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Biochemistry

Iron-independent induction of ferritin H chain by tumor necrosis factor

(muscle/myoblasts/actinomycin D)

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ABSTRACT Iron increases the synthesis of the iron-storage protein, ferritin, largely by promoting translation of preexisting mRNAs for both the H and L ferritin isoforms (H, heavy, heart, acidic; L, light, liver, basic). We have recently cloned and sequenced a full-length cDNA to murine ferritin H and identified ferritin H as a gene induced by tumor necrosis factor α (TNF-α, cachectin). Using primary human myoblasts, we have now examined the relationship between TNF-α and iron in regulating ferritin. Four lines of evidence suggest that TNF-α regulates ferritin independently of iron. First, evaluation of mRNA showed that TNF-α increased ferritin H chain specifically, provoking no change in steady-state levels of ferritin L mRNA; iron, in contrast, increased the mRNA of both isoforms. Second, the increase in ferritin H protein synthesis observed during TNF-α treatment was dependent on an increase in ferritin H mRNA: actinomycin D blocked the TNF-α-induced changes in ferritin H but did not inhibit the translational induction of ferritin seen with iron treatment. Third, equal ferritin mRNA induction was observed in iron-loaded cells and in cells depleted of iron by a permeant chelator, 2,2′-dipyridyl. Fourth, ferritin H induction by TNF-α and iron was additive over the entire range of iron concentrations, even at TNF-α doses known to maximally stimulate ferritin H mRNA levels. Nonetheless, the role of iron in translational regulation of ferritin was retained in TNF-α-treated cells; effective biosynthesis of TNF-α-induced, H-subunit-predominant ferritin protein required iron and could be enhanced by treatment of the cells with additional iron or blocked by 2,2′-dipyridyl. Finally, we observed that the TNF-α-mediated increase in ferritin synthesis peaked at 8 hr and was followed by a decrease in both H and L isoferritin synthesis; the addition of iron, however, reversed the late-occurring depression in ferritin synthesis. This suggests that TNF-α-induced synthesis of H-rich ferritin may reduce the regulatory pool of intracellular iron, secondarily inhibiting iron-mediated translation of ferritin mRNA. We conclude that TNF-α acts independently of iron in its induction of ferritin H mRNA but requires the presence of iron for this effect to be fully expressed at the protein level.

The major sequestration site of intracellular iron is ferritin (reviewed in ref. 1). The mammalian form of this molecule, a protein of ≈450 kDa, is composed of two smaller isoforms, designated H (heavy, heart, acidic) and L (light, liver, basic). Any combination of 24 of the H and L subunits combine within the cytoplasm to form a hollow shell, within which as many as 4500 atoms of ferric iron can be stored (1).

The principal regulator of ferritin synthesis is intracellular iron. This regulation is achieved primarily by increasing translation of preformed mRNA coding for H and L ferritin subunits (2, 3) but also by increasing transcription of the ferritin H- and L-subunit genes in some cell types (4). The translational induction of ferritin protein synthesis is modulated by one or more protein repressors (5–7) and a highly conserved iron response element encoded in the 5′ untranslated region of both H (8, 9) and L (10) ferritin mRNA.

We recently observed that tumor necrosis factor α (TNF-α) induces ferritin H mRNA levels in both mouse TA1 adipocytes and human muscle cells (11). TNF-α, a 17-kDa protein synthesized by stimulated macrophages, is a central mediator of inflammation and septic shock (reviewed in ref. 12). The observation that inflammation (13, 14) and malignancy (15) are associated with changes in iron metabolism, the finding that clinical administration of TNF-α induces hypoferremia (16), and our recent discovery that TNF-α enhances ferritin H production (11) suggest that, through regulation of ferritin, TNF-α may be involved in iron homeostasis.

Critical to such a hypothesis is a definition of the interrelationships between iron- and TNF-α-mediated regulation of ferritin. We now demonstrate that TNF-α specifically induces ferritin H mRNA independently of cellular iron concentration and known iron-dependent regulatory pathways. However, iron continues to mediate translational regulation of TNF-α-induced ferritin H transcripts.

