

Iron-Loading of Cultured Adult Rat Hepatocytes Reversibly Enhances Lactoferrin Binding and Endocytosis

DOUGLAS D. MCABEE* AND YUAN YUAN LING

Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana

Isolated rat hepatocytes bind and internalize bovine lactoferrin (Lf) protein and Lf-bound Fe^{3+} via Ca^{2+} -dependent recycling Lf binding sites (McAbee, 1995, *Biochem. J.*, 311:603–609). In this study, we determined if iron loading of primary cultures of adult rat hepatocytes altered their ability to bind and internalize Lf. Rat hepatocytes were cultured 16–24 h with or without ferric ammonium citrate (FAC) and then assayed for Ca^{2+} -dependent ^{125}I -Lf binding at 4°C or ^{125}I -Lf endocytosis at 37°C. Cells pretreated with FAC (5 $\mu\text{g}/\text{mL}$) internalized two- to sixfold more ^{125}I -Lf than did control cells. The FAC-induced increase in ^{125}I -Lf endocytosis required 4–8 h of culture at 37°C and was fully reversible if cells were incubated an additional 24 h without FAC either in the presence or absence of the Fe^{3+} chelator desferrioxamine. Maximal endocytic rates for untreated and FAC-treated cells were 370 and 2,300 molecules ^{125}I -Lf cell $^{-1}$ sec $^{-1}$, respectively. Both ^{125}I -Lf binding at 4°C and endocytosis at 37°C increased up to sixfold between 0.3–10 $\mu\text{g}/\text{mL}$ FAC, indicating that iron-induced enhancement of ^{125}I -Lf uptake was due to an increase in the number of Lf receptors present on the cells. ^{125}I -Lf bound to untreated and FAC-treated cells at 4°C with similar affinities ($K_d \sim 1.5 \mu\text{M}$). Cycloheximide but not actinomycin D blocked the FAC-induced increase in ^{125}I -Lf binding, indicating that the increase in the number of Lf binding sites required translation but not transcription. Notably, iron loading blocked endocytosis of asialoorosomucoic acid by hepatocytes by up to 80%, reducing the number of active intracellular asialoglycoprotein receptors >65% without altering the number of active cell surface receptors. We conclude from these studies that Lf receptor activity on hepatocytes is regulated posttranscriptionally by the iron status of the cells. **J. Cell. Physiol.** 171:75–86, 1997. © 1997 Wiley-Liss, Inc.

Eukaryotic cells acquire iron by receptor-mediated endocytosis of Fe^{3+} -transport proteins such as transferrin (Klausner et al., 1983) or by facilitated transport of Fe^{3+} or Fe^{2+} at the plasma membrane (Kaplan et al., 1991; Stearman et al., 1996). The steady-state amount of several proteins involved in iron acquisition and storage is regulated by prevailing cellular iron levels. These proteins are encoded by mRNAs that possess one or more iron-regulatory elements (IREs) which form stem-loop secondary structures. When cells are depleted of iron, iron-regulatory proteins (IRPs) bind IREs (for reviews see Klausner et al., 1993; Mascotti et al., 1995; Theil, 1994). Depending on the position of the IRE relative to the mRNA's coding region, binding of the IRP either represses mRNA translation (e.g., ferritin mRNA [Leibold and Munro, 1988; Rouault et al., 1988; Walden et al., 1988]) or stabilizes the mRNA for sustained translation (e.g., transferrin receptor mRNA [Casey et al., 1989]).

The liver is the primary iron-processing site in mammalian systems. Hepatocytes acquire nutrient and recycled iron by endocytosis of iron- and heme-binding proteins—transferrin, serum ferritin, lactoferrin (Lf), hemopexin, and haptoglobin—and by facilitated trans-

port (Morgan and Baker, 1986). We showed that isolated rat hepatocytes take up Lf protein and Lf-bound iron by clathrin-dependent endocytosis of Fe-Lf complexes mediated by a set of Ca^{2+} -dependent Lf binding sites that recycle during endocytosis (McAbee, 1995; McAbee et al., 1993). Circulating levels of Lf increase under a variety of circumstances including episodes of iron overload or inflammation (Levy and Viljoen, 1995). If Lf in blood functions as an iron-scavenging protein, then hepatic Lf clearance mechanisms may be subject to regulation by steady-state iron levels or by inflammatory signals. Recently, Spik and her colleagues reported that iron depletion of HT29 colon carcinoma cells with picolinic acid doubled the number of Lf binding sites on the apical surfaces of these cells (Mikogami et al., 1995). We have found that short-term treatment of isolated rat hepatocytes with the Fe^{2+} che-

Contract Grant sponsor: National Institutes of Health; Contract Grant number DK43355.

*Correspondence to: Douglas D. McAbee, Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556.

Revision received 5 November 1996; Accepted 7 November 1996

lator bathophenanthroline disulfonate enhanced subsequently the uptake of Lf protein and Lf-bound iron (McAbee, 1995). These findings suggest that Lf receptor activity on different cell types may be regulated by the iron status of cells. The purpose of this study, therefore, was to determine if hepatocyte iron status regulates endocytosis of Lf. Cultured hepatocytes from adult rats were loaded with iron by treatment with ferric ammonium citrate (FAC) and assayed subsequently for Ca^{2+} -dependent binding and internalization of Lf protein and Lf-bound iron. We found that hepatocytes treated with FAC increased both the binding and endocytosis of Lf in a reversible manner that required translation but not transcription.

MATERIALS AND METHODS

Materials

Actinomycin D, albumin (bovine fraction V [BSA]), amphotericin B, cycloheximide, desferrioxamine (DFO), dexamethasone, dextran sulfate (5,000 Da), ferric ammonium citrate (FAC), ferritin (rat liver), fetal bovine serum, gentamycin, L-glutamine, Hepes, insulin (bovine), and Lf (bovine colostrum) were purchased from Sigma (St. Louis, MO). Collagenase (Type D) was obtained from Boehringer-Mannheim (Indianapolis, IN). Na^{125}I (~17 Ci/mg of iodine) and $^{59}\text{Fe}(\text{II})$ -citrate were from DuPont New England Nuclear (Boston, MA). FeCl_3 standard solution was obtained from Fisher (Itasca, IL). All other chemicals were reagent grade. Asialoorosomucoid (ASOR), desialylated with neuraminidase as described elsewhere (Schachter et al., 1970), was a generous gift of Dr. P.H. Weigel (University of Oklahoma Health Sciences Center). William's E medium and Basal Medium Eagle's (BME) were obtained from Sigma. Hepes-buffered saline (HBS) contained 150 mM NaCl, 3 mM KCl, 10 mM Hepes, pH 7.4. BME was supplemented with 2.4 g/L Hepes, pH 7.4, and 0.22 g/L NaHCO_3 . BME-BSA is BME containing 0.1% (w/v) BSA.

Hepatocytes

Male Sprague-Dawley rats (150–350 g; Harlan Labs, Indianapolis, IN) were fed standard laboratory chow and water ad libitum. The iron content of the chow (Purina Rodent Laboratory Chow, formula 5001) was 198.0 ppm as assayed by the manufacturer. Hepatocytes were prepared by a modification of a collagenase perfusion procedure (Seglen, 1973) as described previously (McAbee and Esbensen, 1991). Cells were kept at ~30°C during the filtration and differential centrifugation steps. Final cell pellets suspended in ice-cold BME-BSA were ≥85% viable and single cells. Hepatocytes were plated on unmodified tissue culture plates at a density of ~1,000 cells/mm² in William's E medium supplemented with 5% (v/v) fetal bovine serum, 10 mM Hepes, 2 mM L-glutamine, 0.48 µg/mL insulin, 0.5 µM dexamethasone, 2.5 µg/mL amphotericin B, and 50 µg/mL gentamycin and incubated at 37°C in 5% CO_2 . Viability was determined microscopically by trypan blue exclusion. For cultures supplemented with FAC, cells were allowed to adhere to the culture plates in regular medium (~4 h, 37°C, 5% CO_2) and then incubated with culture medium supplemented with FAC as described in the figure legends.

Lactoferrin preparation

Bovine colostrum Lf (commercial preparation >90% pure electrophoretically) was further purified by DE-52 (Whatman, Clifton, NJ) ion-exchange chromatography as described previously (McAbee and Esbensen, 1991). Apo- and holo-Lf were prepared as described previously (McAbee and Esbensen, 1991), dialyzed against HBS, filter-sterilized (0.2 µm), and stored at -20°C prior to use. Lf's iron content determined spectroscopically (Hashizume et al., 1987) from its $A_{465\text{ nm}}:A_{280\text{ nm}}$ ratio was routinely ~0.002 and ~0.04, respectively, for apo-Lf and holo-Lf preparations. ^{125}I -Lf, prepared by the "Iodogen" method (Fraker and Speck, 1978), had specific activities of 12–36 dpm/fmol. ^{59}Fe -Lf was prepared as described previously (McAbee, 1995) and had a specific activity 1,100 dpm/pmol. Homogeneity of radiolabeled and unlabeled Lfs used for all binding studies was confirmed by SDS polyacrylamide gel electrophoresis and autoradiography. ^{59}Fe -Lf preparations were used within 3 weeks after labeling.

Ligand binding and endocytosis assays

Hepatocyte cultures were removed from CO_2 incubation, chilled on ice, and rinsed two or three times with ice-cold BME-BSA. Prior to incubation with radiolabeled Lf, culture surfaces were blocked for 15 min on ice with 8% (w/v) nonfat milk solids in HBS, rinsed thoroughly with cold HBS, and then kept in BME-BSA on ice until use. For Lf binding assays, ~10⁶ hepatocytes per 35 mm well were incubated with ^{125}I -Lf (2–5 µg/mL) at 4°C ≤120 min in 2 mL BME-BSA, buffer 1 (HBS, 5 mM CaCl_2 , 5 mM MgCl_2), or other buffer as designated in the legends. Media were discarded and replaced with 1 mL cold BME. Cells were removed from the dish using a Teflon scraper, transferred into glass tubes on ice, and pelleted by centrifugation (400g, 2 min, 4°C). Unless otherwise indicated, cells were washed free of unbound radiolabeled ligand by centrifugation in excess buffer 2 (buffer 1, 0.3% (w/v) dextran sulfate) twice, 10 min per wash. Washed cells were resuspended in 1.0 ml buffer 1 or buffer 2, transferred to clean plastic tubes, and assayed for cell-associated radioactivity. To measure hepatocyte endocytosis of ^{125}I -Lf and ^{59}Fe -Lf, ~10⁶ hepatocytes per 35 mm dish were incubated with radiolabeled Lf in BME-BSA at 37°C as described in the legends. Uptake was stopped by replacing incubation media with fresh ice-cold BME-BSA and placing the cells on ice. Cells were scraped off the dishes, transferred into glass tubes on ice, and pelleted by centrifugation (400g, 2 min, 4°C). Unless indicated otherwise, total cell-associated (surface and intracellular) Lf was assayed on cells washed by centrifugation twice in 3 ml of cold buffer 1 or buffer 2. Internalized Lf was assayed on cells washed by centrifugation twice, 10 min each wash, in 3 ml of buffer 3 (HBS, 5 mM EGTA, 0.3% (w/v) dextran sulfate) at 4°C. After washing, cells were resuspended in 1.0 ml buffer 2, transferred to clean plastic tubes, and assayed for cell-associated radioactivity. To determine the amount of radiolabeled Lf bound by the culture plates per se, culture dishes without cells were treated similarly and assayed for radioactivity; the amount of ^{125}I -Lf or ^{59}Fe -Lf detected was negligible. ^{125}I -ASOR binding at 4°C and endocytosis at 37°C was determined using proce-

dures similar to that described above for ^{125}I -Lf binding and endocytosis with some modifications. Cultured hepatocytes were incubated with ^{125}I -ASOR in buffer 1 on ice or in BME-BSA at 37°C as described in the figure legends. Following incubations, cells were scraped and washed free of unbound ^{125}I -ASOR by centrifugation in buffer 1 as described above and then assayed for cell-associated radioactivity.

