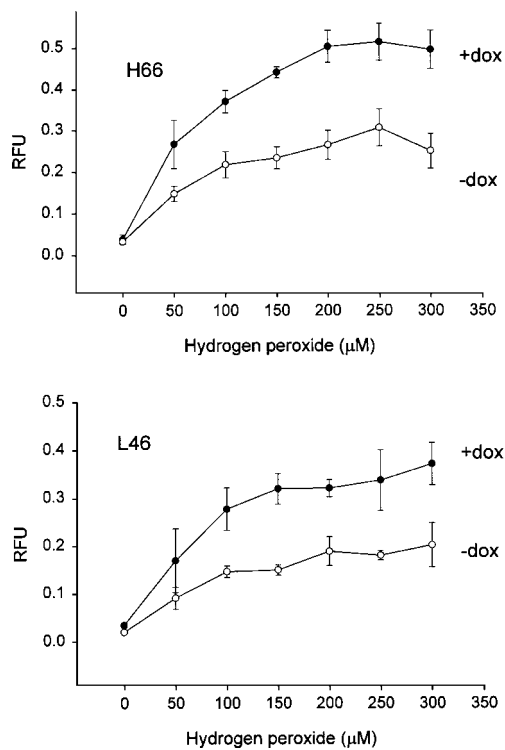
To examine whether ferritin overexpression conferred protection against oxidative stress, HeLa cells were transiently transfected with pUHD15-1neo and ferritin H or control empty vector, and plated into 96 well plates in the presence or absence of tetracycline. After 24 h, cells were treated with H<sub>2</sub>O<sub>2</sub> and effects on viability were measured. In some cases, cells transfected with ferritin H were protected from the cytotoxicity of H<sub>2</sub>O<sub>2</sub> when compared with vector transfectants. However, the protective effect was variable, ranging from a 2–3-fold increased resistance in cells transfected with ferritin H to less than 10% increased resistance (results not shown). Since under conditions of transient transfection a variable number of cells express the transfected gene, we attribute this variability in protective effect to the variability in transfection efficiency, or to the inability of transiently expressed ferritin to efficiently co-assemble with endogenous ferritin [37].

To obtain a population that uniformly overexpressed ferritin, we used HeLa cells that stably express the tetracycline transactivator. These cells were then transfected with expression vectors in which either ferritin H or ferritin L had been cloned downstream of the tet operator. In these transfectants, ferritin synthesis is repressed in the presence of tetracycline or its analogue, doxycycline, but induced following doxycycline withdrawal. Stable clonal transfectants were isolated by selection in hygromycin B, and assessed for their ability to express ferritin following withdrawal of doxycycline. As shown in Figure 6, in clones H46 and H66, withdrawal of doxycycline was accompanied by an induction in ferritin H synthesis. The fold increase in ferritin H in five to six independent experiments was 3.4  $\pm$  0.4 for clone H46, and 2.8  $\pm$  0.5 for clone H66 (means  $\pm$  S.E.M.). Similarly, an induction in ferritin L was observed in two clones



**Figure 6** Ferritin synthesis in stable transfectants is regulated by doxycycline

Stable transfectants (H46, H66, L6 and L46) were grown in culture medium alone (—) or containing doxycycline (+; 5 ng/ml); 48 h after doxycycline withdrawal, cells were incubated with  $\text{Tran}^{35}\text{S}$ -label, and ferritin was immunoprecipitated and analysed by SDS/PAGE [12% (w/v) polyacrylamide]. The L6 cell line was slightly leaky, expressing low levels of transfected ferritin even in the presence of doxycycline. M and H refer to the mouse cell line NIH3T3 and the human cell line MRC5, which were used to identify the mouse (MFL and MFH) and human (HFH and HFL) ferritin H and L subunits respectively.



**Figure 7** Overexpression of ferritin H or ferritin L diminishes ROS production in cells treated with  $\text{H}_2\text{O}_2$

Cells were plated in 96 well microplates at a density of  $5 \times 10^3$  cells/well in culture medium with or without doxycycline (dox; 5 ng/ml). After 48 h, cells were loaded with  $\text{H}_2\text{DCF-DA}$ , treated with the indicated concentrations of  $\text{H}_2\text{O}_2$ , and ROS formation measured as described in the Materials and methods section. Replicate cultures (six) were prepared for each test. Results are presented as means  $\pm$  S.E.M.s of five independent experiments. RFU, relative fluorescence units.

transfected with ferritin L. The fold increase in ferritin L was  $3.2 \pm 1.3$  (mean  $\pm$  S.E.M.,  $n = 3$ ) and  $6.0 \pm 1.3$  (mean  $\pm$  S.E.M.,  $n = 2$ ) in clones L46 and L6 respectively.