MATERIALS AND METHODS

Cell Culture and Treatment. Experiments were performed using a sorted population of human muscle cells (17) kindly provided to us by H. Blau (Stanford, CA). Myoblasts were grown as described (18). At the time of treatment, cells were refed either unsupplemented medium or medium containing recombinant TNF-α (Cetus) (specific activity, 10,000 units/μg) or iron in the form of ferric nitrilotriacetate (FeNTA) (19). In other experiments, treatment with the iron chelator, 2,2′-dipyridyl (150 μM) (Sigma) or actinomycin D (5 μg/ml) (Sigma) was begun 1 hr prior to addition of the TNF-α- or FeNTA-containing medium.

Ferritin Protein Synthesis Analysis. Cells were washed 2.5 hr before cell harvest and placed in methionine-free minimal essential medium (Sigma) containing appropriate supplements (TNF-α, FeNTA, 2,2′-dipyridyl, and/or actinomycin D). Thirty minutes later, [^{35}S]methionine (50 μCi/ml; 1 Ci = 37 GBq) (Tran^{35}S-label, ICN) was added to the cultures. After 2 hr of incubation, cells were lysed, ferritin was

Abbreviations: TNF-α, tumor necrosis factor α; FeNTA, ferric nitrilotriacetate.

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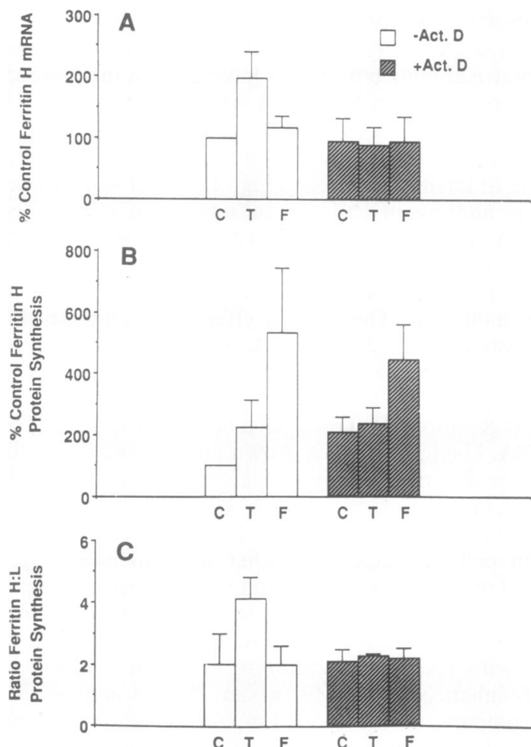

FIG. 1. Actinomycin D prevents TNF-α augmentation of ferritin H synthesis. Myoblasts were treated with either 5 μg of actinomycin D per ml for 1 hr (+Act. D) or did not undergo the actinomycin D pretreatment (−Act. D) before the addition of control medium (C), 10 ng of TNF-α per ml (T), or 100 μM FeNTA (F) for 8 hr. Ferritin protein synthesis and mRNA were assayed and quantified by scanning densitometry. (*A*) Induction of ferritin H mRNA normalized to that seen in control cells (−Act. D; C). (*B*) Induction of ferritin H protein synthesis normalized to that seen in control cells (−Act. D; C). (*C*) Ratio of ferritin H/ferritin L protein synthesis for each treatment. Results are from two experiments. Error bars represent 95% confidence intervals.

immunoprecipitated, and immunoprecipitates were analyzed by denaturing gel electrophoresis as described (11). Dried