Determination of hepatocyte non-heme iron content

Hepatocytes cultured 18 h with and without FAC were rinsed five times with ice-cold HBS on ice and then scraped off dishes in HBS, transferred to glass tubes, and pelleted by centrifugation; supernatants were discarded. The cells ($\sim 10^6$ /sample) were resuspended in 1.0 mL 2.8 N HNO_3 , transferred to microfuge tubes, heated 1 h at 90°C , and then centrifuged (13,000 rpm, 10 min, 4°C) to remove insoluble debris. Clarified supernatants were diluted into water to give a final HNO_3 concentration of 0.11 N and stored at 22°C in polypropylene tubes prior to assay. Non-heme iron content was measured by inductively coupled plasma mass spectroscopy using a VG PlasmaQuad II mass spectrophotometer (Fisons Instruments, Beverly, MA) with FeCl_3 as a standard (Notre Dame Center for Bioengineering and Pollution Control).

General procedures

Protein was determined by the bicinchoninic acid protein assay procedure using BSA as standard (Pierce Chemical Co., Rockford, IL). Hepatocytes contain 1.1 mg protein/ 10^6 cells. Centrifugation of cell suspensions was at 400g for 2 min at 4°C (Sorvall RT6000B centrifuge; DuPont Company, Wilmington, DE). ^{125}I -ASOR was prepared by the "Iodogen" method (Fraker and Speck, 1978) and had a specific activity of 46 dpm/fmol. ^{125}I and ^{59}Fe radioactivity were determined using a Packard Cobra Auto-Gamma Counting System (Model 5002; Packard Instrument Co., Downers Grove, IL). Osmolality was determined using a Wescor 5500 vapor pressure osmometer (Wescor, Inc., Logan, UT). Spectral analysis of Lf was performed using a Beckman (Fullerton, CA) DU-64 spectrophotometer.

RESULTS

Lf binding and endocytosis by cultured hepatocytes

We have documented elsewhere that isolated rat hepatocytes bind and internalize bovine Lf protein and its bound iron (McAbee, 1995; McAbee and Esbensen, 1991; McAbee et al., 1993). Manipulation of cellular iron content with subsequent changes in protein expression, however, cannot be reliably accomplished on nontransformed cells in suspension; this precluded our use of isolated rat hepatocytes in these studies. We chose, therefore, to examine the effects of iron loading on Lf endocytosis using primary cultures of adult rat hepatocytes. Isolated rat hepatocytes bind bovine Lf by Ca^{2+} -dependent and Ca^{2+} -independent sites; Lf bound to the former is endocytosed selectively by the cells (McAbee et al., 1993), whereas Lf bound to the latter is removed by washing with the polyanion dextran sulfate (McAbee and Esbensen, 1991). Thus, we determined if the Lf interaction with cultured hepatocytes resembled

TABLE 1. Lf binding to cultured hepatocytes: effect of divalent cations and dextran sulfate¹

| Binding Buffer | Wash buffer | ^{125}I -Lf bound (fmol/sample) |
|--|---|--|
| HBS | HBS | 300 \pm 28 |
| | HBS, DS | 39 \pm 11 |
| HBS, Ca^{2+} | HBS, Ca^{2+} | 1,376 \pm 352 |
| | HBS, Ca^{2+} , DS | 224 \pm 42 |
| HBS, Mg^{2+} | HBS, Mg^{2+} | 1,168 \pm 272 |
| | HBS, Mg^{2+} , DS | 66 \pm 8 |
| HBS, Ca^{2+} , Mg^{2+} | HBS, Ca^{2+} , Mg^{2+} | 701 \pm 55 |
| | HBS, Ca^{2+} , Mg^{2+} , DS | 218 \pm 44 |
| BME/BSA | HBS, Ca^{2+} , Mg^{2+} | 592 \pm 10 |
| | HBS, Ca^{2+} , Mg^{2+} , DS | 266 \pm 38 |
| | HBS, DS | 78 \pm 27 |

¹Primary cultures of adult rat hepatocytes were incubated with ^{125}I -Lf (2 $\mu\text{g}/\text{mL}$) in the indicated binding buffer for 90 min at 4°C , scraped off dishes, washed in a designated buffer at 4°C , and then assayed for bound radioactivity as described in Materials and Methods. Buffers containing divalent cations were supplemented with CaCl_2 or MgCl_2 at 5 mM. When present, dextran sulfate (DS) was at 0.3% (w/v). BME contains 1.8 mM Ca^{2+} and 0.8 mM Mg^{2+} . Values represent the mean \pm standard deviation of triplicate samples.

its interaction with isolated hepatocytes by assaying ^{125}I -Lf binding at 4°C to 24 h cultures of hepatocytes in various binding and wash buffers (Table 1). We found that $\sim 87\%$ of the ^{125}I -Lf bound in the absence of divalent cations was removed by washing with dextran sulfate. The presence of Ca^{2+} or Mg^{2+} stimulated ^{125}I -Lf binding to cells approximately fourfold, but dextran sulfate-washed cells retained significant amounts of ^{125}I -Lf only when Ca^{2+} was present. These data indicate that cultured hepatocytes, similar to isolated hepatocytes in suspension, bind Lf by Ca^{2+} -dependent and Ca^{2+} -independent sites, and the Ca^{2+} -dependent binding can be assayed specifically by washing the cells with Ca^{2+} and dextran sulfate.

To confirm that cultured hepatocytes internalized Lf, we incubated 24 h primary cultures of rat hepatocytes with ^{125}I -Lf at 4°C and 37°C (Table 2). As seen previously, inclusion of the polyanion dextran sulfate in the wash buffer reduced the total amount of ^{125}I -Lf bound to cells by $\sim 50\%$. For cells incubated with ^{125}I -Lf at 4°C —a temperature that precludes endocytosis—EGTA and dextran sulfate removed $\sim 80\%$ of the ^{125}I -Lf bound to the Ca^{2+} -dependent binding sites, indicating that such wash procedures stripped most of the surface-bound ^{125}I -Lf from these cells. For cells incubated with ^{125}I -Lf at 37°C , EGTA and dextran sulfate removed $\sim 20\%$ of ^{125}I -Lf from cells, indicating that cultured hepatocytes had internalized ^{125}I -Lf at 37°C . These data confirmed that internalized ^{125}I -Lf could be monitored biochemically to assess ^{125}I -Lf endocytosis by cultured hepatocytes following EGTA/dextran sulfate removal of surface-bound ^{125}I -Lf. In other experiments, we measured the amount of Lf bound by the cells over time as well as the evolution of acid-soluble degraded products released by the cells into the culture medium (Fig. 1). When cells were incubated in the continuous presence of ^{125}I -Lf, cell-associated ^{125}I -Lf (surface-bound and internal) increased with saturating kinetics over the 8 h time course. After 2 h, the amount of acid-soluble ^{125}I -degradation products released into the medium increased substantially at a linear rate of 0.88 pmol equivalents 10^6 cells $^{-1}$ h $^{-1}$. The total amount of ^{125}I -Lf processed by the cells (cell-associated and acid-soluble released) was linear throughout the time course

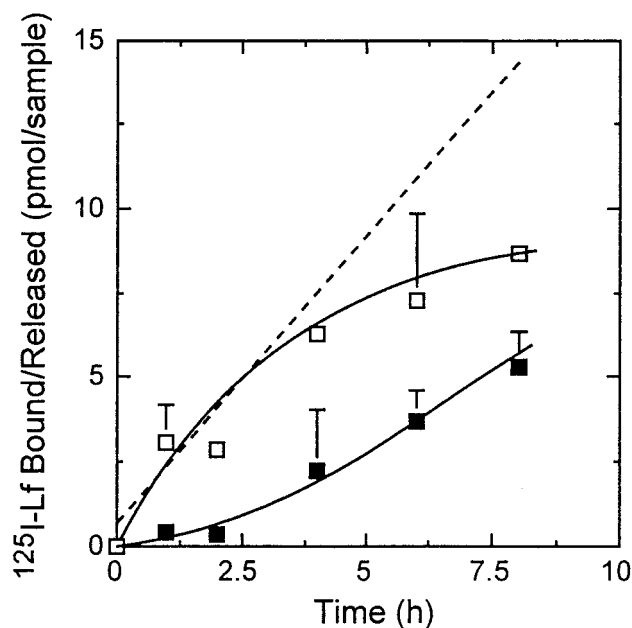


Fig. 1. Kinetics of endocytosis and degradation of ^{125}I -Lf. Overnight cultures of adult rat hepatocytes were incubated with ^{125}I -Lf (30 $\mu\text{g}/\text{mL}$) at 37°C . At the designated times, the cells were chilled on ice and conditioned medium collected. The cells were washed twice with ice-cold buffer 2, 10 min per wash, and then extracted with cold HBS supplemented with 0.1% (v/v) Triton X-100 for 15 min on ice; detergent-soluble material was assayed for radioactivity (\square). To measure degradation products released from the cells, 0.4 mL of conditioned medium was mixed with 0.8 mL of 10% (w/v) phosphotungstic acid in 2 N HCl on ice for ≥ 15 min, after which precipitated material was pelleted by centrifugation (13,000g, 5 min, 4°C); supernatants were assayed for acid-soluble radioactivity (\blacksquare). ^{125}I -Lf nonspecifically bound by the tissue culture plates as determined by parallel incubations carried out as above in cell-free dishes amounted to $\leq 20\%$ of that in the presence of cells and was subtracted from amounts obtained in the presence of cells to give cell-specific ^{125}I -Lf-bound or acid-soluble radioactivity. Total processed ^{125}I -Lf was calculated by adding specific cell-bound and acid-soluble released picomole equivalents (dashed line; $r = 0.99_{(n=5)}$). Values represent the mean of duplicate samples; standard deviations are shown by error bars.

