The role of iron-dependent oxidative metabolism in protecting the oxidable substrates contained in mature adipocytes is still unclear. Because differentiation increases ferritin formation in several cell types, thereby leading to an accumulation of H-rich isoferritins, we investigated whether differentiation affects iron metabolism in 3T3-L1 pre-adipocytes. To this aim, we evaluated the expression of the genes coding for the H and L ferritin subunits and for cytoplasmic iron regulatory protein (IRP) during the differentiation of 3T3-L1 cells in adipocytes induced by the addition of isobutylmethylxanthine, insulin, and dexamethasone. Differentiation enhanced ferritin formation and caused overexpression of the H subunit, thus altering the H/L subunit ratio. Northern blot analysis showed increased levels of H subunit mRNA. A gel retardation assay of cytoplasmic extract from differentiated cells, using an iron-responsive element as a probe, revealed enhanced RNA binding capacity of IRP1, which correlated with the increase of IRP1 mRNA. The observed correlation between differentiation and iron metabolism in adipocytes suggests that an accumulation of H-rich isoferritin may limit the toxicity of iron in adipose tissue, thus exerting an anti-oxidant function.

Ferritin, the intracellular protein required for iron storage, and transferrin, which transports iron into the cells through membrane-specific receptors, are the main proteins controlling cellular iron homeostasis. Ferritin has an approximate mass of 450 kDa and is composed of 24 subunits of two types, namely H and L, in any ratio (1). Changes in iron availability regulate ferritin expression primarily at translational level through specifically regulated protein-RNA interactions between iron regulatory proteins (IRPs)\(^1\) and iron-responsive elements (IREs) contained within the 3'-untranslated region of the H- and L-ferritin mRNA (2). When intracellular concentrations of iron are low, IRP binding to IRE cis-elements represses ferritin translation; and conversely, when intracellular concentrations of iron are high, IRP is unable to bind IRE, and ferritin mRNA is efficiently translated (3).

Two distinct IRPs have been identified: IRP1 and IRP2 (for recent reviews, see Refs. 4–7). IRP1 has been identified as the cytosolic counterpart of aconitase, a key enzyme in the mitochondrial citric acid cycle (8, 9). We have recently shown that its RNA binding activity is inhibited by oxalomalate, a competitive inhibitor of aconitase (10). IRP2 has a different pattern of tissue specificity (11) and binds IRE-containing mRNA with an affinity similar to that of IRP1 (12).

Ferritin synthesis is stimulated during development, cellular differentiation, and inflammation, as well as by some hormones and cytokines (13). With the aim of evaluating the role of iron metabolism on the protection of the highly concentrated oxidable substrates in adipocytes, we investigated the expression of the genes encoding the H- and L-ferritin subunits and IRP1 during differentiation of 3T3-L1 cells to adipocytes. We determined the levels of H- and L-subunits of ferritin, the levels of mRNA coding for the H- and L-subunits of ferritin, as well as the level of mRNA coding for IRP1. The RNA binding activity of IRP1 has been also evaluated. Our data show that the differentiation of 3T3-L1 cells in adipocytes is associated with a consistent increase of ferritin levels and of ferritin mRNA, suggesting a close correlation between iron metabolism and adipocyte differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Treatments—**3T3-L1 fibroblasts obtained from the European Collection of Cell Cultures were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (BioWhittaker), penicillin (50 units/ml), and streptomycin (50 μg/ml). The cells were grown at 37 °C in a humidified 5% CO\(_2\) atmosphere. We treated confluent cultures with 0.5 mM 3-isobutyl-1-methylxantine, 0.25 mM dexamethasone, and 10 μg/ml insulin (all from Sigma) to promote the differentiation of 3T3-L1 cells into adipocytes (14). After 2 days, the 3-isobutyl-1-methylxanthine and dexamethasone were removed, and insulin was continued for another 2 days. The medium containing insulin only was replenished at 2-day intervals until adipocyte differentiation. Iron treatment consisted of incubating 3T3-L1 adipocytes for 20 h with 100 μM ferric ammonium citrate. Intracellular iron was chelated by treating 3T3-L1 adipocytes for 20 h with 100 μM desferrioxamine (Desferal, Ciba-Geigy). For cytoprotection experiments, 3T3-L1 adipocytes were pretreated with 5 μM hemin or with 0.3 mg/ml apoferritin for 18 h.