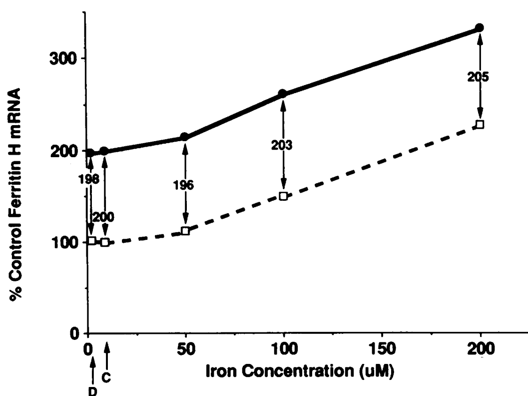

FIG. 2. TNF-α induction of ferritin H mRNA is iron-independent. Myoblasts were treated for 1 hr with either 150 μM 2,2′-dipyridyl (D), control medium (C), or various micromolar amounts of FeNTA. Either no further supplementation (□) or 10 ng of TNF-α per ml (●) was added and cells were continued in culture for another 8 hr. RNA was extracted. Dot blots were hybridized with ferritin H cDNA, washed, and then hybridized with β-actin to control for RNA loading. Induction of ferritin H mRNA was compared to control cells receiving neither iron nor TNF-α. The percentage induction attributable to TNF-α at each iron concentration is indicated between the double-headed arrows.

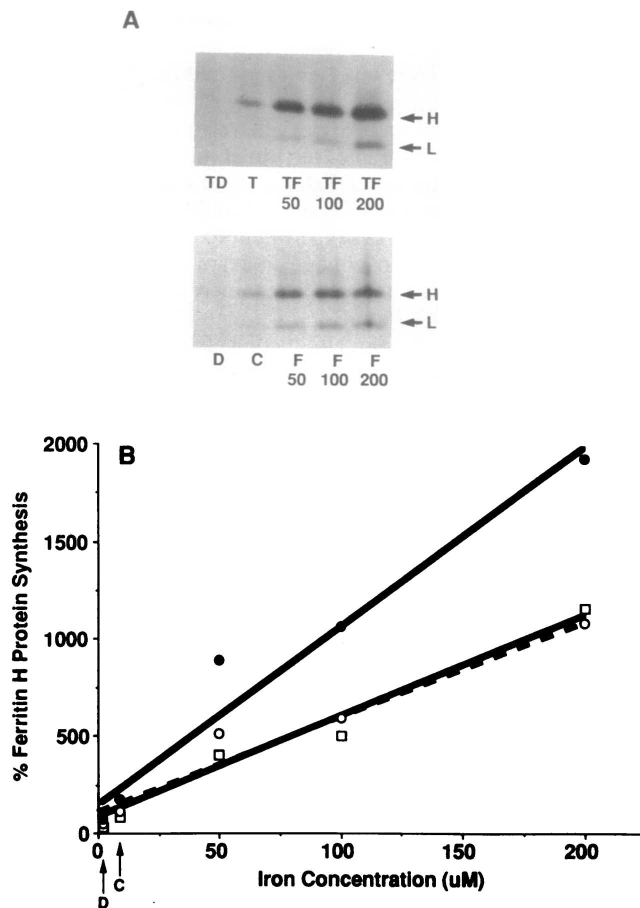

FIG. 3. Iron-mediated synthesis of ferritin is TNF-α independent. (*A*) Myoblasts were treated for 1 hr with either 150 μM 2, 2′-dipyridyl (lane D), control medium (lane C), or various micromolar amounts of FeNTA (lanes F 50, F 100, and F 200). Either no further supplementation or 10 ng of TNF-α per ml (lanes T) was added and cells were incubated for another 8 hr. Cells were labeled with [^{35}S]methionine for the final 2 hr of treatment and immunoprecipitated ferritin was analyzed by SDS/PAGE. The ferritin H and L subunits are indicated. (*B*) Graphic representation of ferritin H data from *A*. □, Changes in ferritin H synthesis due to iron level alone (lanes D, F 50, F 100, and F 200 in *A*) when normalized to control cells (lane C in *A*) receiving neither iron nor TNF-α. ○, Ferritin H synthesis resulting from both TNF-α and iron (lanes TD, TF 50, TF 100, and TF 200 in *A*) as compared to the ferritin H synthetic rate when cells treated with TNF-α (lane T in *A*) are normalized to 100%. ●, Alterations in ferritin H synthesis due to both TNF-α and iron level (lanes TD, TF 50, TF 100, and TF 200 in *A*) when compared to control cells (lane C in *A*) receiving neither iron nor TNF-α.

gels were exposed to x-ray film; relative signal intensities were quantitated by scanning densitometry (Hoefer).