TABLE 2. Binding and internalization of ^{125}I -Lf by cultured hepatocytes at 4 and 37°C ¹

| Temperature ($^\circ\text{C}$) | Wash buffer | ^{125}I -Lf bound (pmol/mg protein) |
|----------------------------------|-------------|--|
| 4 | Buffer 1 | 3.58 ± 0.23 |
| | Buffer 2 | 1.67 ± 0.36 |
| | Buffer 3 | 0.38 ± 0.13 |
| 37 | Buffer 1 | 5.84 ± 1.14 |
| | Buffer 2 | 2.87 ± 0.24 |
| | Buffer 3 | 2.28 ± 0.05 |

¹Primary cultures of adult rat hepatocytes were incubated with ^{125}I -Lf (5 $\mu\text{g}/\text{mL}$) in BME-BSA at the designated temperature for 1 h, after which the cells were scraped off the plates, washed in the designated buffer, and assayed for cell-associated radioactivity as described in Materials and Methods. Buffer 1: HBS, 5 mM CaCl_2 , 5 mM MgCl_2 ; buffer 2: buffer 1, 0.3% (w/v) dextran sulfate; buffer 3: HBS, 5 mM EGTA, 0.3% (w/v) dextran sulfate. Values represent the mean \pm standard deviation of duplicate samples.

(1.71 Lf pmol equivalents $\text{h}^{-1} 10^6$ cells⁻¹) (Fig. 1, dashed line). These data indicated that cultured adult hepatocytes bind, internalize, and degrade Lf in a manner similar to isolated hepatocytes in suspension.

We then determined if an extended incubation of cul-

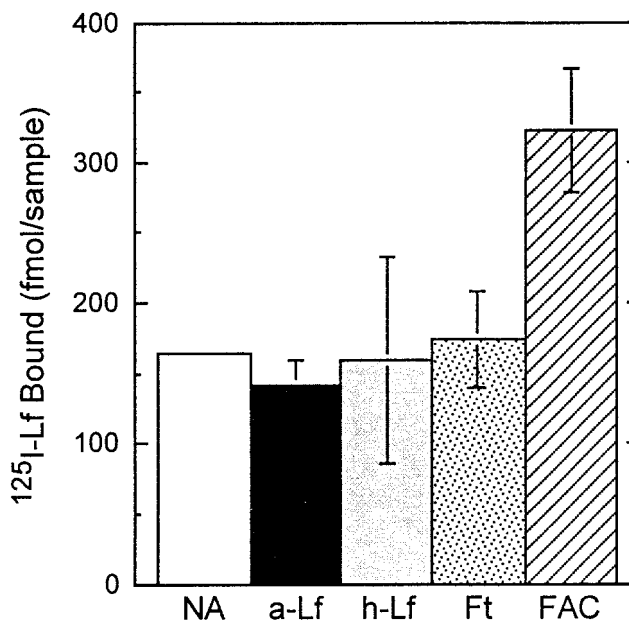


Fig. 2. Effect of Lf, ferritin, and FAC treatment on ^{125}I -Lf endocytosis. Adult rat hepatocytes were cultured 16 h in media with and without (NA, no additions) holo-Lf (h-Lf) (30 $\mu\text{g}/\text{mL}$), apo-Lf (a-Lf) (30 $\mu\text{g}/\text{mL}$), ferritin (Ft) (30 $\mu\text{g}/\text{mL}$), or FAC (5 $\mu\text{g}/\text{mL}$), after which the cells were chilled and washed five times with fresh, cold BME-BSA. The cells were then incubated with ^{125}I -Lf (2 $\mu\text{g}/\text{mL}$) for 60 min at 37°C and assayed for bound cell-associated radioactivity as described in Materials and Methods. Values represent the mean of duplicate samples; standard deviations are shown by error bars.

tured hepatocytes with iron-binding proteins or iron salts altered subsequently the cells' ability to take up Lf. Cells were incubated 16 h in the presence or absence of an excess of Lf, ferritin, or FAC, all of which have been shown to donate iron in significant amounts to rat hepatocytes (Kaplan et al., 1991; McAbee, 1995; Sibille et al., 1989; Wright et al., 1986). Hepatocytes cultured overnight in the presence of either iron-free or diferric Lf accumulated the same amount of ^{125}I -Lf as control cells (Fig. 2). It appears, therefore, that the Lf endocytic capacity of these cells does not undergo ligand-dependent modulation following prolonged incubation with Lf under these conditions. Similarly, overnight ferritin loading did not alter hepatocyte uptake of Lf, but hepatocytes incubated with FAC accumulated twofold more ^{125}I -Lf than did control cells. We found that FAC (5 $\mu\text{g}/\text{mL}$) abruptly increased endocytosis of ^{125}I -Lf in isolated rat hepatocytes after 4–8 h of incubation, after which no further increase in Lf uptake was observed (Fig. 3). When FAC-treated hepatocytes were cultured in FAC-free medium, their FAC-enhanced endocytic activity was partially reversed (Fig. 4). Inclusion of the Fe^{3+} chelator DFO in the FAC-free medium during the recovery incubation completely reversed the FAC-enhanced endocytosis. Notably, cells treated with DFO did not show less ^{125}I -Lf endocytic activity than untreated control cells. In other experiments, we found that incubation of non-iron-supplemented cultured hepatocytes with DFO (≤ 4 mM) for 16–24 h did not reduce ^{125}I -Lf endocytic activity (data not shown). These data, therefore, suggest that even in the absence of iron

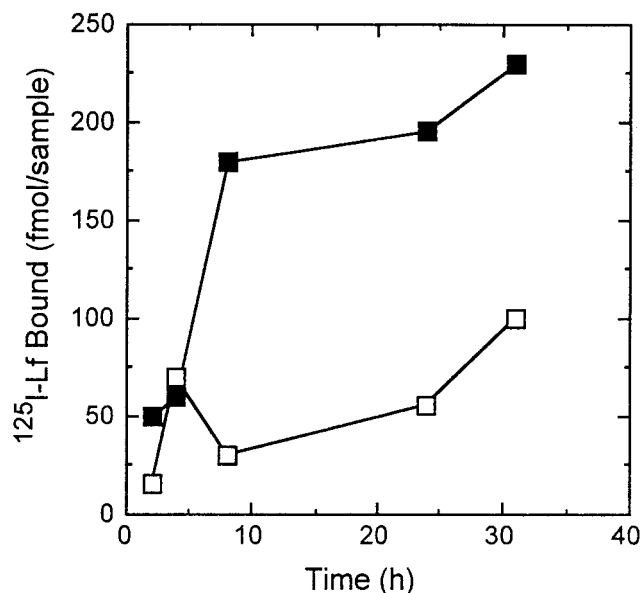


Fig. 3. Kinetics of FAC-induced increase in Lf endocytosis. Isolated hepatocytes were plated onto tissue culture plates ($\sim 10^6$ cells/35 mm well) in the presence (■) or absence (□) of FAC (5 $\mu\text{g}/\text{mL}$) and incubated at 37°C. At the designated times, cells were chilled on ice, washed with cold BME-BSA, and incubated with ^{125}I -Lf (2 $\mu\text{g}/\text{mL}$) in BME-BSA at 37°C for 45 min. Cells were chilled and washed twice, 10 min per wash, with cold buffer 2. Cells were then extracted in HBS, 0.1% (v/v) Triton X-100 15 min on ice; detergent-soluble material was assayed for radioactivity. ^{125}I -Lf nonspecifically bound by the tissue culture plates as determined by parallel incubations carried out as above in cell-free dishes amounted to $\leq 40\%$ of that in the presence of cells and was subtracted from amounts obtained in the presence of cells to give cell-specific ^{125}I -Lf-bound radioactivity. Values represent means of duplicate samples that differed by $\leq 10\%$.

supplementation or following iron depletion hepatocytes maintain a minimal level of Lf endocytic activity.

FAC effect on Lf binding and endocytosis

The foregoing results suggest at least three explanations for the effect of iron loading on Lf endocytosis: (1) FAC treatment stimulates fluid-phase pinocytosis—a prominent vesicular pathway in rat hepatocytes (Oka et al., 1989)—thereby increasing Lf uptake as solute in the coincident volume of pinocytic vesicles; (2) iron loading increases the number of Lf binding sites, which in turn enhance overall Lf endocytosis; (3) iron loading increases the internalization and recycling rates of existing Lf binding sites, thereby enhancing Lf uptake without altering the number of Lf binding sites. To address the first possibility, we determined if hyperosmotic medium, which blocks clathrin-dependent uptake of Lf by isolated rat hepatocytes (McAbee et al., 1993) without altering fluid-phase uptake (Oka et al., 1989), alters the FAC-induced increase in Lf uptake in cultured cells. Cultured hepatocytes were treated with and without FAC and then assayed for ^{125}I -Lf endocytosis in regular media or in media supplemented with 0.4 M sucrose. FAC induced a concentration-dependent increase in ^{125}I -Lf bound by cells, regardless of the presence of sucrose in the incubation medium (Fig. 5A). Hyperosmotic conditions reduced total bound (Fig. 5A) and internalized (Fig. 5B) ^{125}I -Lf endocytosis by $\geq 50\%$

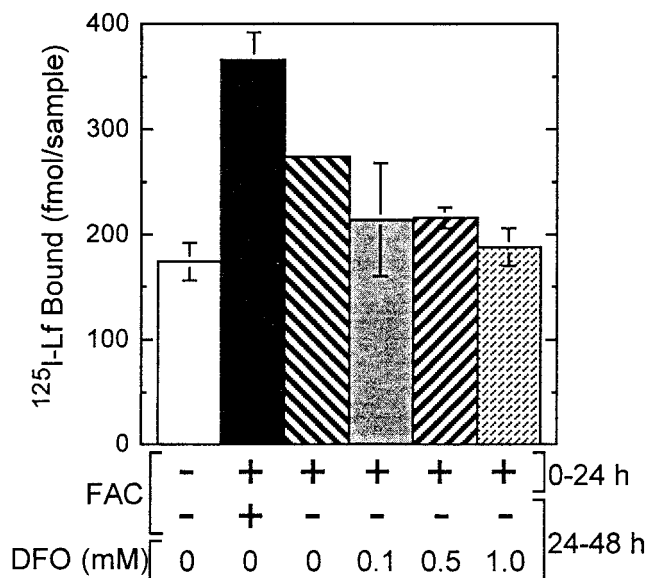


Fig. 4. Reversibility of FAC-induced increase in Lf endocytosis. Adult rat hepatocytes were cultured 24 h in the presence or absence of FAC (5 $\mu\text{g}/\text{mL}$). Cells were then cultured for additional 24 h in media with or without FAC (5 $\mu\text{g}/\text{mL}$) or DFO (0.1–1.0 mM). Cells were chilled, washed in cold BME-BSA, and then incubated with ^{125}I -Lf (2 $\mu\text{g}/\text{mL}$) in fresh BME-BSA at 37°C for 1 h, after which the cells were assayed for bound radioactivity as described in Materials and Methods. Values represent means of duplicate samples; standard deviations are shown by error bars.

at all FAC concentrations tested. These data indicate, therefore, that enhanced Lf uptake induced by iron loading is not due primarily to increased pinocytic activity in these cells.