**Preparation of Cytoplasmic Extracts—**3T3-L1 cells were washed and harvested in PBS containing 1 mM EDTA. To obtain cytoplasmic extracts, cells were treated with lysis buffer (10 mM HEPES, pH 7.5, 3 mM MgCl\(_2\), 40 mM KCl, 5% glycerol, 1 mM dithiothreitol, and 0.2% Nonidet P-40) at 4 °C. Cell debris and nuclei were pelleted by centrifugation at 13,000 rpm for 5 min at 4 °C, and supernatants were stored at −80 °C. The protein concentration was determined by the Bio-Rad protein assay according to the supplier’s manual.

**Western Blot Analysis—**For Western blot analysis, lysate aliquots...
containing 50 or 100 μg of proteins were denatured, separated on a 12% SDS-polyacrylamide gel, and transferred to nitrocellulose membranes using a Bio-Rad Transblot. Proteins were visualized on the filters by reversible staining with Ponceau-S solution (Sigma) and destained in PBS. Filters were blocked in milk buffer (1× PBS, 10% nonfat dry milk, 0.1% Triton X-100) and incubated for 2 h with anti-human ferritin antibodies (Sigma). Subsequently, the membranes were incubated for 1 h with horseradish peroxidase-conjugated donkey anti-rabbit Ig (Amersham Pharmacia Biotech), and the resulting complex was visualized using chemiluminescent Western blotting detection reagents (ECL, Amersham Pharmacia Biotech).

RESULTS

Northern Analysis—Total cellular RNA was isolated by the guanidinium-thiocyanate-phenol-chloroform single-step extraction method (15). For Northern blots, 10 μg of total RNA were fractionated on a 1.5% agarose formaldehyde gel. RNA was transferred by blotting in 3 M NaCl, 0.3 M sodium acetate to Hybond-N filters (Amersham Pharmacia Biotech). The RNA was hybridized using 0.5 μg sodium phosphate buffer, pH 7.2, 1 mM EDTA, pH 8.0, 7% (w/v) SDS for 16 h at 65 °C. Filters were washed twice at room temperature in 40 mM sodium phosphate buffer, pH 7.2, 1% (w/v) SDS (15 min each) followed by a wash at 65 °C for 20 min, and the filters were autoradiographed at −80 °C with an intensifying screen. cDNA fragments (H-ferritin 36.8 clone, L-ferritin L-600 clone, adipisin, IRP1, and C/EBP) were radiolabeled using the random priming method.

Reverse Transcriptase-Polymerase Chain Reaction Amplification and Cloning of cDNA for Mouse IRP1—On the basis of the mouse aconitase/IRP1 sequence (16) (GenBank™ accession number X61147), two oligonucleotide primers were synthesized: (a) 5′-CAATGAGGATCCTATCT-GCTACTGAATTTAGG-3′ and (b) 5′-CAATGAGCATGCGATGCCTC-ATTTGGGAAGTA-3′. These primers were used to amplify a 642-base pair segment of cDNA encoding IRP1 from a cDNA synthesized by reverse transcriptase, using total RNA from 3T3-L1 cells as a template. The specific IRP sequence primers were flanked by BamHI (underlined in primer a) and SphI (underlined in primer b) sites so that the amplified sequence could be cloned in a pGEM-4Z vector.

RNA-Protein Band Shift Assay—Plasmid pSPT-fer containing the sequence corresponding to the HE of the IRE of human ferritin was kindly provided by Prof. G. Cairo (University of Milan). This plasmid was linearized at the BamHI site and transcribed in vitro with T7 RNA polymerase (Promega). The transcription reaction was performed at 38.5 °C for 1 h with 200 ng of plasmid DNA, 50 μCi of [α-32P]CTP (800 Ci/mM) (Amersham Pharmacia Biotech), and 0.5 mM ATP, GTP, and UTP (Promega) in a 20-μl reaction volume. The DNA template was digested with 10 units of RNase-free DNase I for 10 min at 37 °C. Free nucleotides were removed on a Sephadex G-50 column (Roche Molecular Biochemicals).

Band shift analysis was used to measure the interaction between IRPs and IREs using established techniques (17). Briefly, 5 μg of protein extracts were mixed with 0.2 ng of in vitro transcribed 32P-labeled IRS RNA with or without unlabeled competitor RNA. The reaction was performed in lysis buffer (10 mM Hepes, pH 7.5, 3 mM MgCl2, 40 mM NaCl, 5% glycerol, 1 mM dithiothreitol, and 0.07% Nonidet P-40) in a final volume of 20 μl for 30 min at room temperature. To recover the in vitro total IRP1 activity, 2% 2-mercaptoethanol (2-ME) was added to the binding reaction before the addition of 32P-labeled IRS RNA. To degrade unbound probe, the reaction mixture was incubated with 1 unit of RNase T1 (Calbiochem) for 10 min, and the nonspecific RNA-protein interaction was displaced by the addition of 50 μg/ml heparin for 10 min. After the addition of 10 μl of loading buffer containing 30 μl Tris-HCl, pH 7.5, 40% (w/v) sucrose, 0.2% bromophenol blue, the reaction mixtures were electrophoresed for 2 h at 200 V in a 6% nondenaturing polyacrylamide gel, pre-electrophoresed for 20 min at the same voltage. The dried gel was autoradiographed at −80 °C. The IRP:IRE complexes were quantified with a GS-700 imaging densitometer (Bio-Rad).

