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REGULATION OF FERRITIN SYNTHESIS IN MACROPHAGES BY OXYGEN AND A SULFHYDRYL-REACTIVE AGENT

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Summary: Ferritin synthesis is known to be regulated translationally by specific mRNA-protein interactions between an iron-responsive element (IRE) localized in the 5' untranslated region of ferritin mRNA and IRE-binding protein (IRE-BP). Binding of IRE-BP to IRE depresses its translation. In the present study, we demonstrated that ferritin synthesis in macrophages is strongly induced under hypoxic conditions by diethylmaleate, a sulfhydryl-reactive agent. The induction by diethylmaleate decreased as the oxygen tension rose. O_2 is involved in this oxygen effect, because the induction was prevented when O_2-generating agents were present. Actinomycin D did not inhibit the ferritin synthesis induced by diethylmaleate under hypoxi. These results suggest that O_2 is ivolved in post-transcriptional regulation of ferritin sythesis. © 1994 Academic Press, Inc.

Ferritin is a major intracellular iron-storage protein, and occurs as a hollow shell composed of 24 subunits of two types, H and L (1). Its synthesis is regulated in response to changes in available iron primarily at the translational level (2). The mRNA encoding ferritin contains a regulatory structure (IRE), which is responsible for the iron-dependent regulation of translation (3). The IRE functions as a target for the high affinity binding of a cytosolic protein (IRE-BP) (4-6). The IRE-BP has been demonstrated to bind IRE by gel retardation assays (3) and to depress translation of ferritin mRNA in a cell-free system (7). The RNA binding activity of IRE-BP prepared from human K562 cell extract is increased by treatment with 2-mercaptoethanol or dithiothreitol, and decreased by treatment with N-ethylmaleimide or the deazene carbonyl derivative diamide, indicating that sulfhydryl groups are important for the RNA binding (8). To date, it has been concluded that chelatable iron is a direct physiological regulator of ferritin synthesis in cells (9, 10), and changes in cellular

Abbreviations: IRE, iron-responsive element; IRE-BP, iron-responsive element-binding protein; SDS, sodium dodecyl sulfate; DEM, diethylmaleate.

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iron level have been proposed to cause an allosteric redox-sensitive alteration in the IRE-BP molecule (8). Macrophages carry out fundamental protective functions in the inflammatory and immune responses of the host (11, 12). These cells also play a central role in iron recirculation as they are responsible for the catabolism of effete erythrocytes and the return of iron to the circulation. It is known that macrophages actively synthesize ferritin in response to iron (13). We now present evidence that oxygen contributes to the posttranscriptional regulation of ferritin synthesis in macrophages.

MATERIALS AND METHODS

Macrophages were collected by peritoneal lavage from female C57BL/6N mice weighing 20-25 g, which had given an intraperitoneal injection of 2 ml of 4% thioglycollate broth 4 days previously. The lavage medium was RPMI 1640, which had been equilibrated with 5% CO_2-95% N_2, containing 10 unit/ml heparin. The cells were separated by centrifugation, washed once with RPMI 1640, plated at 1 x 10^6 cells/35-mm diameter culture dish in N_2-equilibrated RPMI 1640 containing 10% fetal bovine serum, 50 unit/ml penicillin and 50 μg/ml streptomycin, and incubated at 37°C in 5% CO_2, 2% O_2 in N_2. After 1 h the medium was replaced with fresh, N_2-equilibrated medium in order to remove the nonadherent cells.

Macrophages prepared as described above were exposed to various agents for a given time. Exposure of macrophages to hydrogen peroxide was carried out by incubating the cells with glucose oxidase which generates hydrogen peroxide by reaction with glucose in the culture medium. After exposure to various agents in the given oxygen tension, the cell proteins were radiolabeled by incubating the macrophages for 15 min in 1 ml of methionine- and cysteine-free RPMI 1640 containing 10% dialyzed fetal bovine serum and about 30 μCi ^{35}S-protein labeling mixture (EXPRE^{35}S^{35}S Protein Mix, Du Pont-New England Nuclear, specific activity > 1000 Ci/mmol, containing > 77% L-[^{35}S]methionine and 18% L-[^{35}S]cysteine). The radiolabeled medium was then removed, and the cells were rinsed three times with phosphate-buffered saline and lysed with 0.2 ml of sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 10% glycerol, 3% 2-mercaptoethanol, 50 mM Tris-HCl (pH 6.8) and 0.1 mM phenylmethylsulfonyl fluoride). Gel electrophoresis and autoradiography were performed as described previously (14, 15). Immunoblot analysis was performed as described previously (14).