To address whether or not iron loading increased the number of Lf binding sites on cells, we analyzed ^{125}I -Lf binding at 4°C to control and FAC-treated cells (Fig. 6). We found that FAC-treated hepatocytes bound 3.4 ± 0.7 times more ^{125}I -Lf than did nontreated cells (Fig. 6A). FAC-treated cells bound 24.8 pmol ^{125}I -Lf per 10^6 cells at saturation compared to 12.1 pmol ^{125}I -Lf bound per 10^6 control cells (Fig. 6B). We detected no significant change in the binding affinities (nontreated cells, $K_d = 3.0 \mu\text{M}$; FAC-treated cells, $K_d = 1.7 \mu\text{M}$), and control and FAC-treated cells exhibited a single class of Ca^{2+} -dependent Lf binding sites. When incubated with ^{125}I -Lf at 37°C, we found that cultured hepatocytes internalized ^{125}I -Lf as a function of ^{125}I -Lf concentration in a saturating manner (Fig. 7A). FAC-treated cells, however, exhibited a sixfold higher maximal endocytic rate than did control cells, internalizing 2,370 and 370 molecules ^{125}I -Lf cell $^{-1}$ sec $^{-1}$, respectively (Fig. 7B). Because the FAC-induced increase in ^{125}I -Lf internalization observed in this experiment was greater than the FAC-dependent increase in ^{125}I -Lf binding at 4°C (Fig. 6), it is possible that iron loading also enhanced the rate at which Lf binding sites turn over. Regardless, FAC-dependent enhancement of Lf endocytosis reflects an iron-dependent increase in the number of Lf binding sites with no change in Lf binding affinity and a concomitant increase in Lf endocytosis.

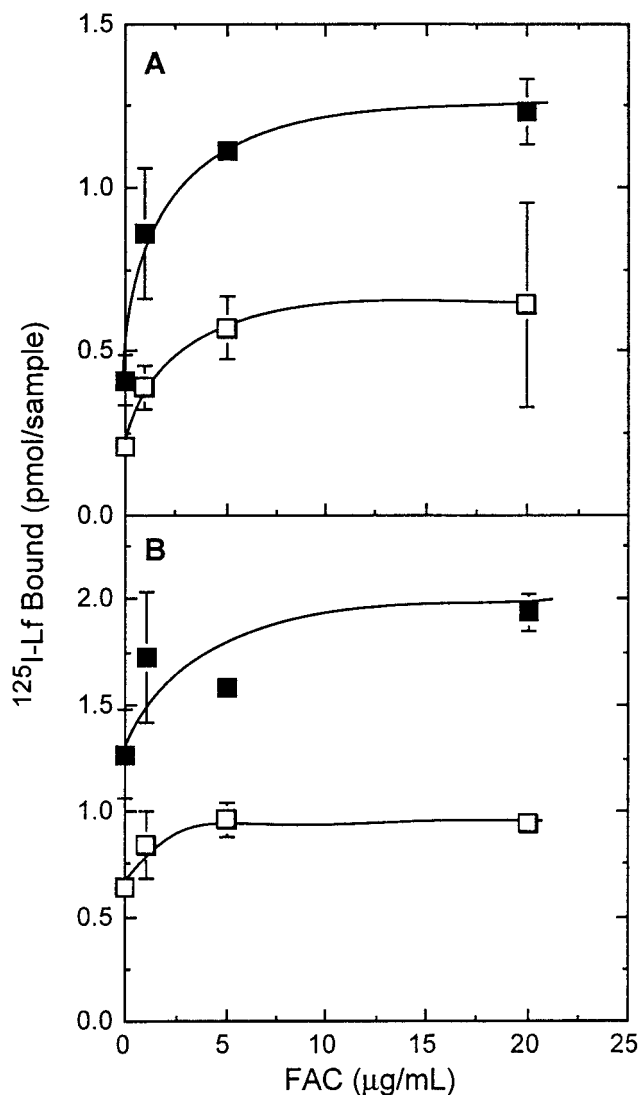


Fig. 5. Effect of hyperosmolality on FAC-induced increase in hepatocyte endocytosis of Lf. Adult rat hepatocytes were cultured 18 h in the presence or absence of FAC at the designated concentrations and then chilled on ice and washed in cold BME-BSA. Cells were then treated with normal BME-BSA (osmolality: 282 mmol/kg) (■) or BSA-BME supplemented with 0.4 M sucrose (osmolality: 699 mmol/kg) (□) for 20 min at 37°C prior to the addition of $^{125}\text{I-Lf}$ (2 $\mu\text{g/mL}$). The cells were incubated an additional 60 min (A) or 45 min (B) at 37°C and then chilled on ice. Cells were assayed for total bound $^{125}\text{I-Lf}$ following washing in buffer 2 (A) or internalized $^{125}\text{I-Lf}$ following washing in buffer 3 (B) as described in Materials and Methods. Values represent means of triplicate samples; standard deviations are shown by error bars. Experiments shown in A and B were performed with different hepatocyte preparations.

Effects of iron loading on Lf and ASOR receptor activity

We examined cultured hepatocyte iron content and Lf binding and endocytic activity as a function of FAC concentration used during the iron-loading phase. As seen in Figure 8, hepatocyte non-heme iron content increased linearly over the range of FAC concentration tested. Parallel incubations of cultured plates with FAC-containing medium in the absence of cells showed no significant

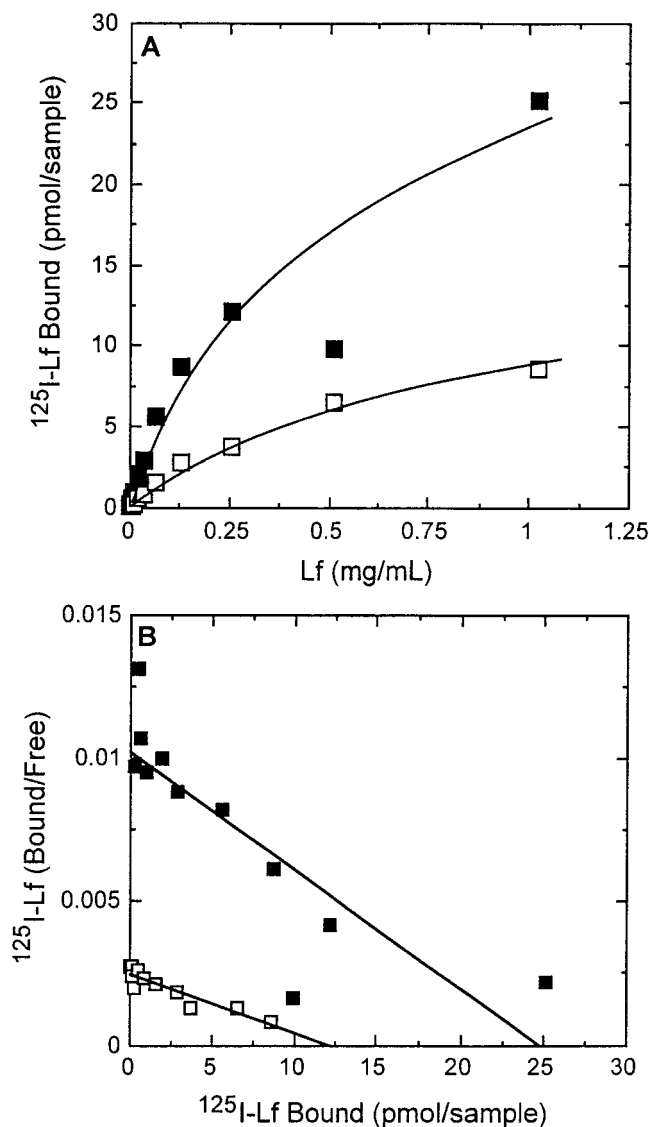


Fig. 6. Equilibrium binding of $^{125}\text{I-Lf}$ to control and FAC-treated hepatocytes. **A:** Adult rat hepatocytes were cultured 20 h in the presence (■) or absence (□) of FAC (5 $\mu\text{g/mL}$) and then chilled on ice and washed in cold BME-BSA. The cells were then incubated with $^{125}\text{I-Lf}$ (0.002–1.024 mg/mL) for 90 min at 4°C, after which the incubation medium was removed and assayed for unbound radioactivity. The cells were scraped off the dishes and washed twice, 10 min per wash, with cold buffer 2 and assayed for cell-associated radioactivity as described in Materials and Methods. **B:** Equilibrium binding constants for binding data in A were determined according to the method of Scatchard (1949) for control (□) and FAC-treated (■) cells. Isotherms were calculated by linear regression analysis; control cells: $\text{binding}_{\text{max}} = 5.7 \times 10^6 \text{ sites cell}^{-1}$, $K_d = 3.0 \mu\text{M}$, $r = -0.75_{(n=10)}$; FAC-treated cells: $\text{binding}_{\text{max}} = 11.8 \times 10^6 \text{ sites cell}^{-1}$, $K_d = 1.7 \mu\text{M}$, $r = -0.92_{(n=10)}$. Slopes of isotherms were analyzed for covariance and found not to be significantly different ($F_{S_{0.005(1,18)}}$).

iron content, which indicated that the FAC-dependent increase in cellular iron content did not arise from non-specific absorption of iron to culture plates. Titration of FAC on cultured hepatocyte binding and endocytosis of $^{125}\text{I-Lf}$ showed that FAC-induced increase in Lf endocytosis at 37°C was accompanied by an increase in Lf binding