RNA Preparation and Analysis. RNA was harvested after lysis in guanidine isothiocyanate (20) from cells treated identically to those collected for protein synthesis. Denatured RNA was applied to nitrocellulose with a dot blot apparatus (Schleicher & Schuell). Baked filters were hybridized with a full-length cDNA to mouse ferritin H (11, 18); the 650-base-pair 3′ *Pst* I-defined fragment of a human L subunit cDNA (21), kindly supplied by J. W. Drysdale; and a full-length human β-actin cDNA (22), generously provided by L. Kedes.

RESULTS

TNF-α-Mediated Induction of Ferritin Contrasts Qualitatively and Quantitatively with Iron-Mediated Induction of Ferritin. Time course experiments demonstrated that the

induction of ferritin H mRNA was maximal at 24 hr at a TNF-α dose of 10 ng/ml and resulted in a 3- to 6-fold increase in steady-state mRNA levels (see Fig. 4 and ref. 11). At early time points the increases in ferritin H protein synthesis and ferritin H mRNA level were approximately proportionate; for example, by 8 hr, induction of ferritin H mRNA was 2.1-fold (95% confidence interval of 2.0–2.1 in 15 experiments) and ferritin H protein synthesis was 2.8-fold (95% confidence interval of 2.3–3.3 in 12 experiments). Under the same conditions, however, no change in ferritin L mRNA was noted, and there was no associated increase in L subunit protein synthesis (see Fig. 4).

In contrast to the specific effects of TNF-α on the H chain of ferritin, iron increased both ferritin L- and H-subunit mRNA: the increase in ferritin L mRNA (2.5-fold with 200 μM FeNTA) at 8 hr (data not shown) in response to iron was comparable to the increase in ferritin H mRNA (2.1-fold) (see Fig. 2). Furthermore, unlike TNF-α, iron induced the synthesis of both isoferritins out of proportion to its effect on ferritin mRNA: in four experiments, average ferritin synthetic rates increased 6- to 9-fold for both ferritin H and ferritin L after 8 hr of treatment with 200 μM FeNTA (see Fig. 3).

Augmentation of Ferritin H by TNF-α Does Not Proceed in the Presence of Actinomycin D. Iron increases ferritin synthesis primarily by causing a redistribution of preexisting ferritin mRNA from monosomes to polyribosomes (2, 3). This effect is resistant to actinomycin D (3, 19), a potent inhibitor of DNA-dependent RNA polymerase. To further compare the response of ferritin to iron and TNF-α, we added actinomycin D (5 μg/ml) to myoblast cultures for 1 hr prior to the addition of either TNF-α or iron for another 8 hr. In the absence of actinomycin D, TNF-α provoked a 2-fold increase in the ratio of ferritin H/L synthetic rates that mirrored the 2-fold increase in ferritin H mRNA (Fig. 1). However, with actinomycin D present, the elevation in H/L protein synthesis was blocked, as was any increase in ferritin H mRNA. In contrast, iron provoked a 5-fold increase in both ferritin H and ferritin L protein synthesis and did not alter the ferritin H/L subunit ratio in either the presence or absence of actinomycin D (Fig. 1). Although not directly addressing whether TNF-α acts transcriptionally in augmenting ferritin H mRNA, these data suggest different mechanisms for ferritin induction by TNF-α and iron.

Equal TNF-α Induction of Ferritin H mRNA Occurs over a Wide Range of Iron Concentrations. To evaluate an interaction between iron and TNF-α, myoblasts were pretreated with either 2,2′-dipyridyl, a permeant iron chelator (23), received no additional iron over that present in growth medium (≈7 μM iron), or were supplemented with various amounts (50, 100, or 200 μM) of FeNTA for 1 hr before TNF-α was added. A second set of myoblasts received the same pretreatments but did not have TNF-α introduced into the cultures. Ferritin H mRNA was analyzed after 8 hr of incubation.