RESULTS

In mouse peritoneal macrophages exposed to 100 μM diethylmaleate (DEM), two proteins with molecular masses of 34 and 23 kDa were induced (Fig. 1). As shown previously, the 34-kDa protein is heme oxygenase (14), which catalyzes the oxidative degradation of heme to form biliverdin, and the 23-kDa protein is a stress protein inducible by oxidative and sulfhydryl-reactive agents (15). When the cells were incubated with DEM in 2% O_2, two other proteins with molecular masses of approximately 22 and 21 kDa were induced (Fig. 1). The synthesis of these proteins was depressed as O_2 concentration increased. Without DEM, enhanced synthesis of these

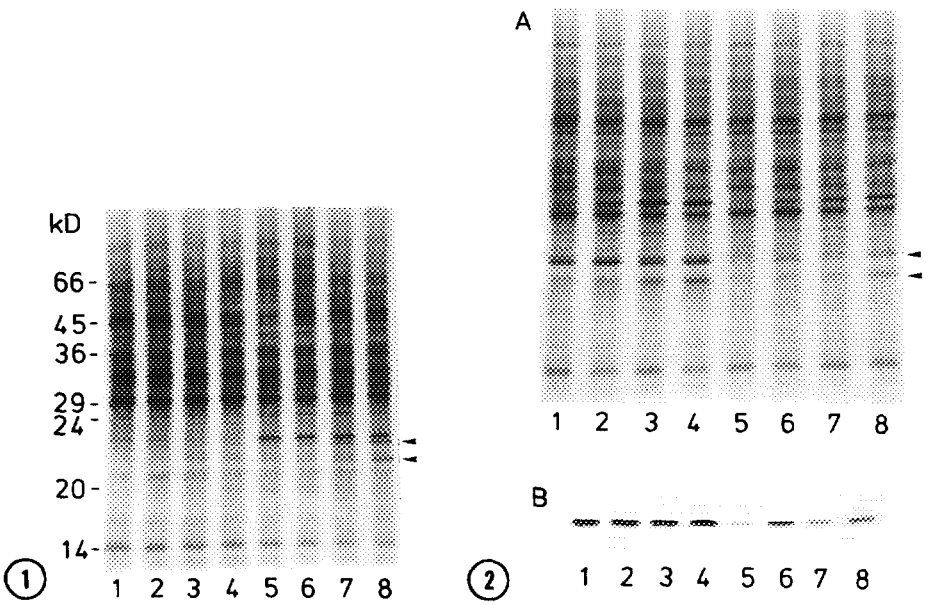

Fig. 1. Induction of synthesis of 22 and 21 kDa proteins in macrophages incubated with DEM in various O_2 conditions. The cells were incubated for 11 h with (lanes 5-8) or without (lanes 1-4) 100 μM DEM in 20% (lanes 1, 5), 10% (lanes 2, 6), 4% (lanes 3, 7), or 2% (lanes 4, 8) O_2. They were radiolabeled for 15 min with [^{35}S]methionine-cysteine mixture, and proteins were then analyzed by 12.5% SDS-polyacrylamide gel electrophoresis and autoradiography. The positions of the 22 and 21 kDa proteins are indicated by the arrows on the right. Molecular mass markers are indicated on the left.

Fig. 2. Induction of synthesis of the 22 and 21 kDa proteins in macrophages incubated with iron or hemin. A, autoradiograph; B, immunoblots. The cells were incubated for 11 h with 100 μM iron (ammonium iron citrate) (lanes 1, 2), 100 μM hemin (lanes 3, 4), 20 μM iron (lanes 5, 6), or 20 μM hemin (lanes 7, 8) in 20% O_2 (lanes 1, 3, 5, 7) or in 2% O_2 (lanes 2, 4, 6, 8). They were then pulse-labeled and analyzed as described in the legend to Fig. 1. The positions of the 22 and 21 kDa proteins are indicated by the arrows.

proteins was not observed in 2% O_2, indicating that induction of the synthesis of these proteins requires both the presence of DEM and hypoxic conditions.

In macrophages incubated with iron as ammonium iron citrate or hemin, synthesis of 22 and 21 kDa proteins was stimulated, depending on the concentration of iron or hemin (Fig. 2A). The synthesis of these proteins in response to iron or hemin was augmented under hypoxic conditions. A polyclonal antibody to mouse liver ferritin has been shown to recognize the heavier protein (Fig. 2B). From these results, we have concluded that the apparently heavier protein is liver-type ferritin subunit (mouse L subunit). It was reported that in mouse cells the two subunits had very similar molecular masses, 20.9 kDa and 20.6 kDa for H and L, respectively, which are calculated from isolated cDNA clones, yet the H subunit had a faster electrophoretic mobility than that of the L subunit in denaturing gel (16).