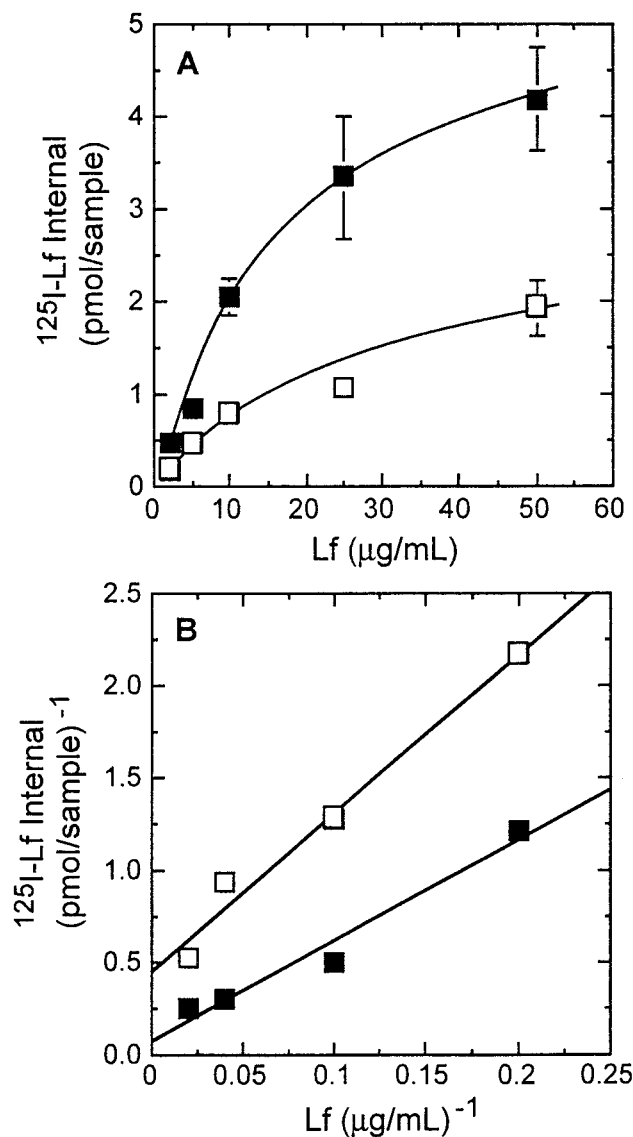


Fig. 7. Effect of iron loading on maximal Lf uptake rates. **A:** Adult rat hepatocytes were cultured 20 h in the absence (□) or presence (■) of FAC (5 µg/mL), after which the cells were chilled and washed in cold BME-BSA. The cells were incubated with ¹²⁵I-Lf at the designated concentrations for 1 h at 37°C, chilled on ice, scraped off the dishes, and washed twice, 10 min per wash, in cold buffer 3 to remove surface-bound ¹²⁵I-Lf. The cells were assayed for internalized radioactivity as described in Materials and Methods. Values represent the mean of duplicate samples; standard deviations are shown by error bars. **B:** Double-reciprocal analysis of data shown in A to determine maximal internalization rates. Isotherms for control (□) and FAC-treated (■) cells were calculated by linear regression analysis. Control cells: $r = 0.98$ ($n=5$), $\text{internalization}_{\text{max}} = 373 \text{ molecules cell}^{-1} \text{ sec}^{-1}$; FAC-treated cells: $r = 0.99$ ($n=5$), $\text{internalization}_{\text{max}} = 2,312 \text{ molecules cell}^{-1} \text{ sec}^{-1}$.

at 4°C (Fig. 9A,B). Increases in ¹²⁵I-Lf binding at 4°C and endocytosis at 37°C occurred at FAC concentrations ≥ 0.3 µg/mL (Fig. 9A) and plateaued at FAC concentrations ≥ 25 µg/mL (Fig. 9B). Under these conditions, ¹²⁵I-Lf binding at 4°C increased less than fourfold, and endocytosis at 37°C increased <4.8 -fold (Fig. 9B). Moreover, FAC-induced enhancement of ¹²⁵I-Lf endocytosis was mirrored

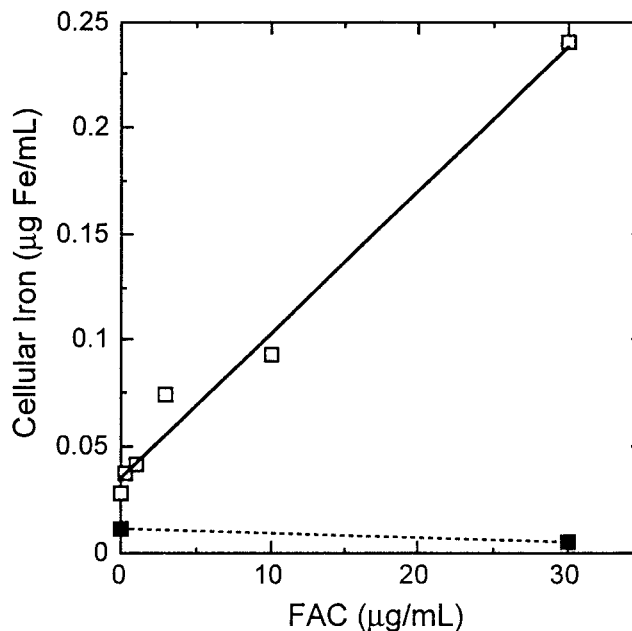


Fig. 8. Effect of FAC treatment on non-heme hepatocyte iron content. Adult rat hepatocytes were cultured 18 h with the designated concentrations of FAC and assayed for non-heme iron content (□) as described in Materials and Methods. Isotherm was calculated by linear regression analysis ($r = 0.99$ ($n=5$)). To determine the amount of iron nonspecifically absorbed onto tissue culture dishes, parallel samples prepared without cells were assayed for iron content (■).

by a commensurate increase in uptake and retention of Lf-bound ⁵⁹Fe (Fig. 10).

To determine if iron loading enhanced cultured hepatocyte endocytic activity in general, we examined the effects of FAC on the cultured hepatocyte binding and uptake of ASOR, a high-affinity ligand for the mammalian hepatocyte Gal/GalNAc receptor (Breitfeld et al., 1985; Hardy et al., 1985). Unexpectedly, we found that between 1 and 25 µg/mL FAC progressively reduced ASOR endocytosis $\leq 80\%$ (Fig. 9C,D). In addition, ¹²⁵I-ASOR binding to cells at 4°C was essentially unaltered regardless of the FAC concentration used even though ASOR uptake was reduced dramatically (Fig. 9C,D). Notably, reduction of ¹²⁵I-ASOR uptake occurred over the same FAC concentrations that induced an increase in ¹²⁵I-Lf binding and endocytosis (compare Fig. 9A,C).

The differential effects of iron loading on ¹²⁵I-Lf and ¹²⁵I-ASOR endocytosis were underscored when we examined binding of both ligands at 4°C on intact and digitonin-permeabilized cultured hepatocytes (Fig. 11). Digitonin permeabilization of isolated rat hepatocytes allows detection of intracellular pools of Gal/GalNAc receptors (McAbee and Weigel, 1987) but solubilizes $\leq 16\%$ of Ca^{2+} -dependent Lf binding sites from these cells (McAbee et al., 1993). FAC-treated hepatocytes bound approximately threefold more ¹²⁵I-Lf than did control cells (Fig. 11A). Intact and permeable cells bound similar amounts of ¹²⁵I-Lf following FAC treatment, consistent with our previous observations that the large majority of Ca^{2+} -dependent Lf binding sites on isolated hepatocytes are on the cell surface (McAbee et al., 1993). In contrast, permeable cells bound severalfold more ¹²⁵I-ASOR than did intact cells,

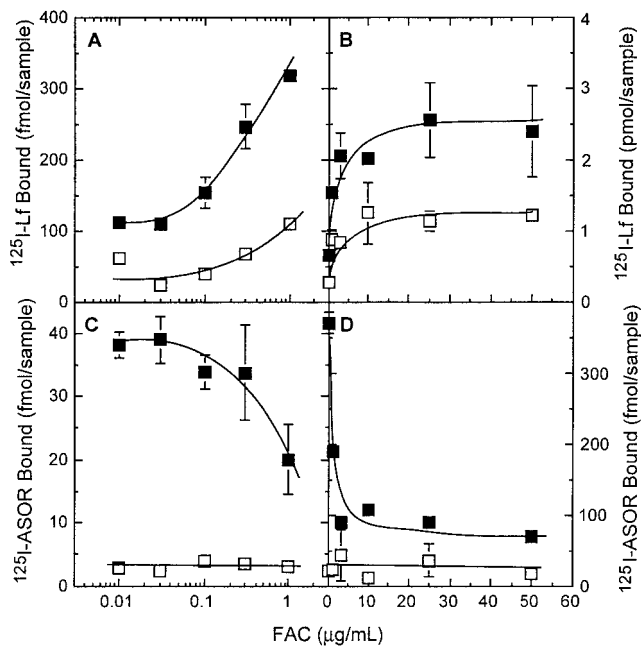


Fig. 9. Effect of iron loading on Lf and ASOR binding and endocytosis. Adult rat hepatocytes were cultured 16 h with the designated concentrations of FAC, chilled on ice, and washed with cold BME-BSA. Cells were then incubated with either ^{125}I -Lf (2 $\mu\text{g/mL}$) (A,B) or ^{125}I -ASOR (1 $\mu\text{g/mL}$) (C,D) for 90 min at 4°C (□) or 1 h at 37°C (■). Cells were scraped off dishes, washed, and assayed for cell-associated ^{125}I -Lf and ^{125}I -ASOR as described in Materials and Methods. Values represent means of duplicate samples; standard deviations are shown by error bars. Cultured hepatocytes used in A and C vs. B and D were from different hepatocyte preparations.

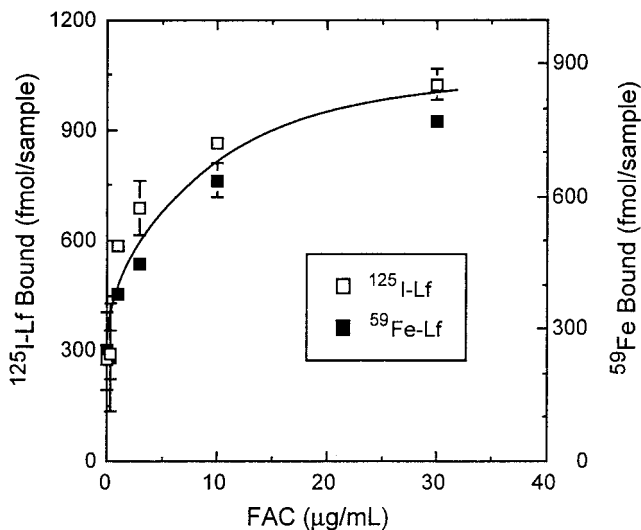


Fig. 10. Effect of iron loading on ^{125}I -Lf and ^{59}Fe -Lf endocytosis. Adult rat hepatocytes were cultured 18 h with the designated concentrations of FAC, chilled on ice, and washed with cold BME-BSA. The cells were then incubated with ^{125}I -Lf (□) (2 $\mu\text{g/mL}$) and ^{59}Fe -Lf (■) (2 $\mu\text{g/mL}$) for 1 h at 37°C, after which the cells were chilled, scraped off dishes, washed in buffer 2, and assayed for cell-associated radioactivity. ^{125}I radioactivity was measured between 15 and 75 keV, and ^{59}Fe radioactivity was measured between 940 and 1,400 keV. Values represent means of duplicate samples; standard deviations are shown by error bars.