In response to iron, myoblasts showed a dose-dependent increase in ferritin H mRNA (Fig. 2). At all iron concentrations, the addition of TNF-α resulted in an increase in ferritin H mRNA additive to that seen with iron alone. This strictly additive relationship of TNF-α and iron occurred even at doses of TNF-α (10 ng/ml) that are saturating for ferritin H mRNA induction. The TNF-α effect was undiminished in cells treated with 2,2′-dipyridyl, indicating that iron is not required for TNF-α to exert an influence on ferritin H mRNA.

Iron Is Required for Translation of TNF-α-Induced Ferritin H mRNA. Using myoblasts grown and treated concurrently with those described in Fig. 2, we sought to understand how the combined effects of TNF-α and iron would alter the translation of TNF-α-modulated ferritin mRNA.

When cells subjected to either iron chelation by 2,2′-dipyridyl or iron supplementation were compared to control cells (Fig. 3A, lane C), an iron-dependent change in ferritin H protein synthesis was observed (Fig. 3B, □). Myoblasts treated with TNF-α in addition to iron (Fig. 3B, ●) demonstrated enhanced synthesis of an H-predominant ferritin when compared to the control myoblasts (Fig. 3A, lane C). Iron translationally amplified the TNF-α-induced increase in the H subunit. 2,2′-Dipyridyl virtually blocked ferritin synthesis, indicating that iron is required for the final cellular expression of the changes induced by TNF-α. When ferritin H synthesis in cells receiving both iron and TNF-α was normalized to ferritin synthesis seen in cells treated only with TNF-α (Fig. 3A, lane T), an identical dependence on iron dose was seen (compare □ and ○ in Fig. 3B). This indicates that the presence or absence of TNF-α in the cultures had no impact on the iron-dependent changes in translation and that translational control of ferritin by iron is independent of TNF-α.

Prolonged TNF-α Treatment Causes a Decrease in Both H and L Ferritin Synthesis That Is Reversible with Iron. While examining prolonged treatment of myoblasts with TNF-α, we noted that, despite a >3-fold increase in ferritin H mRNA and no change in ferritin L mRNA, synthesis of both H and L proteins had fallen markedly by 24 hr (Fig. 4).

Because iron maintains translational control of ferritin in TNF-α-treated cells (Fig. 3), we conjectured that TNF-α might promote iron sequestration by newly synthesized ferritin and that the resulting decrease in cytosolic iron could