Lipid Peroxidation Assay—Lipid peroxidation products from mature adipocytes were measured by the thiobarbituric acid colorimetric assay (18). Briefly, after hemin or apoferritin treatment, cells were washed three times with 1× PBS, incubated with 20 μg/ml ferric ammonium citrate for 2 h at room temperature, then washed once more and scraped in 1× PBS containing 0.5 mM EDTA and 1.13 mM butylated hydroxytoluene. Cell lysis was performed by means of six cycles of freezing and thawing. To 450 μl of cellular lysate was added 1 ml of 10% (w/v) trichloroacetic acid. After centrifugation at 3000 rpm for 10 min, 1.3 ml 0.5% (w/v) thiobarbituric acid were added, and the mixture was heated at 100 °C for 20 min. After cooling, malondialdehyde formation was recorded at 532 nm (nm) and 550 nm in a PerkinElmer LS-5B spectrophotometer. The results are presented as picomoles of malondialdehyde/mg of cell protein, determined by the Lowry method. The ferritin content was determined using a fluorometric enzyme immunoassay system according to the supplier’s manual (Enzymum test, Roche Molecular Biochemicals). The results are expressed as ng of ferritin/mg of cell protein.

RESULTS

Differentiation of 3T3-L1 Cells to Adipocytes—3T3-L1 pre-adipocytes are among the most fully characterized models of in vitro cell differentiation. Exposure to 3-isobutyl-1-methylxanthine, insulin, and dexamethasone induces 3T3-L1 cells to differentiate into mature adipocytes (14). This process is accompanied by the expression of C/EBP, a family of transacting factors involved in the coordinated expression of adipocyte genes during differentiation (15).

In our study, 3T3-L1 cells, following treatment with 3-isobutyl-1-methylxanthine, insulin, and dexamethasone, acquired the rounded morphology characteristic of adipose cells and accumulated lipids, as observed by phase-contrast microscopy and by lipid staining of cultures (data not shown). We then isolated total RNA from adipocytes and analyzed it by Northern blot using C/EBP and adipisin cDNA probes, the latter protein being an adipocyte differentiation-dependent serine protease (20). The β-actin probe was used to standardize the amounts of mRNA in each lane. cDNA fragments were radiolabeled by the random priming method. The position of the ribosomal 18 and 28 S RNA are shown. These data are typical of three experiments.
Hybridized with random-primed 32P-labeled cDNA for L-ferritin and were electrophoresed on a 1.5% agarose-formaldehyde gel and blotted with antiserum at a 1:500 dilution, and proteins were detected by chemiluminescence Western blotting detection reagents. The iron metabolism in adipocytes which contains a high level of oxygen radical formation. Because IRPs are involved in the translational regulation of ferritin genes in various cell types, we measured IRP1 mRNA levels in mature adipocytes and its binding capacity to the specific mRNA. Fig. 3A shows the results of a Northern blot analysis of mRNA extracted from adipocyte precursors and differentiated adipocytes using a mouse IRP1 cDNA fragment as a probe (see “Experimental Procedures”). IRP1 mRNA was present in both samples, but its levels were clearly increased in differentiated adipocytes.

We also evaluated the IRP-IRE binding activity using an RNA band shift assay. A 32P-labeled ferritin IRE RNA transcript, containing the 5’-untranslated region of the human apoferritin H-chain (bases 31–58), was incubated with cytoplasmic extracts from undifferentiated and differentiated adipocytes; the results are shown in Fig. 3B. Only an RNA-protein band corresponding to the IRP1-IRE complex was observed. The binding activity of IRP1 was higher in differentiated adipocytes, thus correlating with the increased levels of the mRNA (see Fig. 3A). Moreover, a longer autoradiographic exposure of a gel (not shown) revealed a faster migrating band, which corresponded to the IRP2-IRE complex. The IRP2-IRE complex was more abundant in differentiated adipocytes, although it was about 10% of the IRP1-IRE in the same lane. To verify that the faster migrating band was indeed IRP2, we treated adipocytes with iron and desferrioxamine, a potent iron chelator. The IRP2-IRE band disappeared in iron-replete cells (Fig. 3C, lane 4) and was present in desferrioxamine-treated cells (lane 3), which is in accordance with iron regulation of IRP2 (21).