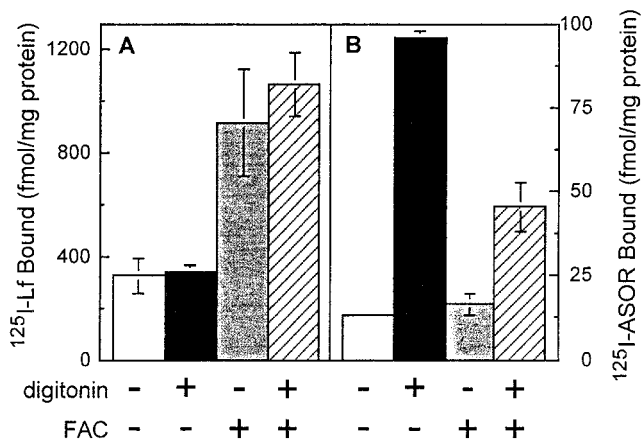


Fig. 11. Effect of iron loading on cell surface and total Lf and ASOR binding activity. Adult rat hepatocytes were cultured 14 h with and without FAC (5 $\mu\text{g/mL}$), after which the cells were chilled and washed in cold BME-BSA. Half of the cells were permeabilized with 0.055% digitonin (McAbee et al., 1993). Intact and permeable cells were incubated in BME-BSA containing ^{125}I -Lf (A) (2 $\mu\text{g/mL}$) or ^{125}I -ASOR (B) (1 $\mu\text{g/mL}$) for 90 min at 4°C, after which the cells were scraped off dishes, washed, and assayed for cell-associated radioactivity and cell protein. Values represent the mean of duplicate samples; standard deviations are shown by error bars.

which is consistent with previous reports that rat hepatocytes possess a large intracellular pool of Gal/GalNAc receptors (Ashwell and Harford, 1982). Following FAC treatment, however, the amount of ^{125}I -ASOR bound to permeable cells was reduced by 53% with essentially no change in ^{125}I -ASOR binding to intact cells, indicating that iron loading resulted in a loss of ligand-binding activity of intracellular but not cell surface Gal/GalNAc receptors. Taken together, these data suggest that the effects of iron loading of hepatocytes does not induce a general increase in the clathrin-dependent endocytic activity of these cells but rather is selective for the Lf uptake pathway.

Effect of protein synthesis inhibitors on FAC-dependent increase in Lf receptor activity

The iron-induced increase in the number of Lf binding sites on hepatocytes resembles the kinetics and extent of the iron-dependent increase in steady-state levels of ferritin heavy and light chains following FAC treatment (Toth et al., 1995). If expression of the hepatocyte Lf receptor is regulated by an IRE/IRP-based mechanism, then one would predict that translation inhibitors but not transcription inhibitors would block the iron-dependent increase in Lf receptor number. To address this possibility, we examined the effect of the translation inhibitor cycloheximide and the transcription inhibitor actinomycin D on Lf binding and endocytosis on control and FAC-treated cultured hepatocytes. We found that cycloheximide treatment of non-iron-supplemented cells did not alter their ^{125}I -Lf binding at 4°C but did reduce their endocytosis of ^{125}I -Lf at 37°C by ~50% (Fig. 12). These data suggest that hepatocytes maintained a basal level of cell surface Lf receptor activity independent of de novo protein synthesis even though endocytosis was somewhat impaired by the prolonged block in translation. In contrast, cycloheximide

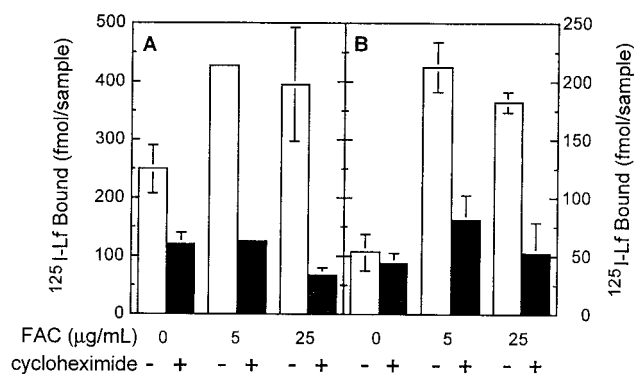


Fig. 12. Effect of cycloheximide on FAC-induced increase in Lf binding and endocytosis. Adult rat hepatocytes were incubated 18 h with the designated concentrations of FAC in the presence or absence of 20 μ M cycloheximide, after which the cells were chilled on ice and washed with cold BME-BSA. The cells were then incubated with 125 I-Lf (2 μ g/mL) for 1 h at 37°C (A) or 2 h at 4°C (B). The cells were chilled, scraped off dishes, washed, and assayed for cell-associated radioactivity as described in Materials and Methods. We detected \leq 5% loss of cell viability following overnight treatment with cycloheximide as measured by trypan blue exclusion. Values represent the mean of duplicate samples; standard deviations are shown by error bars.

blocked the FAC-induced increase in 125 I-Lf binding at 4°C, confirming that iron loading induced the translation of new Lf receptors rather than exposing previously latent binding sites at the cell surface.

To determine if transcription was required for FAC-induced expression of Lf binding activity, we incubated control and FAC-treated cells with and without actinomycin D prior to analysis of 125 I-Lf binding and endocytosis (Fig. 13). We found that actinomycin D \leq 5 μ g/mL neither blocked the FAC-induced increase in 125 I-Lf binding nor reduced 125 I-Lf binding to non-iron-supplemented cells (Fig. 13A). When we assayed hepatocytes for 125 I-Lf endocytosis at 37°C, we found that actinomycin D progressively reduced the amount of 125 I-Lf endocytosis to levels approaching that for non-iron-supplemented cells (Fig. 13B). Somewhat surprisingly, we observed no significant actinomycin D-dependent reduction in 125 I-Lf endocytosis by cells not treated with FAC. Nevertheless, these data provide strong evidence that iron-induced expression of Lf receptor activity does not require new transcription, suggesting Lf receptor mRNA already present in the cells is translated following iron loading of the cells.

DISCUSSION

The purpose of this study was to determine if the iron status of hepatocytes altered their ability to endocytose Lf, an iron-binding protein that may help scavenge or recycle iron in blood and exocrine fluids. We found that iron loading of cultured hepatocytes with 0.3–25 μ g/mL FAC for at least 8 h resulted in a fully reversible two- to sixfold increase in 125 I-Lf binding to Ca^{2+} -dependent Lf binding sites at 4°C and endocytosis at 37°C. Ca^{2+} -dependent Lf binding sites on nontreated and FAC-treated cells bound Lf with the same affinity ($K_d \sim 2 \mu\text{M}$). Furthermore, the iron-dependent increase in the number of Lf binding sites required translation of existing mRNA, but de novo transcription of new Lf receptor mRNA was not required. mRNAs for mamma-

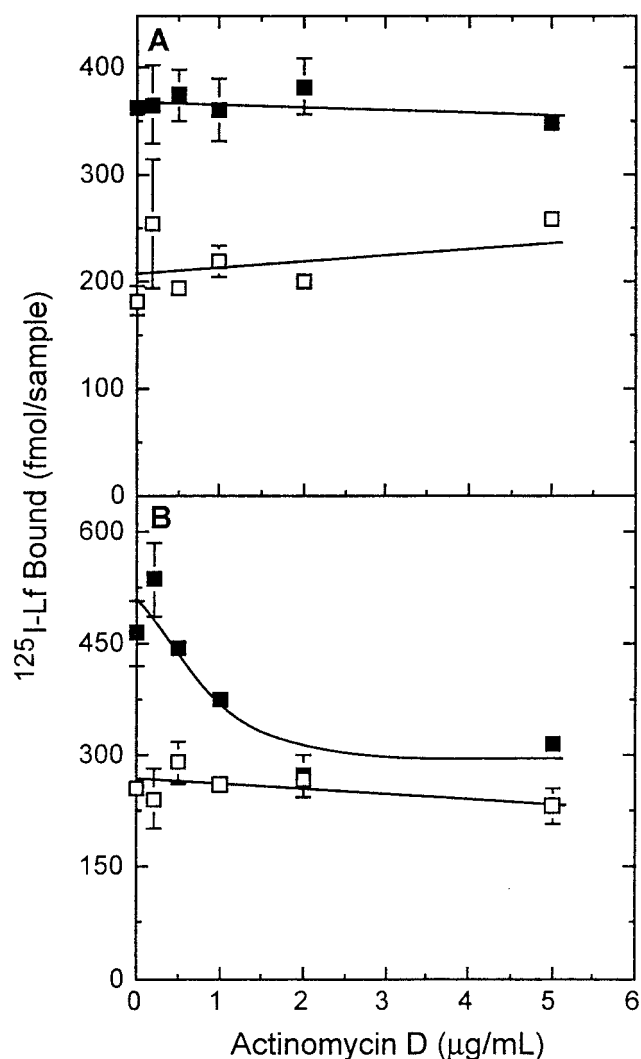


Fig. 13. Effect of actinomycin D on FAC-induced increase in Lf binding and endocytosis. Adult rat hepatocytes were plated onto tissue culture dishes for 4–5 h at 37°C, incubated for 30 min at 37°C with the designated concentration of actinomycin D, and then cultured an additional 12 h (A) or 8 h (B) with (■) and without (□) FAC (15 μ g/mL, A; 5 μ g/mL, B). The cells were chilled on ice and washed in cold BME-BSA and then incubated with 125 I-Lf at 4°C for 90 min (A) or at 37°C for 1 h (B). The cells were then chilled, scraped off dishes, washed with buffer 2, and assayed for cell-associated radioactivity as described in Materials and Methods. We detected \leq 5% loss of cell viability following overnight treatment with actinomycin D as measured by lactate dehydrogenase activity released into conditioned medium. Values represent mean of duplicate samples; standard deviations are shown by error bars.

lian ferritin heavy and light chains (Leibold and Munro, 1988), erythroid 5-aminolevulinic synthase (Bhasker et al., 1993), and *Drosophila* succinate dehydrogenase subunit b (Gray et al., 1996; Kohler et al., 1995) contain functional IREs in their 5'-untranslated regions, and expression of these proteins is enhanced under conditions of iron repletion. Toth et al. (1995) have shown that the steady-state levels of ferritin in human hepatoma cell lines Hep3B and HepG2 increased four- to sixfold following 16 h incubation with

10 $\mu\text{g}/\text{mL}$ FAC, reflecting a fiftyfold increase in ferritin mRNA translation. Our findings presented here (Figs. 3, 6, 7, 9) indicate that the iron-induced increase in Lf receptor activity follows a similar pattern. Our working hypothesis, therefore, is that Lf receptors on hepatocytes are regulated posttranscriptionally by cellular iron levels, possibly by an IRE/IRP-mediated mechanism.

