Proteomic analysis of iron overload in human hepatoma cells

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Iron is indispensable for virtually all aspects of life. As an integral part of numerous proteins, iron serves as a reaction center for many metabolic processes. The chemical reactivity of iron, critical to its normal biochemical functions, is potentially cytotoxic. Ferrous iron is capable of the generation of reactive oxygen species (ROS) such as the hydroxyl radical and superoxide. Free radicals initiate lipid peroxidation of cellular membranes and oxidative damage of proteins, which in turn cause changes in membrane fluidity, disruption of microsomes and lysosomes, and accumulation of peptide fragments and cross-linked protein aggregates (21). This ultimately leads to dysregulation of cellular processes, cell dysfunction, and eventually to apoptosis or necrosis.

Toxicity of iron requires tight regulation of iron metabolism. Because there is no active or regulated mechanism of iron elimination from the body, systemic iron concentrations are regulated at the level of intestinal absorption. The regulation of iron absorption and cellular iron metabolism is achieved by controlling expression of genes directly engaged in iron uptake, storage, and recycling. Most proteins involved in the processes are regulated posttranslationally via iron-responsive elements and iron-regulatory proteins (10). However, iron can also influence gene and protein expression indirectly via oxidative stress, nitric oxide production, cytokine signaling, and many other, so far unknown, pathways.

Disruption of iron metabolism can lead to iron deficiency anemia or to iron overload. In iron overload diseases, such as hereditary hemochromatosis, dangerous surplus iron accumulates in parenchymal organs, namely, in the liver. Untreated iron overload leads to liver fibrosis, cirrhosis, and cancer (13). Our understanding of the molecular mechanisms of liver cell response to iron overload and iron-mediated oxidative stress is severely limited and requires employment of new and progressive techniques. Therefore, we used a proteomic approach, combining two-dimensional (2-D) electrophoresis and mass spectrometry to assess changes in protein expression initiated by iron excess in liver-derived HepG2 cells.

MATERIALS AND METHODS

All chemicals and reagents used were from Sigma-Aldrich unless stated otherwise.

Cell culture and induction of iron overload. Human hepatoma HepG2 cells (atcc no. HB-8065) were grown in Iscove’s modified Dulbecco’s medium (IMDM; Gibco) supplemented with 10% fetal calf serum in 75-cm² plastic flasks (Costar) at 37°C in an incubator with a controlled humidified atmosphere containing 5% CO₂. To induce iron overload, cells were treated with ferrous sulfate (1 mM FeSO₄) added to the media for 72 h. Cells were harvested and washed in PBS. For the measurement of cellular iron by atomic absorption spectrometry, cells were washed once in PBS, then in washing buffer containing 500 μM desferrioxamine (DFO; Desferal, Novartis) and 1.5 mM EDTA in PBS, and again in PBS buffer to remove all noninternalized iron. The viability of cells was determined by trypan blue exclusion.

Determination of cellular iron content by atomic absorption spectrometry. The cellular iron content was determined by atomic absorption spectrometry with acetylene-air flame atomization. The analysis was performed with the Varian atomic absorption spectrometer (Mulgrave, Australia) with a deuterium background correction. Measurements were performed with the analytical line 248.3 nm in the spectral interval of 0.2 nm. The iron concentration was determined by the standard addition method. Sample decomposition was accomplished in the MDS 2000 microwave sample preparation system (CEM) in Teflon cartridges by a mixture of nitric acid (5 ml) and hydrogen peroxide (2 ml) (both from Merck, ultrapure grade) for 20 min at a pressure of 120 psi. The resulting product was analyzed directly in the Teflon cartridges.

Thiobarbituric acid-reactive substances assay. The levels of reactive lipid peroxides and aldehydes in the iron-loaded and control cells were measured by the thiobarbituric acid (TBA) assay, as previously described (6, 16). To ensure that no lipid oxidation occurred during the assay, the free radical scavenger 2,6-di-tert-butyl-4-methylphenol [butylated hydroxytoluene (BHT)] and EDTA were added to the sample before precipitation with trichloroacetic acid (TCA) (6, 16).