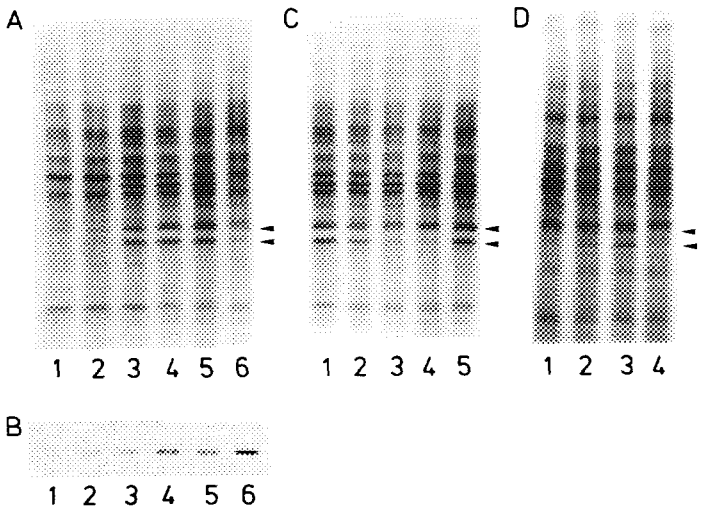

Fig. 3. Time course of ferritin synthesis in macrophages incubated with DEM in 2% O_2 and changes of ferritin synthesis in macrohpages with changes of O_2 conditions. A, autoradiograph; B, immunoblots. The cells were incubated with 100 μM DEM in 2% O_2 before (lane 1) or after the addition of DEM for 2 h (lane 2), 5 h (lane 3), 8 h (lane 4), 11 h (lane 5), or 23 h (lane 6). C, The cells were incubated with 100 μM DEM in 2% O_2 for 8 h (lane 1), and then the cells were transferred to 20% O_2 and incubated for 1 h (lane 2), 2 h (lane 3), 3 h (lane 4), or maintained in 2% O_2 for 3 h (lane 5). D, The cells were incubated with DEM in 20% O_2 for 8 h (lane 1), and then the cells were transferred to 2% O_2 and incubated for 3 h (lane 2), 6 h (lane 3), or maintained in 20% O_2 for 6 h (lane 4). They were then pulse-labeled and analyzed as described in the legend to Fig. 1. The arrows indicate the 22 and 21 kDa proteins.

The time course of ferritin synthesis was measured in macrophages incubated in 2% O_2 with 100 μM DEM (Fig. 3A). Accumulation of the L subunit of ferritin was observed using the antibody to mouse liver ferritin (Fig. 3B). When macrophages that had been incubated with DEM in 2% O_2 were transferred to 20% O_2, the synthesis of ferritin was gradually depressed after the transfer (Fig. 3C). Conversely, when cells that had been incubated with DEM in 20% O_2 were transferred to 2% O_2, enhanced synthesis of ferritin was detected 3 h after the transfer (Fig. 3D).

When menadione and methylviologen, which are known to generate O_2^-, were added to macrophages that had been incubated with DEM in 2% O_2 for 8 h, the synthesis of ferritin was depressed even under hypoxic conditions (Fig. 4). On the other hand, the addition of hydrogen peroxide itself, or the addition of glucose oxidase had no effect on ferritin synthesis. These results suggest that the depression of ferritin synthesis by oxygen occurs via an oxygen tension-dependent production of O_2^- by the cell.

We examined the effect of actinomycin D, an inhibitor of transcription, on the induction of ferritin synthesis by DEM under hypoxic conditions (Fig. 5). Enhanced synthesis of ferritin was observed in cells incubated with DEM in 2%