In order to determine whether overexpression of ferritin reduced formation of ROS in oxidatively challenged cells, ferritin synthesis was induced by withdrawal of doxycycline for 48 h. Controls were maintained in the presence of doxycycline.

The ability of cells to respond to acute oxidative challenge was then measured by assessing the rate of accumulation of ROS in cells challenged with graded doses of  $\text{H}_2\text{O}_2$ . ROS were detected by pre-loading cells with  $\text{H}_2\text{DCF}$  (produced intracellularly from  $\text{H}_2\text{DCF-DA}$ ) prior to exposure to  $\text{H}_2\text{O}_2$ . Oxidation of intracellular  $\text{H}_2\text{DCF}$  by ROS produces DCF, an oxidized and fluorescent product [34,38]. As shown in Figure 7,  $\text{H}_2\text{O}_2$  treatment led to a dose-dependent increase in the formation of ROS in both ferritin H and ferritin L transfectants. In clones expressing either ferritin H or ferritin L, induction of ferritin by withdrawal of doxycycline dampened the formation of ROS. The presence of doxycycline did not induce the formation of ROS as assessed in control untransfected HeLa cells or in cells transfected with the tet transactivator alone (results not shown). Thus overexpression of either ferritin H or ferritin L reduces, but does not prevent, the formation of ROS in response to oxidative challenge.

## DISCUSSION

Ferritin has a large capacity for iron storage, and the suggestion that this protein may serve to detoxify excess iron surfaced early on in the study of ferritin biochemistry [39]. However, only recently has a critical evaluation of the role of ferritin in protection from oxidative stress begun. The picture that has emerged is complex. Several studies have indeed suggested that ferritin protects against oxidative stress; for example in endothelial [7], tumour [40] and leukaemia [41] cells pretreatment with haemin and consequent induction of ferritin led to protection against subsequent oxidant challenge. This protective effect could be reversed by antisense oligonucleotides directed at the H subunit of ferritin [41], suggesting the direct involvement of ferritin H in protection against oxidant injury.

Ferritin synthesis is subject to transcriptional, as well as post-transcriptional, regulatory mechanisms. Ferritin translation proceeds when iron is abundant, and is repressed under conditions of iron depletion. This effect is mediated by the binding of IRPs to an IRE present in the 5' untranslated region of ferritin mRNA. IRP-1 can switch from an IRE-binding to a non-binding form depending on intracellular iron and redox status. Somewhat paradoxically, it has been reported that oxidants, such as  $\text{H}_2\text{O}_2$ , activate IRP-1 binding to the IRE. This would have the effect of decreasing ferritin synthesis under conditions of oxidative stress. However, some reports have shown opposite effects: ischaemia/reperfusion *in vivo* leads to inactivation of IRP [21]; and similarly, superoxide and  $\text{H}_2\text{O}_2$  down-regulate the RNA-binding of IRP in a cell-free system [22]. We observed that both of these effects

occurred: in HeLa cells, treatment with  $H_2O_2$  had a short-lived activating effect on IRP activity that was not apparent after 4 h (Table 1). Thus as we previously observed in BNLCL2 cells [25], activation of IRP binding in HeLa cells was transient. As a consequence, ferritin protein synthesis was augmented despite an initial activation of IRP by  $H_2O_2$ . Although our assay did not distinguish between IRP-1 and IRP-2, others have reported that  $H_2O_2$  preferentially activates IRP-1 [15]. We have seen a similar preferential induction of IRP-1 in cultured BNLCL2 liver cells [25].

Although our experiments did not address the mechanism of stimulation of IRP binding activity by  $H_2O_2$ , models suggested by others include direct disassembly of the iron-sulphur cluster [14], or indirect signal transduction pathways involving protein kinases [15,42]. Similarly, several mechanisms may underlie the return of IRP binding activity to its basal state. For example, peroxide-mediated release of iron from haem and other intracellular iron-containing proteins, including ferritin itself [8], may elevate the labile iron pool and reduce IRP binding activity. Since activated IRP stabilizes transferrin receptor mRNA, the decline in IRP binding at later time points may be due to an increase in transferrin receptor number and a consequent increase in iron import. Alternatively, signal transduction pathways may activate phosphatases to restore IRP binding activity to its original state.