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Briefly, HepG2 cell pellets were resuspended in 1 ml PBS supplemented with 1.5 mM EDTA + 5 μl of 0.1% BHT stock in ethanol, frozen, thawed, and disintegrated in the MSE Soniprep 150 by two 10-s pulses (amplitude: 10 μm). Homogenates were centrifuged at 4°C for 15 min at 15,000 g. Supernatants were collected, and the protein concentration was determined by the Bradford method (Bio-Rad; Hercules, CA). Protein concentrations were equalized to 5 mg/ml with dilution by BHT- and EDTA-supplemented PBS. One milliliter of each sample was precipitated by the addition of 100 μl of 100% TCA for 15 min on ice and then centrifuged at 4°C for 15 min at 15,000 g. Supernatants (1 ml) were then mixed with 100 μl of 6.7% (wt/vol) TBA in 50% glacial acetic acid and heated for 30 min at 95°C. The relative quantity of TBA-reactive substances (TBARS) was measured by absorbance of the sample at 535 nm.

Sample preparation for 2-D electrophoresis. Cell pellets were thawed and lysed in lysis buffer [7 M urea, 2 M thiourea, 4% CHAPS, 60 mM DTT, and 1% ampholytes (IPG buffer pH 4–7, Amersham)] containing protease inhibitor cocktail (EDTA Free, Roche Diagnostics) for 20 min at room temperature. Samples were centrifuged at 14,000 g for 20 min at room temperature. Supernatants were collected, and the protein concentration was determined as above. The protein concentration in all samples was equalized to 3 mg/ml by dilution with the lysis buffer.

2-D electrophoresis. Isoelectric focusing was performed with the Bio-Rad Protein IEF cell using 18-cm IPG strips (pH 4–7, Amersham). Samples were applied by rehydration loading. Each strip (4 for control samples and 4 for iron-overloaded cells) was rehydrated overnight in 350 μl of sample, representing 2.0 mg protein. Isoelectric focusing was performed for 75 kV-h, with maximum voltage not exceeding 5 kV, current limited to 50 μA/strip, and temperature set at 18°C. Focused strips were stored at −80°C. For the SDS electrophoresis, strips were thawed, equilibrated, and reduced in equilibration buffer A [6 M urea, 50 mM Tris (pH 8.8), 30% glycerol, 2% SDS, and 450 mg DTT per 50 ml buffer] for 15 min and then alkylated in equilibration buffer B [6 M urea, 50 mM Tris (pH 8.8), 30% glycerol, 2% SDS, and 1.125 mg iodoacetamide per 50 ml buffer]. Equilibrated strips were placed on the top of a 10% PAGE and secured in place by molten agarose. Electrophoresis was performed in a Tris-glycine-SDS system using a Bio-Rad Protean II apparatus with external cooling (20°C). Gels were run at constant voltage, starting at 50 V for 30 min, then 200 V for 1 h, and 300 V for 3.5 h. After electrophoresis, gels were washed twice for 15 min in deionized water to remove redundant SDS. Washed gels were stained in colloidal Coomassie blue (Simply Blue SafeStain, Invitrogen) overnight and then destained in deionized water.

Gel image analysis. Coomassie blue-stained gels were scanned with a GS 800 calibrated densitometer (Bio-Rad). Image analysis was performed with Phoretix 2-D software (Nonlinear Dynamics) in semimanual mode with gel quadruplicates. Normalization was based on total spot density. Protein spots differentially expressed were considered for protein identification.

Mass spectrometry and protein identification. Differentially expressed proteins were excised from gels, cut into small pieces, and washed several times with 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (MeCN). After complete destaining, the gel was washed with deionized water, shrunk by dehydration in MeCN, and reswollen again in water. The supernatant was removed, and the gel was partly dried in a SpeedVac concentrator. Gel pieces were then reconstituted in cleavage buffer containing 0.01% 2-mercaptoethanol, 0.1 M 4-ethylmorpholine acetate, 10% MeCN, and sequencing grade trypsin (50 ng/μl, Promega; Madison, WI). After overnight digestion, the resulting peptides were extracted with 40% MeCN-0.5% trifluoroacetic acid (TFA).