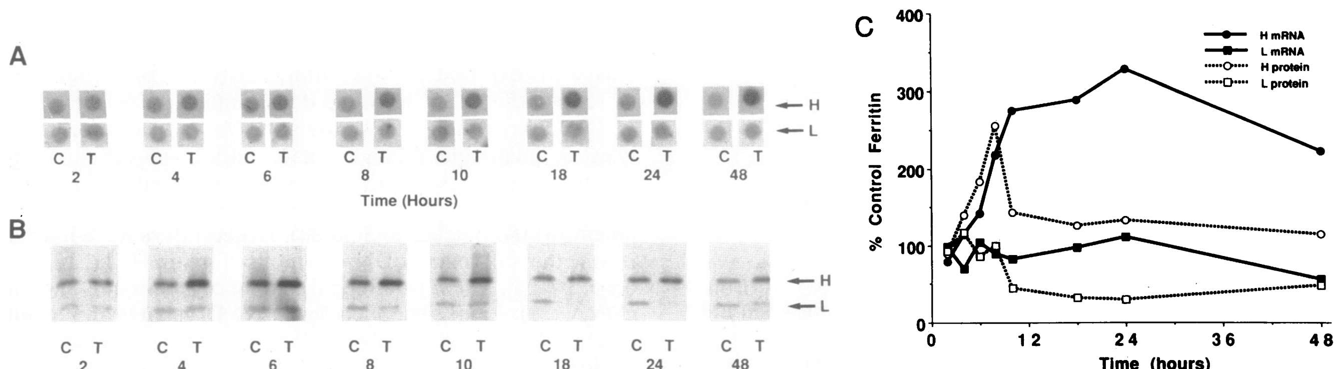

FIG. 4. Time course of TNF-α treatment. Myoblasts were treated with 10 ng of TNF-α per ml (lanes T) or control medium (lanes C) for the indicated times. The ferritin H and L subunits are indicated. (A) RNA was extracted. Dot blots were prepared and hybridized with ferritin H or L cDNA, washed, and then hybridized with β-actin. Dots of equivalent β-actin signal intensity are shown. (B) Cells were labeled with [^{35}S]methionine during the final 2 hr of treatment. Immunoprecipitated ferritin was analyzed by SDS/PAGE. (C) Graph of data from A and B showing TNF-α induction of ferritin protein synthesis and ferritin mRNA levels when normalized to control values at each time point. Ferritin H (●) and L (■) mRNA levels, and ferritin H (○) and L (□) protein synthetic rates are shown.

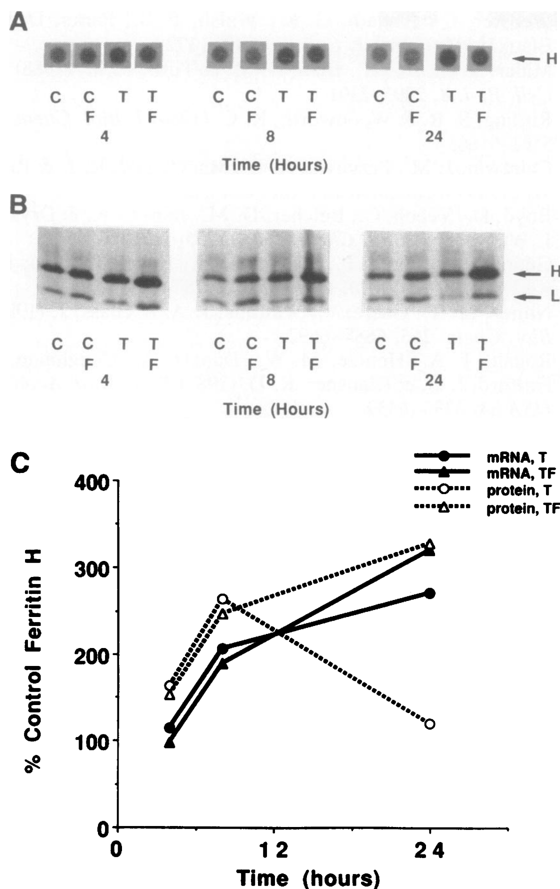

FIG. 5. Iron reverses the TNF-α-induced modulation of ferritin H synthesis observed at later time points. Myoblasts were treated with 10 ng of TNF-α per ml (lanes T) or control medium (lanes C) for the indicated times or also had 200 μM FeNTA (lanes F) added for the last 3 hr of treatment. The ferritin H and L subunits are indicated. (*A*) RNA was extracted. Dot blots were prepared and hybridized with ferritin H cDNA, washed, and rehybridized with β-actin cDNA. Dots of approximately equivalent β-actin signal are shown. (*B*) Cells were labeled with [^{35}S]methionine during the final 2 hr of treatment. Immunoprecipitated ferritin was analyzed by SDS/PAGE. (*C*) Graph of data from *A* and *B* showing TNF-α induction of ferritin H protein synthesis and ferritin H mRNA levels when normalized to control values at each time point. mRNA levels from cells treated with T (●) or TF (▲) and ferritin H protein synthesis from cells receiving T (○) or TF (△) are shown.