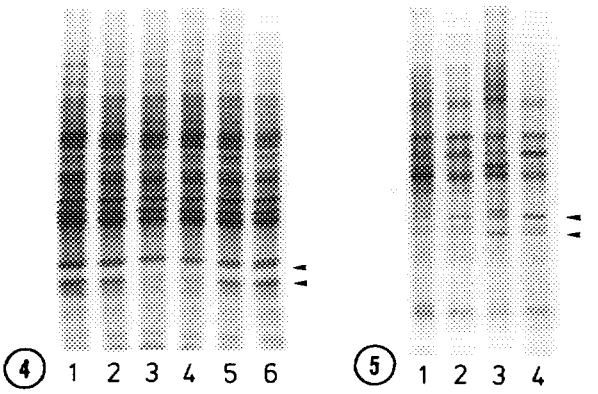

Fig. 4. Effects of O_2^- and hydrogen peroxide on ferritin synthesis in macrophages incubated with DEM in 2% O_2. The cells were incubated with 100 μM DEM in 2% O_2 for 8 h (lane 1), and then incubated in 2% O_2 for 3 h with no additives (lane 2), 5 μM menadione (lane 3), 200 μM methylviologen (lane 4), 50 μM hydrogen peroxide (lane 5), or 50 mU/ml glucose oxidase (lane 6). They were then pulse-labeled and analyzed as described in the legend to Fig. 1. The arrows indicate the 22 and 21 kDa proteins.

Fig. 5. Effects of actinomycin D on ferritin synthesis in macrophages incubated with DEM in 2% O_2. The cells were incubated for 11 h in 2% O_2 with no additives (lane 1), 0.1 μg/ml actinomycin D (lane 2), 100 μM DEM (lane 3), 0.1 μg/ml actinomycin D plus 100 μM DEM (lane 4). They were then pulse-labeled and analyzed as described in the legend to Fig. 1. The arrows indicate the 22 and 21 kDa proteins.

O_2 irrespective of the presence of actinomycin D. In contrast, synthesis of the 34-kDa (heme oxygenase) and 23-kDa protein was depressed by actinomycin D. The effect of DEM and hypoxia on the ferritin synthesis is thus post-transcriptional.

DISCUSSION

The cDNA of IRE-BP has been isolated from some species, revealing that it has a sequence homology to a mitochondrial aconitase, which is an iron sulfur (Fe-S) enzyme in the Krebs cycle catalyzing the stereospecific conversion of citrate to iso-citrate (17-21). There is another aconitase in the cytosol. Recently, IRE-BP and cytosolic aconitase have been shown to be identical (22). Klausner et al. have proposed as a working model the idea that both the Fe-S cluster and the aconitase substrate might contribute to maintaining the overall structure of IRE-BP, which has aconitase activity but does not have IRE-binding activity. On the other hand, the peculiar vulnerability of the aconitase-type cluster to disassembly and loss of the substrate presumably allows significant structural changes, resulting in the conformation which possesses IRE-binding activity but not aconitase activity (23).

The present study demonstrated that DEM was a strong inducer of ferritin synthesis under hypoxic conditions and that the induction occurred posttranscriptionally. These results suggest that DEM reacts with IRE-BP and reduces the binding activity of IRE-BP in intact cells cultured under hypoxia. In a cell-free binding assays, it has been shown that sulfhydryl-blocking agents inhibit binding of IRE-BP to IRE, and sulfhydryl-generating agents increase the binding activity (8). These are consistent with our results obtained in the intact cells. Recently it has also been found in the cell-free system that one of the cysteine residues (Cys-437) predicted to coordinate the Fe-S cluster in the IRE-BP is the primary target for inactivation of RNA binding by the sulfhydryl-blocking agents (24). Our data suggest that DEM reacts with this sulfhydryl group of the cysteine, resulting in the loss of the binding activity in the cells cultured under hypoxia.

The present experiments showed that oxygen depressed induction of ferritin synthesis by DEM and that O_2^- was involved in this depression. Traces of O_2^- is inevitably generated in aerobic metabolism of cells, including macrophages, and the amount of O_2^- produced is reasonably assumed to depend on the oxygen tension. O_2^- is presumably involved in the regulation of IRE-binding activity of IRE-BP by interacting with the Fe-S cluster of the protein. The induction of ferritin synthesis by DEM under hypoxic conditions is interpreted as that the induction requires both the block of the sulfhydryl group(s) of IRE-BP by DEM and the dissociation of O_2^- from the Fe-S cluster of the protein. Gradner and Fridovich have recently demonstrated that bacterial aconitase, which is also an Fe-S enzyme, particularly sensitive to inactivation by O_2^- (25). They have also reported that O_2^- is a major determinant of aconitase activity and that aconitase can be used as an indicator of intracellular steady-state O_2^- level (26). Recently, it is reported that nitric oxide produced by macrophages may modulate the post-transcriptional regulation of genes involved in iron homeostasis (27,28). Nucleotide sequences corresponding to IREs have also been found in mRNAs encoding the human transferrin-receptor (29), murine δ-aminolevulinate synthase, and porcine heart aconitase (30). O_2^- may function as an effector that regulates the synthesis of these proteins.

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