MALDI mass spectrometry and protein identification. A saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in aqueous 50% MeCN-0.2% TFA was used as a matrix-assisted laser desorption ionization (MALDI) matrix; 0.5 μl sample and 0.5 μl matrix solution were placed on the sample target and allowed to dry at room temperature. Positive-ion MALDI mass spectra were measured on a Bruker BIFLEX II reflectron time of flight (TOF) mass spectrometer (Bruker-Franzen; Bremen, Germany) equipped with a SCOUT 26 sample inlet, a gridless delayed extraction ion source, and a nitrogen laser (337 nm, Laser Science; Cambridge, MA). The ion acceleration voltage was 19 kV, and the reflector voltage was set to 20 kV. The spectrometer was calibrated externally using the monoisotopic [M + H] ions of peptide standards angiotensin II and insulin (Sigma). Proteins were identified by searching of peptide mass maps in the National Center for Biotechnology Information (NCBI) nr database (release date: 2005/06/01) using the search program ProFound (http://129.85.19.192/profound_bin/webProFound.exe). The search criteria were Taxonomy: Homo sapiens, molecular mass: 0–200 kDa, pI: 3–11, enzyme: trypsin, missed cleavage: 1, complete modification: Cys (iodoacetamide), partial modification: methionine oxidation, and mass tolerance: 0.5 Da.

RNA isolation, cDNA, and RT-PCR. Cells were lysed by a direct addition of RNA isolation reagent RNA-Beet (TEL-TEST). RNA isolation was done according to the manufacturer’s manual. cDNA was produced using oligo-dT and SuperScriptII reverse transcriptase (Invitrogen). Real-time quantification was done three times using SYBR green (Roche) and RotorGene 3000 equipment (Corbett Research). The expression of GAPDH was used as an internal standard (forward primer 5'-AACACGCGACACTCCTC-3' and reverse primer 5'-GGTCTCTCTTCTCGTGATC-3'). Primers for the α-subunit of prolyl-4-hydroxylase (P4H) were as follows: forward primer 5'-GCTCCCTTTTCTCTCATG-3' and reverse primer 5'-AACCTGCGCTTTTCTGTG-3'. PCR conditions were as follows: initial denaturation (95°C/5 s/min) was followed by 40 cycles of denaturation (95°C/15 s), primer annealing (60°C/15 s), and elongation (72°C/20 s).

RESULTS

Iron status. In preliminary experiments, we first determined the maximum ferrous sulfate concentration that can be tolerated for short-term cultivation of HepG2 cells (not shown). We found that HepG2 cells can be grown in media containing as much as 1 mM FeSO4 for 72 h without significantly affecting their viability and growth. Iron-treated cells exhibited viability (measured by trypan blue exclusion) comparable with control cells (87% and 91%, respectively). After growth for 3 days in 1 mM ferrous sulfate, HepG2 cells contained almost 13 times more intracellular iron than control cells (98 μg Fe/g wet cell pellet vs. 7.6 μg Fe/g) as measured by atomic absorption spectrometry (Fig. 1A). To eliminate any cell surface-bound (noninternalized) iron complexes, cellular iron concentrations were measured after careful washing of the cells with PBS containing two strong iron chelators: EDTA (1.5 mM) and DFO (500 μM). The measurements were performed in triplicate.

Oxidative stress and lipid peroxidation. Iron is well-known initiator of oxidative stress (3). To determine the relative extent of oxidative stress in the iron-loaded HepG2 cells, we measured lipid peroxidation in the cell extracts. TBA forms spectrophotometrically detectable adducts with the products of peroxidative alteration of membrane phospholipids. Quantification of the adduct serves as a relative indicator of oxidative lipid damage. Lipid peroxidation in iron-treated HepG2 cells was ~20-fold increased compared with control cells as determined by relative quantification of TBARS (Fig. 1B), confirming the presence of significant oxidative stress in iron-loaded HepG2 cells.
FeSO₄ were used as the starting material. We analyzed sample electrophoresis. HepG2 cells treated with or without 1 mM iron overload in HepG2 cells was performed using 2-D gel pressed proteins by mass spectrometry. Measurements were performed with sample quadruplicates. Thiobarbituric acid-reactive substances were determined in iron-overloaded and control HepG2 cells. Measurements were performed with sample quadruplicates.