eventually inhibit translation of both ferritin H and L subunits. To examine this hypothesis we performed a second time course experiment. Control and TNF-α-treated cells were either harvested without additional treatment or were supplemented with 200 μM FeNTA during the 3 hr before harvesting (Fig. 5). In cells not loaded with iron during the [^{35}S]methionine labeling period, the previously observed fall in ferritin production occurred by 24 hr. This contrasted with those TNF-α-treated cells receiving iron during the time of [^{35}S]methionine incorporation; in these cells, the ferritin synthetic rate, as compared to that of iron-enhanced control cells, matched that predicted by the mRNA induction. Iron thus appeared able to reverse the decrease in ferritin synthesis provoked by TNF-α at later times. Loss of ferritin-bound iron via TNF-α-induced ferritin secretion at later times cannot serve as an alternative explanation; immunoprecipitation of conditioned medium from myoblasts treated with TNF-α for 24 hr failed to reveal any labeled ferritin in the medium (unpublished data). Pulse–chase experiments at 24 hr also demonstrated no enhanced degradation of labeled ferritin in TNF-α-treated myoblasts (data not shown).

DISCUSSION

These studies indicate that TNF-α and iron are quite different in their effects on ferritin. Iron enhances ferritin production by two mechanisms: it increases both ferritin H and L mRNA levels and also amplifies the synthesis of ferritin H and L by a direct translational effect that is resistant to actinomycin D. In contrast, TNF-α increases ferritin H protein synthesis through an actinomycin D-sensitive induction that is restricted to ferritin H mRNA. Furthermore, the TNF-α-associated increase in ferritin H mRNA is iron-independent, occurring to the same degree in iron-deprived or iron-supplemented cells. These data suggest that alterations in ferritin after TNF-α treatment do not result because TNF-α increases intracellular iron, which then promotes ferritin production.

TNF-α, by increasing ferritin H mRNA, broadens the dynamic range over which intracellular iron can act to promote ferritin H translation. Iron remains critical to the full expression of the TNF-α effect on ferritin H because TNF-α does not alter translational efficiency in response to iron. This result confirms the finding that the iron-responsive translational repressor is not limiting (24), because higher levels of ferritin H mRNA in TNF-α-treated myoblasts are as available for repression as ferritin mRNA in control cells.

Following an initial increase in ferritin H synthesis in response to TNF-α treatment, the synthesis of both ferritin H and ferritin L protein subunits eventually declines. However, iron supplementation during the last 3 hr of TNF-α treatment can restore ferritin protein production to a level proportional to the levels of ferritin mRNA. This suggests that the TNF-α effect on ferritin has functional consequences—i.e., that TNF-α-induced ferritin sequesters iron, resetting the level or activity of ferritin relative to the amount of intracellular iron. Supporting this hypothesis are previous results demonstrating that the greater the level of intracellular ferritin, the higher the fraction of iron that is incorporated into ferritin, regardless of the actual amount of iron delivered to the cell (25).

Our observation of the TNF-α effect on ferritin H mRNA and reports that thyroid-stimulating hormone increases ferritin H mRNA in thyroid cell lines (26, 27) suggest that, in addition to iron (4, 28, 29), paracrine and endocrine factors may also influence ferritin composition. The degree to which cells respond to TNF-α with ferritin H induction as a part of normal homeostasis or during overt inflammation remains to be documented. However, studies of whole rat organ homogenates (liver, spleen, lung, kidney, and small bowel) reveal evidence of TNF-α transcripts even in the absence of inflammatory stimulation (30), suggesting the possibility of endogenous TNF-α production by tissue macrophages that may have a local impact on ferritin subunit composition.

The consequences of the selective action of TNF-α on ferritin H require further elucidation, but evidence available to date does suggest different roles for the two ferritin isoforms; H subunits appear to confer a greater capacity for rapid iron uptake (31–33). TNF-α could therefore act to promote rapid sequestration of iron in H-rich ferritin, an effect that may be relevant to the increases in ferritin and ferritin iron seen following inflammatory stimulation of monocytes (34) or enterocytes (35). Sequestration of iron might serve the beneficial function of making iron unavailable to invading organisms (reviewed in ref. 36) or of lessening free-radical tissue damage (37). Further studies of the role of TNF-α and interleukin 1 (38) in ferritin regulation may provide an understanding of the alterations in iron homeostasis that occur during such diverse processes as infection, inflammation, and malignancy.

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