Blood Lf originates in neutrophils which release it during exocytosis of specific granules. The physiological role of lactoferrin in blood is unclear at present, but Lf possesses immunoregulatory (Brock, 1995; Zucali et al., 1989), mitogenic (Kohn et al., 1993), and myelopoietic regulatory (Broxmeyer et al., 1987; Hangoc et al., 1987) activities. It is likely, therefore, that hepatic clearance of Lf protein helps regulate these activities by maintaining a low steady-state concentration of Lf. One clear implication of our findings here, however, is that Lf may also participate in iron scavenging, particularly under conditions of iron overload. Hepatocytes receive and recycle iron and heme—recovered from the turnover of senescent erythrocytes—from serum ferritin, transferrin, haptoglobin, or hemopexin (Morgan and Baker, 1986; Osterloh and Aisen, 1989; Sibille et al., 1988). During episodes of iron overload, hepatocytes sequester iron from the circulation to minimize its toxic effects (Whitaker et al., 1994). Moreover, inflammation and iron overload elevate the amount of Lf released into the blood (Levay and Viljoen, 1995). Our findings suggest that hepatocytes upregulate Lf receptors so that endocytosis of blood Lf during iron overload is unrestricted. In this way, hepatic uptake of Lf accomplishes both clearance of bioactive Lf protein and scavenging of Lf-bound iron.

Our findings also indicate that Lf receptors on various cell types are regulated differentially by iron status of cells. It has been proposed that Lf regulates iron internalization by intestinal mucosal cells (Britton and Koldovsky, 1987; de Vet and van Gool, 1974), possibly by delivering iron directly into intestinal epithelia. Two polarized colon carcinoma cell lines, CaCo-2 cells and HT29-18-C₁ cells, acquire ⁵⁹Fe from Lf by endocytosis of ⁵⁹Fe-Lf at the apical plasma membrane (Mikogami et al., 1994; Sanchez et al., 1996). These cells degrade internalized Lf protein but retain Lf-delivered iron. Moreover, HT29-18-C₁ cells depleted of cellular iron by treatment with picolinic acid increased Lf binding and endocytosis in a translation-dependent manner (Mikogami et al., 1995). In this way, intestinal cells enhance their acquisition of nutrient iron by upregulating their Lf receptors. Thus, Lf receptor activity in intestinal cells and hepatocytes is regulated differentially such that iron depletion and iron repletion, respectively, both increase the number of Lf receptors. One explanation of these data is that while both intestinal and hepatic Lf receptors are regulated by an IRE/IRP-mediated mechanism, the intestinal Lf receptor mRNA may contain an IRE that stabilizes the mRNA under conditions of cellular iron depletion. Regardless, it appears that the underlying strategy in intestinal epithelia and hepatocytes is to upregulate their Lf receptors when iron import is necessary, either to fulfill nutrient iron requirements or to scavenge excess iron from the circulation.

Presently, the identity of the iron-regulated Lf recep-

tor on cultured hepatocytes is not clear. It has been reported that isolated rat hepatocytes bind human Lf via the low-density lipoprotein receptor-related protein/ α_2 -macroglobulin receptor (LRP) in a Ca^{2+} -independent manner (Meilinger et al., 1995; Willnow et al., 1992; Ziere et al., 1992), but it has not been determined if LRP's mRNA contains an IRE in the 5'-UTR. A recent report has shown that chondroitin sulfate on the surfaces of isolated rat hepatocytes binds large amounts of human Lf with low affinity and that cell surface proteoglycan and a yet to be defined lipoprotein remnant receptor may mediate Lf binding and endocytosis (Ziere et al., 1996). During the course of this investigation, we found that isolated rat hepatocytes bind and internalize Lf via the RHL-1 subunit of the Gal/GalNAc receptor (Bennatt and McAbee, manuscript in preparation). Other evidence suggests, however, that RHL-1 is not the hepatocyte Lf receptor that undergoes iron-induced upregulation. First, we find that asialofetuin, a high-affinity ligand for the Gal/GalNAc receptor (Rice et al., 1990), competes poorly with ¹²⁵I-Lf for binding to iron-supplemented cultured rat hepatocytes but shows strong competition with ¹²⁵I-Lf for binding to isolated rat hepatocytes (unpublished findings). Second, iron loading of cultured hepatocytes induces opposite effects on Lf and ASOR endocytosis (Figs. 9, 11). In addition, the absolute molar amounts of Lf and ASOR bound by cultured hepatocytes varied approximately tenfold (compare Fig. 9A,C), whereas the molar quantities of ASOR and Lf bound to isolated rat hepatocytes are similar (Bennatt and McAbee, manuscript in preparation). Third, the RHL-1 gene does not contain sequences in its 5'-flanking region that encode a consensus stem-loop IRE structure (Leung et al., 1985; Theil, 1994). Besides RHL-1 and LRP, the only other Lf receptor that has been cloned is the endothelial receptor which binds advanced glycation endproducts complexed to Lf (Schmidt et al., 1992, 1994, 1996), but the mRNA for this receptor does not possess an IRE. Cultured hepatocytes maintained a basal level of Lf receptor activity that was not affected by prolonged incubation with DFO, cycloheximide, and actinomycin D (Figs. 4, 12, 13), suggesting that these cells possess both iron-dependent and -independent binding sites. We are currently investigating the nature of the Ca^{2+} -dependent Lf binding sites in cultured adult hepatocytes whose expression is amplified following iron loading.

We were surprised to find that iron loading of cultured hepatocytes induced a dramatic loss of ASOR endocytosis. Cultured hepatocytes maintained a constant number of ASOR binding sites on their surfaces following FAC treatment, but FAC induced a large reduction in the number of intracellular ASOR binding sites (Figs. 10, 11). We have not found significant differences in the amount of immunodetectable RHL-1 in control and FAC-treated cultured hepatocytes (unpublished findings), suggesting that iron loading of cultured hepatocytes does not enhance the rate at which the steady-state levels of RHL subunits are reduced in hepatocytes during primary culture (Chu and Doyle, 1985). Based on these observations we conclude that iron loading may enhance the accumulation of inactive Gal/GalNAc receptors intracellularly. A large subpopulation of these receptors recycles constitutively, and they reversibly lose their ability to bind desialylated

glycoconjugates (McAbee et al., 1991; McAbee and Weigel, 1988). Receptor inactivation is ATP-dependent (Medh and Weigel, 1991) and constitutes part of an inactivation-reactivation cycle that corresponds to reversible loss of receptor subunit palmitoylation (Weigel et al., 1994; Weigel and Oka, 1993; Zeng et al., 1996; Zeng and Weigel, 1995). It remains to be determined if palmitoylation of Gal/GalNAc receptors is reduced under conditions of iron repletion. Regardless, these findings suggest that the function of Gal/GalNAc receptors in liver may be impaired under conditions of acute or chronic iron overload.

ACKNOWLEDGMENTS

We thank Dr. Douglas Fishkind and Dr. David Hyde for helpful discussions during the course of the preparation of the manuscript.