2-D electrophoresis and identification of differentially expressed proteins by mass spectrometry. Protein analysis of the iron overload in HepG2 cells was performed using 2-D gel electrophoresis. HepG2 cells treated with or without 1 mM FeSO₄ were used as the starting material. We analyzed sample quadruplicates (4 gels, each originating from an individual sample) for each group (4 gels for the control cells and 4 gels for the iron-overloaded cells). With high protein load (2.0 mg protein/strip) combined with colloidal Coomassie blue staining, we reproducibly detected 1,060 (±30) spots on the individual gels (Fig. 2).

Gel analysis with Phoretix 2D software (Nonlinear Dynamics) in semimansual mode revealed 21 spots to be differentially expressed (selection criteria: average normalized spot volume difference 1.5-fold; statistical significance of the change determined by the t-test, P < 0.005). Eleven proteins were upregulated by iron treatment, whereas ten proteins were downregulated. Relative expression changes ranged from 1.5-fold to as much as 10-fold. All differentially expressed spots/proteins were excised from the gels, digested with trypsin, and analyzed by peptide mass fingerprint with MALDI-TOF mass spectrometry. An example spectrum is presented in Fig. 3.

Out of the 21 differentially expressed proteins, the identity of 19 proteins was determined (Table 1). Only two very small and faint spots (spots 15 and 16) failed to provide enough protein material for successful identification. The identified differentially expressed proteins are involved in various cellular functions including iron storage, response to stress, protein folding, energy metabolism, transcriptional regulation, apoptosis, and other processes (Table 2).

Spot 3 and 5, which were upregulated in response to iron overload, both represent protein disulfide isomerase (PDI), a multifunctional protein identical with the β-subunit of collagen P4H. P4H is a heterotetrameric 2α2β enzyme responsible for the maturation of collagen. The enzymatic activity of P4H is exclusive to the α-subunit; the β-subunit is PDI (17).

Multifunctional PDI has a theoretical molecular mass of 57 kDa and pI of 4.8. Spot 5, which migrated at ∼60 kDa with an observed pI of 4.7–4.9, represents intact PDI. Spot 3, with an observed molecular mass of ∼40 kDa and pI of 5.2–5.4, contains PDI processed from the COOH terminus, as suggested by the total absence of COOH-terminal tryptic fragments from the mass spectra. This is also supported by the calculated theoretical pI of the 40-kDa NH₂-terminal fragment, which is 5.2.

Increased expression of PDI in response to iron overload can reflect two different processes. It can indicate either increased activity of collagen modifying the P4H holoenzyme or an upregulation of PDI itself, as a disulfide rearranging enzyme and chaperone. Considering the fact that the P4H α-subunit is an insoluble protein (32) and therefore most likely escapes 2-D electrophoretic separation, we determined the relative expression of the α1-subunit (the main form of the α-subunit in most cell types) at the mRNA level by quantitative PCR. The P4H α1-subunit mRNA level was not increased in response to iron; it was even markedly (2.5-fold) decreased compared with controls. Therefore, we assume that upregulation of PDI in iron-overloaded HepG2 cells indicates an increased demand of the disulfide rearranging activity of PDI itself, independent of P4H activity.

In addition to the truncated form of PDI, we found evident discrepancies between the observed molecular mass and pI and their theoretical counterparts of several other proteins. α-Glucosidase (spot 2) seemed to be markedly truncated from the COOH terminus, because its mass spectra contained only signals from NH₂-terminal peptides (not shown). Peptides corresponding to ∼490 amino acids from the COOH terminus were missing. The observed molecular mass (58 kDa) of the protein confirmed such a profound truncation of the peptide compared with the calculated theoretical molecular mass of intact α-glucosidase (107 kDa). The detected differences between the apparent and calculated isoelectric points in some of the identified proteins may reflect a posttranslational modification. Among them, phosphorylation is the most common and may be responsible for the pI discrepancy of TRIM28 or SLC25A13.