In order to directly assess whether this observed induction of ferritin reduced the formation of ROS in response to oxidative stress, and to assess the roles of the H and L subunits in this process, we used transfection with ferritin H or L. Ferritin introduced into cells by transient transfection was unable to reproducibly protect cells from oxidative stress despite robust expression (Figure 5). Since the efficiency of transient transfection is variable, and a protective effect cannot be expected unless the majority of cells express the transfected gene, the heterogeneity in response to oxidative stress we observed in transient transfectants may be attributable to variability in transfection efficiency. The reported inability of transiently expressed ferritin to efficiently co-assemble with endogenous ferritin may also underlie these results [37].

We therefore utilized a system of stable transfection to overexpress ferritin. Because the isolation of clonal stable transfectants can lead to artifacts created by clonal variation, we used an inducible system. This allowed ferritin gene expression to be induced by doxycycline withdrawal, and thus the effect of overexpression of ferritin to be dissociated from variations in phenotype resulting from clonal selection. SDS/PAGE analysis revealed that ferritin synthesis was indeed inducible in this system: following withdrawal of doxycycline, ferritin H levels were augmented approximately 3-fold in clones H66 and H46, and ferritin L levels rose 3–6-fold in clones L46 and L6. In both cases, increased levels of ferritin reduced the accumulation of ROS in cells challenged with  $H_2O_2$  (Figure 7). The fact that cells expressing different levels of ferritin responded to identical levels of  $H_2O_2$  by accumulating different levels of oxidized DCF suggests that ferritin overexpression affects the formation and/or degradation of intracellular ROS. These results are consistent with the reduction in ROS formation observed in erythroid cells stably transfected with ferritin H [27].

Our previous results with recombinant proteins have demonstrated that mouse and human ferritin H subunits exhibit strong functional overlap, since both subunits possess a ferroxidase activity that is lost following mutation of amino acid residues Glu-62 and His-65 [6]. The results presented in the current paper further suggest that mouse and human ferritin H function similarly when expressed in mammalian cells, since over-

expression of either human ferritin H [26] or mouse ferritin H (Figure 7) reduces the formation of ROS.

What are the relative contributions of the H and L subunits to the reduction in ROS? Our data indicate that synthesis of both subunits is induced following challenge with pro-oxidants. Induction may occur at a transcriptional level, since an electrophilic response element has been described in both L [43] and H [25] ferritin genes of the mouse. Overexpression of either ferritin H or ferritin L by withdrawal of doxycycline in clonal transfectants led to a reduction in the formation of ROS in response to acute challenge with  $H_2O_2$ . Since ferritin H and L subunits co-assemble, increasing levels of either subunit may suffice to increase overall ferritin levels, leading to an enhanced intracellular capacity to sequester intracellular iron and a reduction in formation of ROS via Fenton-like reactions. However, although H and L subunits of ferritin co-assemble to form the apoferritin protein, these subunits are not identical in function. The H subunit of ferritin contains a ferroxidase activity responsible for the oxidation of iron, whereas the L subunit contributes to overall ferritin protein stability and iron mineralization [3]. It has been reported that although overexpression of wild-type ferritin H protected HeLa cells from  $H_2O_2$  toxicity, overexpression of a ferroxidase-deficient, double point mutant of ferritin H did not [26]. Our results suggest that overexpression of ferritin L may be different from overexpression of a ferroxidase-deficient mutant of ferritin H. Since HeLa cells are rich in ferritin H subunits {approximate H/L ratio of 10:1 ([26] and Figure 2)}, and since ferritins with an H ferritin content ranging from 60 to 100% exhibit a similar ferroxidase activity [6], the similar reduction in ROS we observed in clones overexpressing ferritin H and L may stem from the fact that, in both cases, ferritin species that exhibit a similar overall catalytic efficiency in iron oxidation are formed.

The cellular response to oxidant challenge is probably dictated by a complex interplay of events. For example, the amount of iron in ferritin at the moment of stress induction, levels of ascorbate (which enhances ferritin translation [44]), or the availability of alternative mechanisms to buffer oxidant stress (e.g. glutathione, superoxide dismutase and catalase) may all contribute to the final cellular outcome. Thus we observed that transfected ferritin could mitigate the formation of ROS in HeLa cells challenged with  $H_2O_2$ . However, the protective effect of ferritin overexpression was insufficient to completely prevent ROS formation. Although a greater reduction in ROS accumulation might be achieved in transfectants expressing greater levels of ferritin than those attained in the present study, an alternative interpretation is that ferritin induction is one component of a multi-component anti-oxidant response, and that there are limits to the ability of ferritin induction to buffer oxidant stress. Our results also suggest that underlying some of the disparate observations in the recent literature are real differences in cell-type-specific responses, and a complex interplay between transcriptional and translational control. This closely mimics the situation in the whole organism, in which individual tissues display different responses to oxidant injury.

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