To evaluate the correlation between IRP1 mRNA level and IRP1 RNA binding activity, we assayed cytoplasmic extracts in the presence of 2-ME. The addition of high concentrations of this reducing agent to cell extracts converts s-actinase, when present, into the fully activated IRP1 form, thereby revealing the total amounts of IRP1 (22). The results of RNA band shift analysis performed with and without 2% 2-ME are shown in Fig. 3D. The addition of the reducing agent enhanced the RNA binding capacity of either undifferentiated or differentiated 3T3-L1 lysates. Imaging densitometry (Fig. 3D, lower panel) revealed that the total level of IRP1 in the differentiated adipocytes was also increased, thus correlating with the increased level of its mRNA.

**Ferritin Protects Adipocytes from Lipid Peroxidation**—To establish whether the increased ferritin expression observed following adipocyte differentiation was related to endogenous iron sequestration, as part of an antioxidant strategy we evaluated lipid peroxidation, assessed by quantification of thiobarbiturate-reactive material in mature adipocytes exposed to iron challenge. Mature adipocytes were pre-incubated with apoferritin (0.3 mg/ml), which is easily pinocytosed by the cell, or with hemin (5 μM), a known inducer of ferritin synthesis (23), and were then treated with ferric ammonium citrate (20 μg/ml). Treatment with iron for 2 h caused a remarkable degree of lipid peroxidation (Fig. 4), whereas preincubation of the cells with exogenous apoferritin and with hemin reduced the iron-induced lipid peroxidation to 15 and 20%, respectively. Consequently, the increased synthesis of the iron-scavenging ferritin in adipocytes is indeed related to antioxidant cellular defense, since ferritin interrupts the reaction sequence leading to lipid peroxidation and cell damage by removing the iron ions from the site of oxygen radical formation.

**DISCUSSION**

The role of iron metabolism in adipocytes which contain a high concentration of oxidation-sensitive substrates, is obscure. In the present study, we have evaluated the modifications in the
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expression of the genes encoding proteins involved in the regulation of iron metabolism. We demonstrate that differentiation of 3T3-L1 cells to adipocytes increases H- and L-ferritin subunit mRNA levels and the expression of the protein; however, accumulation of the H subunit seems to occur preferentially. These findings are in agreement with reports showing that H-ferritin mRNA is increased in various differentiation processes (24–27), as a consequence of a selective transcriptional regulation of the H subunit gene needed to produce ferritin with a structure appropriate to a differentiated cell type. In fact, it has been proposed that the accumulation of H-rich isoferritin during the maturation of erythropoietic cells affects the intracellular distribution and availability of iron for heme synthesis (25, 26). Furthermore, the differential transcriptional regulation of H-ferritin in absence of a change in L-ferritin, as observed in pre-adipocytes and premyoblasts in response to tumor necrosis factor (27), results in a change in the total amount of ferritin, mainly in its subunit composition. Also, differentiation of 3T3-L1 pre-adipocytes leads to an up-regulation of the H-ferritin expression similar to that observed in tumor necrosis factor-stimulated cells. It is possible to predict that in these cells the H-ferritin may contribute to a rapid chelation of iron, thus protecting cells from iron-induced oxidative injury. It has been demonstrated that cultured endothelial cells briefly pulsed with heme became highly resistant to oxidant-mediated injury and to the accumulation of endothelial lipid peroxidation products (28). The results of our experiments with exogenously added apoferritin or with hemin-induced ferritin (see Fig. 4) demonstrate that ferritin remarkably decreased iron-induced lipid peroxidation. It is feasible that the overexpressed ferritin plays a role as an iron cytoprotective agent by limiting the reactivity of intracellular iron on lipids in adipocytes themselves, where lipids are biosynthesised. Thus, H-ferritin overexpression may represent an adaptive adipocyte response to iron-induced oxidative stress.