**DISCUSSION**

Iron-mediated organ damage is common in patients with iron overload diseases, namely, hereditary hemochromatosis. To obtain deeper insight into the poorly understood and complex cellular response to iron overload and consequent oxidative stress, we studied HepG2 cells, which are a well-established model of human hepatocytes. The human hepatoma cell
line HepG2 was exposed to a high concentration of iron, and the changes in protein expression were studied. In iron-loaded HepG2 cells, a 13-fold increase of cellular iron concentration was detected. Not surprisingly, such an iron burden initiates significant oxidative stress, as demonstrated by a substantial increase in oxidative damage of membrane lipids. This in turn triggers defense mechanisms of cell protection involving numerous proteins. We detected significant expression changes in

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**Fig. 2.** Two-dimensional electrophoretic separation of cell lysates from iron-loaded and control HepG2 cells. Isoelectric focusing performed on IPG strips pH 4–7 was followed by denaturing SDS-PAGE in a Tris-glycine buffer system. Gel analysis was performed with gel quadruplicates.

**Fig. 3.** Example of matrix-assisted laser desorption ionization (MALDI)-TOF mass spectra and identification of mitochondrial aldehyde dehydrogenase from spot 20.
21 proteins of 1,060 proteins observed on our gels and identified 19 of them. Eleven identified proteins were upregulated in response to iron overload, and eight proteins were downregulated. Interestingly, some of the identified proteins have not been previously implicated in iron overload and/or oxidative injury. All of the identified proteins are relatively hydrophilic, soluble molecules. Highly hydrophobic membrane proteins such as transferrin receptor or ferroportin precipitate during separation by the conventional 2-D electrophoresis and therefore are not present on the 2-D gels (26).

As expected, the expression of L-ferritin was substantially increased by iron overload in HepG2 cells. Most of the cellular iron can thus be safely stored in its ferric form inside the ferritin shell, and the iron-mediated cell injury is therefore partially buffered. However, even massively increased ferritin synthesis did not prevent oxidative stress and lipid peroxidation in iron-loaded HepG2. This was clearly demonstrated by the >20-fold increase in the levels of reactive lipid peroxides and aldehydes (TBARS).

PDI (the β-subunit of collagen P4H), which was found to be upregulated in response to iron overload in HepG2 cells, is a multifunctional protein. In addition to its role in collagen helix maturation, as already mentioned in the RESULTS, PDI plays important roles in corrective protein folding and as a cellular chaperone (33). Increased collagen secretion is a landmark of liver fibrosis in liver injury; therefore, we originally hypothesized that increased levels of PDI may reflect iron-mediated injury.

Table 2. Functional clustering of identified differentially expressed proteins

<table>
<thead>
<tr>
<th>Function</th>
<th>Proteins</th>
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<tbody>
<tr>
<td>Iron storage</td>
<td>L-Ferritin</td>
</tr>
<tr>
<td>Cell stress and defense, chaperones, or heat shock proteins</td>
<td>PDI, PDI5A, cytokeratin 8, cytokeratin 19, and lamin B1</td>
</tr>
<tr>
<td>Gene expression</td>
<td>TRIM28 and Lamin B</td>
</tr>
<tr>
<td>Energy metabolism</td>
<td>Enolase 1, enolase 2, alcohol dehydrogenase, and dihydrolipoamide dehydrogenase-binding protein</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>Laminin-binding protein</td>
</tr>
<tr>
<td>Cell organization</td>
<td>α-Actin</td>
</tr>
<tr>
<td>Protein metabolism and processing</td>
<td>PDI, PDI5A, α-glucosidase and 26S proteasome subunit 9</td>
</tr>
<tr>
<td>Cell cycle and apoptosis</td>
<td>Anamorsin</td>
</tr>
<tr>
<td>Other</td>
<td>Septin 2 and citrin</td>
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P4H induction. We tested the expression of the enzymatically active α1-subunit of P4H at the mRNA level in control and iron-overloaded HepG2 cells. However, α1-subunit mRNA was markedly decreased in response to iron overload, disproving our hypothesis. Therefore, we assume that the observed upregulation of PDI in iron-loaded human hepatoma cells reflects intrinsic functions of PDI, independent of P4H activity, namely, rearrangement of disulfide bridges. PDI is capable of catalyzing oxidation, reduction, and corrective disulfide isomerization (33). In mammalian cells, PDI is mostly present in its reduced form, which is consistent with a need for reducing or isomerase activity (33, 22). Under oxidative stress induced by iron overload, proteins are highly susceptible to cysteine oxidation and incorrect formation of disulfide bridges. Therefore, we assume that increased PDI concentrations compensate for a higher need of corrective disulfide isomerization in iron-overloaded HepG2 cells, where oxidative stress is significant. In concordance with our hypothesis, we found PDI-related protein 5 to be also upregulated by iron overload. This PDI homolog also resides in the endoplasmic reticulum and has disulfide isomerase activity (12).