LITERATURE CITED

- Ashwell, G., and Harford, J. (1982) Carbohydrate-specific receptors of the liver. *Annu. Rev. Biochem.*, *51*:531–554.
- Bhasker, C.R., Burgiel, G., Neupert, B., Emery-Goodman, A., Kuhn, L.C., and May, B.K. (1993) The putative iron-responsive element in the human erythroid 5-aminolevulinic synthase mRNA mediates translational control. *J. Biol. Chem.*, *268*:12699–12705.
- Breitfeld, P.P., Simmons, C.F.J., Strous, G.J.A.M., Geuze, H.J., and Schwartz, A.L. (1985) Cell biology of the ASGP-R system: A model of receptor mediated endocytosis. *Int. Rev. Cytol.*, *97*:47–95.
- Britton, J.R., and Koldovsky, O. (1987) Luminal digestion of lactoferrin in suckling and weanling rats. *Am. J. Physiol.*, *253*:G397–G403.
- Brock, J. (1995) Lactoferrin: A multifunctional immunoregulatory protein? *Immunol. Today*, *16*:417–419.
- Broxmeyer, H.E., Williams, D.E., Hangoc, G., Cooper, S., Gentile, P., Shen, R.N., Ralph, P., Gillis, S., and Bicknell, D.C. (1987) The opposing actions in vivo on murine myelopoiesis of purified preparations of lactoferrin and the colony stimulating factors. *Blood Cells*, *13*:31–48.
- Casey, J.L., Koeller, D.M., Ramin, V.C., Klausner, R.D., and Harford, J.B. (1989) Iron regulation of transferrin receptor mRNA requires iron-responsive elements and a rapid turnover determinant in the 3'-untranslated region of the mRNA. *EMBO J.*, *8*:3693–3699.
- Chu, F.F., and Doyle, D. (1985) Turnover of plasma membrane proteins in rat hepatoma cells and primary cultures of rat hepatocytes. *J. Biol. Chem.*, *260*:3097–3107.
- de Vet, B.J.C.M., and van Gool, J. (1974) Lactoferrin and iron absorption in the small intestine. *Acta Med. Scand.*, *196*:393–402.
- Fraker, P.J., and Speck, J.C. (1978) Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.*, *80*:849–857.
- Gray, N.K., Pantopoulos, K., Dandekar, T., Ackrell, B.A.C., and Hentze, M.W. (1996) Translational regulation of mammalian and *Drosophila* citric acid cycle enzymes via iron-responsive elements. *Proc. Natl. Acad. Sci. U.S.A.*, *93*:4925–4930.
- Hangoc, G., Lu, L., Oliff, A., Gillis, S., Hu, W., Bicknell, D., Williams, D., and Broxmeyer, H.E. (1987) Modulation of Friend virus infectivity in vivo by administration of purified preparations of human lactoferrin and recombinant murine interleukin-3 to mice. *Leukemia*, *1*:762–764.
- Hardy, M.R., Townsend, R.R., Parkhurst, S.M., and Lee, Y.C. (1985) Different modes of ligand binding to the hepatic galactose-N-acetyl-galactosamine lectin on the surface of rabbit hepatocytes. *Biochemistry*, *24*:22–28.
- Hashizume, S., Kuroda, K., and Murakami, H. (1987) Cell culture assay of biological activity of lactoferrin and transferrin. *Methods Enzymol.*, *147*:302–314.
- Kaplan, J., Jordan, I., and Sturrock, A. (1991) Regulation of the transferrin-independent iron transport system in cultured cells. *J. Biol. Chem.*, *266*:2997–3004.
- Klausner, R.D., Renswoude, J.V., Ashwell, G., Kempf, C., Schechter, A.N., Dean, A., and Bridges, K.R. (1983) Receptor-mediated endocytosis of transferrin in K562 cells. *J. Biol. Chem.*, *258*:4715–4724.
- Klausner, R.D., Rouault, T.A., and Harford, J.B. (1993) Regulating the fate of mRNA: The control of cellular iron metabolism. *Cell*, *72*:19–28.
- Kohler, S.A., Henderson, B.R., and Kuhn, L.C. (1995) Succinate dehydrogenase b mRNA of *Drosophila melanogaster* has a functional iron-responsive element in its 5'-untranslated region. *J. Biol. Chem.*, *270*:30781–30786.
- Kohno, Y., Shiraki, K., Mura, T., and Ikawa, S. (1993) Iron-saturated lactoferrin as a co-mitogenic substance for neonatal rat hepatocytes in primary culture. *Acta Paediatr.*, *82*:650–655.
- Leibold, E.A., and Munro, H.N. (1988) Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5' untranslated region of ferritin heavy- and light-subunit mRNAs. *Proc. Natl. Acad. Sci. U. S. A.*, *85*:2171–2175.
- Leung, J.O., Holland, E.C., and Drickamer, K. (1985) Characterization of the gene encoding the major rat liver asialoglycoprotein receptor. *J. Biol. Chem.*, *260*:12523–12527.
- Levay, P.F., and Viljoen, M. (1995) Lactoferrin: A general review. *Haematologica*, *80*:252–267.
- Mascotti, D.P., Rup, D., and Thach, R.E. (1995) Regulation of iron metabolism: Translational effects mediated by iron, heme, and cytokines. *Annu. Rev. Nutr.*, *15*:239–261.
- McAbee, D.D. (1995) Isolated rat hepatocytes acquire iron from lactoferrin by endocytosis. *Biochem. J.*, *311*:603–609.
- McAbee, D.D., and Esbensen, K. (1991) Binding and endocytosis of apo- and holo-lactoferrin by isolated rat hepatocytes. *J. Biol. Chem.*, *266*:23624–23631.
- McAbee, D.D., and Weigel, P.H. (1987) ATP depletion causes a reversible redistribution and inactivation of a subpopulation of galactosyl receptors in isolated rat hepatocytes. *J. Biol. Chem.*, *262*:1942–1945.
- McAbee, D.D., and Weigel, P.H. (1988) ATP-dependent inactivation and reactivation of constitutively recycling galactosyl receptors in isolated rat hepatocytes. *Biochemistry*, *27*:2061–2069.
- McAbee, D.D., Lear, M.C., and Weigel, P.H. (1991) Total cellular activity and distribution of a subpopulation of galactosyl receptors in isolated rat hepatocytes are differentially affected by microtubule drugs, monensin, low temperature, and chloroquine. *J. Cell. Biochem.*, *45*:59–68.
- McAbee, D.D., Nowatzke, W., Oehler, C., Sitaram, M., Sbaschnig, E., Opferman, J.T., Carr, J., and Esbensen, K. (1993) Endocytosis and degradation of bovine apo- and holo-lactoferrin by isolated rat hepatocytes are mediated by recycling calcium-dependent binding sites. *Biochemistry*, *32*:13749–13760.
- Medh, J.D., and Weigel, P.H. (1991) Reconstitution of galactosyl receptor inactivation in permeabilized rat hepatocytes is ATP-dependent. *J. Biol. Chem.*, *266*:8771–8778.
- Meilinger, M., Haumer, M., Szakmary, K.A., Steinbock, F., Scheiber, B., Goldenberg, H., and Huettinger, M. (1995) Removal of lactoferrin from plasma is mediated by binding to low density lipoprotein receptor-related protein alpha(2)-macroglobulin receptor and transport to endosomes. *FEBS Lett.*, *360*:70–74.
- Mikogami, T., Heyman, M., Spik, G., and Desjeux, J.F. (1994) Apical-to-basolateral transepithelial transport of human lactoferrin in the intestinal cell line HT-29cl.19A. *Am. J. Physiol.*, *267*:G308–G315.
- Mikogami, T., Marianne, T., and Spik, G. (1995) Effect of intracellular iron depletion by picolinic acid on expression of the lactoferrin receptor in the human colon carcinoma cell subclone HT29-18-C-1. *Biochem. J.*, *308*:391–397.
- Morgan, E.H., and Baker, E. (1986) Iron uptake and metabolism by hepatocytes. *Fed. Proc.*, *45*:2810–2816.
- Oka, J.A., Christensen, M.D., and Weigel, P.H. (1989) Hyperosmolarity inhibits galactosyl receptor-mediated but not fluid phase endocytosis in isolated rat hepatocytes. *J. Biol. Chem.*, *264*:12016–12024.
- Osterloh, K., and Aisen, P. (1989) Pathways in the binding and uptake of ferritin by hepatocytes. *Biochim. Biophys. Acta*, *1011*:40–45.
- Rice, K.G., Weisz, O.A., Barthel, T., Lee, R.T., and Lee, Y.C. (1990) Defined geometry of binding between triantennary glycopeptide and the asialoglycoprotein receptor of rat hepatocytes. *J. Biol. Chem.*, *265*:18429–18434.
- Rouault, T.A., Hentze, M.W., Caughman, S.W., Harford, J.B., and Klausner, R.D. (1988) Binding of a cytosolic protein to the iron-responsive element of human ferritin messenger RNA. *Science*, *241*:1207–1210.
- Sanchez, L., Ismail, M., Liew, F.Y., and Brock, J.H. (1996) Iron transport across Caco-2 cell monolayers. Effect of transferrin, lactoferrin and nitric oxide. *Biochim. Biophys. Acta*, *1289*:291–297.
- Scatchard, G. (1949) The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.*, *51*:660–672.
- Schachter, H., Jabbal, I., Hudgin, R.L., Pinteric, L., McGuire, E.J., and Roseman, S. (1970) Intracellular localization of liver sugar nucleotide glycoprotein glycosyltransferases in a Golgi-rich fraction. *J. Biol. Chem.*, *245*:1090–1100.
- Schmidt, A.M., Vianna, M., Gerlach, M., Brett, J., Ryan, J., Kao, J., Esposito, C., Hegarty, H., Hurley, W., Clauss, M., Wang, F., Pan,

- Y.C.E., Tsang, T.C., and Stern, D. (1992) Isolation and characterization of 2 binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. *J. Biol. Chem.*, *267*:14987–14997.
- Schmidt, A.M., Mora, R., Cao, R., Yan, S.D., Brett, J., Ramakrishnan, R., Tsang, T.C., Simionescu, M., and Stern, D. (1994) The endothelial cell binding site for advanced glycation end products consists of a complex—an integral membrane protein and a lactoferrin-like polypeptide. *J. Biol. Chem.*, *269*:9882–9888.
- Schmidt, A.M., Hori, O., Cao, R., Yan, S.D., Brett, J., Wautier, J.L., Ogawa, S., Kuwabara, K., Matsumoto, M., and Stern, D. (1996) RAGE: A novel cellular receptor for advanced glycation end products. *Diabetes*, *45*:S77–S80.
- Seglen, P.O. (1973) Preparation of rat liver cells. III. Enzymatic requirements for tissue dispersion. *Exp. Cell Res.*, *82*:391–398.
- Sibille, J.-C., Kondo, H., and Aisen, P. (1988) Interactions between isolated hepatocytes and Kupffer cells in iron metabolism: A possible role for ferritin as an iron carrier protein. *Hepatology*, *8*:296–301.
- Sibille, J.-C., Kondo, H., and Aisen, P. (1989) Uptake of ferritin and iron bound to ferritin by rat hepatocytes: Modulation by apotransferrin, iron chelators, and chloroquine. *Biochim. Biophys. Acta*, *1010*:204–209.
- Stearman, R., Yuan, D.S., Yamaguchi-Iwai, Y., Klausner, R.D., and Dancis, A. (1996) A permease-oxidase complex involved in high-affinity iron uptake in yeast. *Science*, *271*:1552–1557.
- Theil, E.C. (1994) Iron regulatory element (IREs): A family of mRNA non-coding sequences. *Biochem. J.*, *304*:1–11.
- Toth, I., Rogers, J.T., McPhee, J.A., Elliott, S.M., Abramson, S.L., and Bridges, K.R. (1995) Ascorbic acid enhances iron-induced ferritin translation in human leukemia and hepatoma cells. *J. Biol. Chem.*, *270*:2846–2852.
- Walden, W.E., Daniels-McQueen, S., Brown, P.H., Gaffield, L., Russell, D.A., Bielser, D., Bailey, L.C., and Thach, R.E. (1988) Translational repression in eukaryotes: Partial purification and characterization of a repressor of ferritin mRNA translation. *Proc. Natl. Acad. Sci. U. S. A.*, *85*:9503–9507.
- Weigel, P.H., and Oka, J.A. (1993) Regulation of asialoglycoprotein receptor activity by a novel inactivation/reactivation cycle. Receptor reactivation in permeable rat hepatocytes is mediated by fatty acyl coenzyme A. *J. Biol. Chem.*, *268*:27186–27190.
- Weigel, P.H., Medh, J.D., and Oka, J.A. (1994) A novel cycle involving fatty acyl-coenzyme A regulates asialoglycoprotein receptor activity in permeable hepatocytes. *Mol. Biol. Cell*, *5*:227–235.
- Whitaker, P., Chanderbhan, R., Calvert, R., and Dunkel, V. (1994) Cellular and molecular responses in the Sprague-Dawley rat to chronic iron overload. *J. Tr. Elem. Exp. Med.*, *7*:19–31.
- Willnow, T.E., Goldstein, J.L., Orth, K., Brown, M.S., and Herz, J. (1992) Low density lipoprotein receptor-related protein and gp330 bind similar ligands, including plasminogen activator-inhibitor complexes and lactoferrin, an inhibitor of chylomicron remnant clearance. *J. Biol. Chem.*, *267*:26172–26180.
- Wright, T.L., Brissot, P., Ma, W.-L., and Weisiger, R.A. (1986) Characterization of non-transferrin-bound iron clearance by rat liver. *J. Biol. Chem.*, *261*:10909–10914.
- Zeng, F.-Y., and Weigel, P.H. (1995) Hydroxylamine treatment differentially inactivates purified rat hepatic asialoglycoprotein receptors and distinguishes two receptor populations. *J. Biol. Chem.*, *270*:21388–21395.
- Zeng, F.-Y., Oka, J.A., and Weigel, P.H. (1996) The human asialoglycoprotein receptor is palmitoylated and fatty deacylation causes inactivation of state 2 receptors. *Biochem. Biophys. Res. Commun.*, *218*:325–330.
- Ziere, G.J., Vandijk, M.C.M., Bijsterbosch, M.K., and Vanberkel, T.J.C. (1992) Lactoferrin uptake by the rat liver—characterization of the recognition site and effect of selective modification of arginine residues. *J. Biol. Chem.*, *267*:11229–11235.
- Ziere, G.J., Kruijt, J.K., Bijsterbosch, M.K., and van Berkel, T.J. (1996) Recognition of lactoferrin and aminopeptidase-M-modified lactoferrin by the liver: Involvement of proteoglycans and the remnant receptor. *Biochem. J.*, *313*:289–295.
- Zucali, J.R., Broxmeyer, H.E., Levy, D., and Morse, C. (1989) Lactoferrin decreases monocyte-induced fibroblasts production of myeloid colony-stimulating activity by suppressing monocyte release of interleukin-1. *Blood*, *74*:1531–1536.