We also observed an increased expression of the IRP1 gene in mature adipocytes, which correlates with the increase of the RNA binding activity of IRP1 to IRE in ferritin mRNA. We also show that IRP2 occurs in 3T3-L1 adipocytes, albeit at much lower levels than IRP1. To our knowledge, this is the first demonstration that adipose cells contain IRPs. We found that the IRP2-IRE complex was more relevant in differentiated adipocytes (Fig. 3C, lane 3). Because iron enhances IRP2 degradation (21), we hypothesize that the increased expression of IRP2 could be consequent to the intracellular iron sequestration by H-ferritin, as well being overexpressed in differentiated adipocytes from lipid peroxidation. 3T3-L1 adipocytes were incubated with 5 μM hemin or with 0.3 mg/ml apoferritin for 18 h at 37 °C before exposure to 20 μg/ml desferrioxamine (lane 3) or treated for 20 h with 100 μg/ml FeSO₄ (lane 4). Arrows indicate IRP1, IRP2, and free RNA. Panel D, cytosolic extract (5 μg) prepared from undifferentiated and differentiated 3T3-L1 cells were incubated with 0.2 ng of 32P-labeled IRE RNA for 30 min at room temperature. To reveal the total activable amounts of IRP1, 2% 2-ME was added to the RNA binding reaction 10 min before the addition of labeled IRE probe. The IRP-IRE complexes were resolved by electrophoretic mobility shift assay and quantified with an imaging densitometer. The relative activities are plotted as arbitrary units. The data represent typical results obtained in at least four independent experiments. The error bars indicate standard deviations.

FIG. 3. IRP1 mRNA level (A) and RNA binding activity (B–D) in 3T3-L1 differentiation to adipocytes. Panel A, Northern blot analysis of 10 μg of cytoplasmic RNA isolated from undifferentiated (U) and differentiated (D) 3T3-L1 cells. The blot was sequentially hybridized with IRP1 cDNA and β-actin cDNA probes. The position of the ribosomal 18 and 28 S RNA are shown. Panels B–D, RNA-band shift assays were performed with 5 μg of cytoplasmic proteins and a excess of 32P-labeled IRE probe. After the addition of RNase T1, and heparin, RNA-protein complexes were resolved on nondenaturing 6% polyacrylamide gel. Extracts for the experiments shown in panel B were prepared from undifferentiated 3T3-L1 cells and differentiated 3T3-L1 cells. Extracts for the experiments shown in panel C were from differentiated 3T3-L1 cells treated for 20 h with 100 μg/ml desferrioxamine (lane 3) or treated for 20 h with 100 μg/ml FeSO₄ (lane 4). Arrows indicate IRP1, IRP2, and free RNA. Panel D, cytosolic extract (5 μg) prepared from undifferentiated and differentiated 3T3-L1 cells were incubated with 0.2 ng of 32P-labeled IRE RNA for 30 min at room temperature. To reveal the total activable amounts of IRP1, 2% 2-ME was added to the RNA binding reaction 10 min before the addition of labeled IRE probe. The IRP-IRE complexes were resolved by electrophoretic mobility shift assay and quantified with an imaging densitometer. The relative activities are plotted as arbitrary units. The data represent typical results obtained in at least four independent experiments. The error bars indicate standard deviations.

FIG. 4. Ferritin protects 3T3-L1 adipocytes from lipid peroxidation. 3T3-L1 adipocytes were incubated with 5 μM hemin or with 0.3 mg/ml apoferritin for 18 h at 37 °C before exposure to 20 μg/ml ferric ammonium citrate (FAC) for 2 h. Cells were lysed, and lipid peroxidation was measured by a thiobarbituric acid colorimetric assay (see “Experimental Procedures”). Ferritin concentration was determined by the enzyme immunosassay system; the results are expressed as ng of ferritin/mg of cell protein. The data represent results obtained in at least three independent experiments; the error bars indicate standard deviations.
adipocytes. The results obtained in cells treated with dexferrioxamine support this hypothesis (Fig. 3C).

The RNA binding activity assays in the presence of 2-ME (see Fig. 3D) confirmed that differentiation results in an increase IRP1 protein expression. In addition, the RNA binding activity in the presence of the reducing agent may include a fraction of IRP1 resulting from the conversion of a pre-existing c-aconitase form in differentiated adipocytes.

It is generally agreed that increased IRP1 binding activity prevents ferritin biosynthesis. We have shown that there is a concomitant increase of ferritin and IRP1 RNA binding activity in differentiated adipocytes. These apparently contradictory data can be explained in the light of the following considerations: (i) increased transcription of the IRP1 gene induced by differentiation leads to major expression of the protein; (ii) 3T3-L1 cells, which undergo adipose conversion in vitro, possess an NADPH-dependent \( \text{H}_2\text{O}_2 \)-generating system in their plasma membranes (29). Activation of IRP1 binding capacity by \( \text{H}_2\text{O}_2 \) is a well documented phenomenon (30); (iii) overexpression of H-ferritin subunits leads to rapid chelation of the intracellular iron pool, thereby preventing the formation of a [4Fe-4S] cluster and thus contributing to the maintenance of IRP1 RNA binding activity.

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