Keratins are intermediary filament (IF) proteins, which can be denoted as “guardians of liver cells,” protecting this organ against mechanical and nonmechanical stress and injury (20). Hepatocytes normally express keratin 8 and keratin 18, but in human hepatoma cells, the presence of nonhepatocytic keratin 19 has been demonstrated in addition to the keratin 8-keratin 18 pair (34). Keratin mutations represent a risk factor in various diseases (14, 37), and overexpression and also overphosphorylation of liver keratins are considered to be markers of liver disease progression (30). Keratins play a vital, although not yet explained, role in prevention of toxic injury including oxidative damage. Mutations in keratin 18 have been clearly shown to predispose the mouse liver to oxidative injury and to increase levels of the lipid peroxidation product malondialdehyde (39). In response to iron overload, we observed increased expression of keratin 8 and keratin 19 in HepG2 cells. This is fully in accordance with the known protective role of keratins against oxidative stress. However, our results are the first direct evidence of increased keratin expression in response to iron overload in liver-derived cells. We hypothesize that keratins may play an important role in prevention of liver injury in iron overload diseases such as hereditary hemochromatosis.

The laminin receptor (nonintegrin 67-kDa laminin receptor) is a high-affinity laminin-binding protein. Laminins are a family of extracellular matrix proteins and represent the major noncollagenous glycoprotein found in the basement membrane. Laminins and the laminin receptor are involved in many physiological processes, such as cell attachment, cell migration, growth, and differentiation. Laminin (either intrinsically expressed or added to media) has been recently demonstrated to induce the expression of keratin 19 in hepatoma cells in culture. This process is thought to involve the laminin receptor (29). We observed increased expression of the laminin receptor as well as of keratin 19 in response to iron overload. Therefore, we presume that there is a direct link of signal events connecting iron overload with increased expression of the laminin receptor and increased production of the cellular antistress protein keratin 19 in iron-loaded HepG2 cells.

Along with keratins, other components of the cytoskeleton, γ-actin and lamin B1, showed increased expression in iron-overloaded HepG2 cells. Expression of γ-actin has been recently shown to be stimulated in response to liver and pancreas injury in mice (28, 38), suggesting that along with keratins, actin induction may serve important protective role in liver and pancreatic injury. Our observation adds more evidence for the presumed protective function of γ-actin in the liver and suggests its involvement in the defense against oxidative stress.

Lamin B1 belongs to the lamin family of cytoskeletal IF proteins. Lamins support the nuclear envelope and provide anchorage sites for chromatin. Recently, laminas have also been shown to be involved in a number of other functions including transcription, apoptosis (8), and the heat shock response (40). So far, laminas have not been connected with iron metabolism.

The endoplasmic reticulum enzyme α-glucosidase (glucosidase II α-subunit) was upregulated by iron overload. Glucosidase II plays a key role in processing of NH2-linked oligosaccharide chains of glycoproteins and seems to be involved in the quality control mechanism of glycoprotein folding (23). So far, nothing is known about the α-glucosidase connection with iron overload or oxidative stress. However, increased demand for glycoprotein folding control in oxidatively stressed cells can be, at least, envisioned. On the other side, no obvious connection can be linked between iron overload and another upregulated protein, SLC25A13, also known as citrin. Citrin is an aspartate/glutamate mitochondrial carrier protein mutated in patients with adult-onset type II citrullinemia (25).

In addition to the 11 proteins with increased expression, we identified 8 proteins that were downregulated by iron overload. Among them was the transcriptional regulator KAP-1 (Trim28, TIF-1b). This protein is a universal coexecutor for a large family of transcription factors, the KRAB domain-containing zinc finger proteins (7). KAP-1 exerts its corepressive function in association with various proteins, for instance, with the chromatin remodeling protein HP-1 (24), histone deacetylase complexes (31), and von Hippel-Lindau-associated KRAB-A domain-containing protein (15). We demonstrated that expression of KAP-1 was decreased in iron-loaded cells. Because KAP-1 is a versatile molecule engaged in several regulatory processes, the meaning of its downregulation in response to iron and oxidative stress may be very complex and remains to be elucidated.

A very exciting finding in the present study was the identification of protein spot 14, which was downregulated by iron. In this spot, we identified a human homolog of anamorsin, a cytokine-induced apoptosis inhibitor recently discovered in mice. So far, nothing is known directly about the human anamorsin function and expression; we can only analogize based on the mouse homolog. Shibayama et al. (27) showed that mouse anamorsin is a critical antiapoptotic protein essential for hematopoiesis. We presume that reduced expression of the human anamorsin homolog in response to iron and oxidative stress can signal an increased proapoptotic trend in oxidatively damaged cells. To our knowledge, our observation is the first direct piece of information provided on human anamorsin protein expression and function.

During periods of oxidative stress, irreversible protein oxidation may become a threat to cell survival, unless the altered proteins are rapidly recognized and degraded by the proteasome. On the other hand, oxidative stress rapidly inactivates the proteasome in intact cells (4). The molecular mechanism of the proteasome inactivation is not clear. However, oxidative
stress induced in vivo by treatment with ferric nitritoacetate in the kidney resulted in an impairment of proteasome function caused by covalent binding of reactive products of lipid peroxidation to the proteasome (19). Our observation of decreased expression of 26S proteasome subunit 9 in response to the iron overload in HepG2 cells can reflect another mechanism (or a consequence) of 26S proteasome inactivation by iron-related oxidative stress.

Dihydrolipoamide dehydrogenase-binding protein (DDBP) was found to be downregulated by the experimental iron overload. DDBP is required for anchoring dihydrolipoamide dehydrogenase (E₃) to the dihydrolipoamide transacetylase (E₂) core of the pyruvate dehydrogenase complexes of eukaryotic mitochondria. DDBP deficiency causes pyruvate dehydrogenase deficiency, which results in delayed development and lactic acidosis in patients (OMIM 608769). We hypothesize that the observed decreased expression of protein DDBP in iron-overloaded HepG2 cells leads to a partial pyruvate dehydrogenase deficiency that causes depletion of NADH, which in turn can lead to decreased ATP production by mitochondria. This is in agreement with the disturbances of mitochondrial oxidative metabolism and decreased ATP production observed in cells exposed to excess iron (1).

Decreased expression of mitochondrial aldehyde dehydrogenase (ALDH2) in HepG2 cells exposed to excess of iron is most likely documents one of the key mechanisms involved in iron-mediated liver injury. ALDH2 plays a major role in acetaldehyde detoxification, and its deficiency increases vulnerability to oxidative stress (18). The lipid peroxidation product malondialdehyde, which is normally metabolized by ALDH2, has been shown to be also a potent inhibitor of the enzyme (11). We showed that iron overload substantially increases levels of TBARS, including malondialdehyde, in HepG2 cells and causes a decline of ALDH2. Our result are in agreement with previously observed decreased activities of ALDH2 in iron-overloaded liver cells (2).

The important glycolytic enzymes enolase 1 and enolase 2 were downregulated in iron-loaded HepG2 cells. Expression of human enolase-1 has been previously shown to be iron regulated (35, 36). Enolase 2 is considered to be neuron specific; however, evidence for non-neural expression exists in tumor tissues including liver carcinoma (5). The fact that levels of the two glycolytic enzymes are decreased in iron-overloaded cells indicates iron-related changes in glucose metabolism.

In spot 21, septin 2 was shown to be downregulated by iron. Septins are GTP-binding and filament-forming proteins implicated in diverse roles including microtubule and actin function, membrane association and vesicle trafficking, exocytosis, and even apoptosis (9). No obvious link is known between iron overload and septins, but any of the above-mentioned cellular events involving septins can be theoretically altered by iron overload or oxidative stress.

In summary, by employing proteomic approaches, we identified 19 proteins differentially expressed by iron overload and the consequent oxidative stress in human hepatoma cells. To our knowledge, this is the first attempt to use modern proteomic techniques for gaining a deeper understanding of iron overload in mammalian cells and its cellular consequences and molecular pathophysiology.

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GRANTS
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