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Regulation of Ferritin Synthesis and Iron Regulatory Protein 1 by Oxygen in Mouse Peritoneal Macrophages

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Ferritin is an intracellular iron storage protein whose synthesis is regulated post-transcriptionally by a mechanism that involves binding of cytoplasmic iron regulatory protein (IRP) to iron-responsive element (IRE) in the 5′ untranslated region of ferritin mRNA. In this study, we have shown that in mouse peritoneal macrophages, the synthesis of ferritin was enhanced and the IRE binding activity of IRP-1 was diminished when the oxygen tension was decreased. Iron is known to induce ferritin synthesis and even in the presence of a low concentration of iron, synthesis of ferritin was enhanced and the activity of IRP-1 was decreased under hypoxia. The enhanced synthesis of ferritin under hypoxia was abolished by the addition of O_2^--generating agents but not H_2O_2. The decreased activity of IRP-1 under hypoxia was reversed by adding O_2^--generating agents. These data suggest that O_2^- generated in the cell is involved in alterations of ferritin synthesis and the activity of IRP-1 by oxygen.

Cellular iron homeostasis is maintained by the coordinate regulation of expression of various proteins including the intracellular iron storage protein, ferritin (1). Recent studies have demonstrated that the synthesis of ferritin is post-transcriptionally regulated in response to changes in iron availability (2, 3). The regulatory mechanism includes the iron-responsive element (IRE) in the 5′ untranslated region of ferritin mRNA and cytosolic proteins designated iron regulatory proteins (IRP-1 and IRP-2) which bind to the IRE. IRP-1 is an iron-sulfur protein containing a cubane [4Fe-4S] cluster and exhibits aconitase activity, whereas IRP-2 neither possesses the iron-sulfur cluster nor exhibits aconitase acitivity. Binding activity of IRP-1 is modulated by intracellular iron levels. In iron-replete cells, IRP-1 has a low affinity for the IRE, resulting in enhanced synthesis of ferritin. In iron-depleted cells, IRP-1 binds to the IRE with high affinity, resulting in suppression of ferritin mRNA translation. Similar mechanisms are involved in the synthesis of erythroid -aminolevulinate synthase, an enzyme that catalyzes the first step of heme biosynthesis (4). On the other hand, several IREs are present in the 3′-untranslated region of transferrin receptor (TfR) mRNA (5, 6). When IRP binds to these IREs, TfR mRNA is protected from nuclease attack, causing the enhanced expression of TfR.

Although IRP-1 is regulated mainly by intracellular iron levels, nitric oxide (NO), which is produced from L-arginine in many cell types including macrophages, has been recently demonstrated to raise IRE binding activity of IRP-1 and thus represses ferritin synthesis (7, 8). It has been also demonstrated that H_2O_2 can raise IRE-binding activity of IRP-1 (9, 10) and that peroxinitrite modulates the IRE-binding activity of IRP (11).

In bacteria, the iron-sulfur protein, fumarate nitrate reduction protein (FNR) can act as an oxygen sensor (12). In the absence of oxygen, FNR binds to promoters of the anaerobic metabolism genes which activate transcription (13). Aconitase of E. coli is an another iron-sulfur protein which is sensitive to O_2^-. These observations suggest that the IRE-binding activity of IRP-1 is also regulated by oxygen. We have previously reported in mouse peritoneal macrophages that ferritin synthesis is enhanced under low oxygen conditions in the presence of diethylmaleate, a sulfhydryl-reactive agent (14). In mouse peritoneal macrophages, the band of IRE/IRP complex in the gel retardation assay seemed to be single and aconitase activity was also detected (7). Therefore, IRP in these cells is considered IRP-1.

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After completion of this work, a paper has been published [Hanson and Leibold (1998) *J. Biol. Chem.* **273,** 7588–7593] describing the regulation of IRP-1 by oxygen tension in rat hepatoma cell line and primary cardiac myocytes. Their observations are similar to some parts of the results of the present study.

Abbreviations used: IRE, iron-responsive element; IRP, iron regulatory protein; TfR, transferrin receptor; SDS, sodium dodecyl sulfate.

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Qi, et al. also reported that enhanced ferritin synthesis occurs under hypoxia in neonatal rat oligodendrocytes and human oligodendroglioma cells (15, 16). However, it has not been experimentally demonstrated that both synthesis of ferritin and the activity of IRP are simultaneously modulated in response to changes in oxygen tension. In the present study, we demonstrate in macrophages that synthesis of ferritin is enhanced and the binding of IRP-1 to IRE is attenuated by hypoxia. It has been suggested that the changes in IRP-1 binding activity by oxygen is mediated by O_2^-.

MATERIALS AND METHODS

Macrophages were collected by peritoneal lavage from female C57BL/6N mice weighing 20-25 g, that had been given an intraperitoneal injection of 2 ml of 4% thioglycollate broth 4 days previously. The lavage medium was RPMI1640 containing 10 unit/ml heparin. The cells were separated by centrifugation, washed once with RPMI 1640, placed at 1×10^6 cells/35-mm diameter culture dish in 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 in air. After 1 h the medium was replaced with fresh medium in order to remove the nonadherent cells, the cells were cultured for a further 11 h and then used for all experiments.

To investigate effects of hypoxia, cells were cultured in a gas-tight chamber flushed with 95% N_2 and 5% CO_2 and the concentration of O_2 was monitored by the oxygen meter. In some experiments, various agents were added before the cells were put into the chamber.

The cell proteins were radiolabeled by incubating the macrophages for 15 min under normoxic conditions in 1 ml of methionine- and cysteine-free RPMI 1640 containing 10% dialyzed fetal bovine serum and about 20 μCi ^{35}S-protein labeling mixture (EXPRE^{35}S^{35}S [^{35}S] Protein Labeling Mix, NEN Life Science Product, Inc., specific activity > 1000 Ci/mmol, containing 73% L-[^{35}S] methionine and 23% L-[^{35}S] cysteine). The radiolabeled medium was then removed, and the cells were rinsed three times with ice-cold 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 (17). Immunoblot analysis was performed using a polyclonal antibody to mouse liver ferritin as described previously (18).

Cytosolic extracts were prepared from the macrophages treated as described above and gel retardation assays were performed as

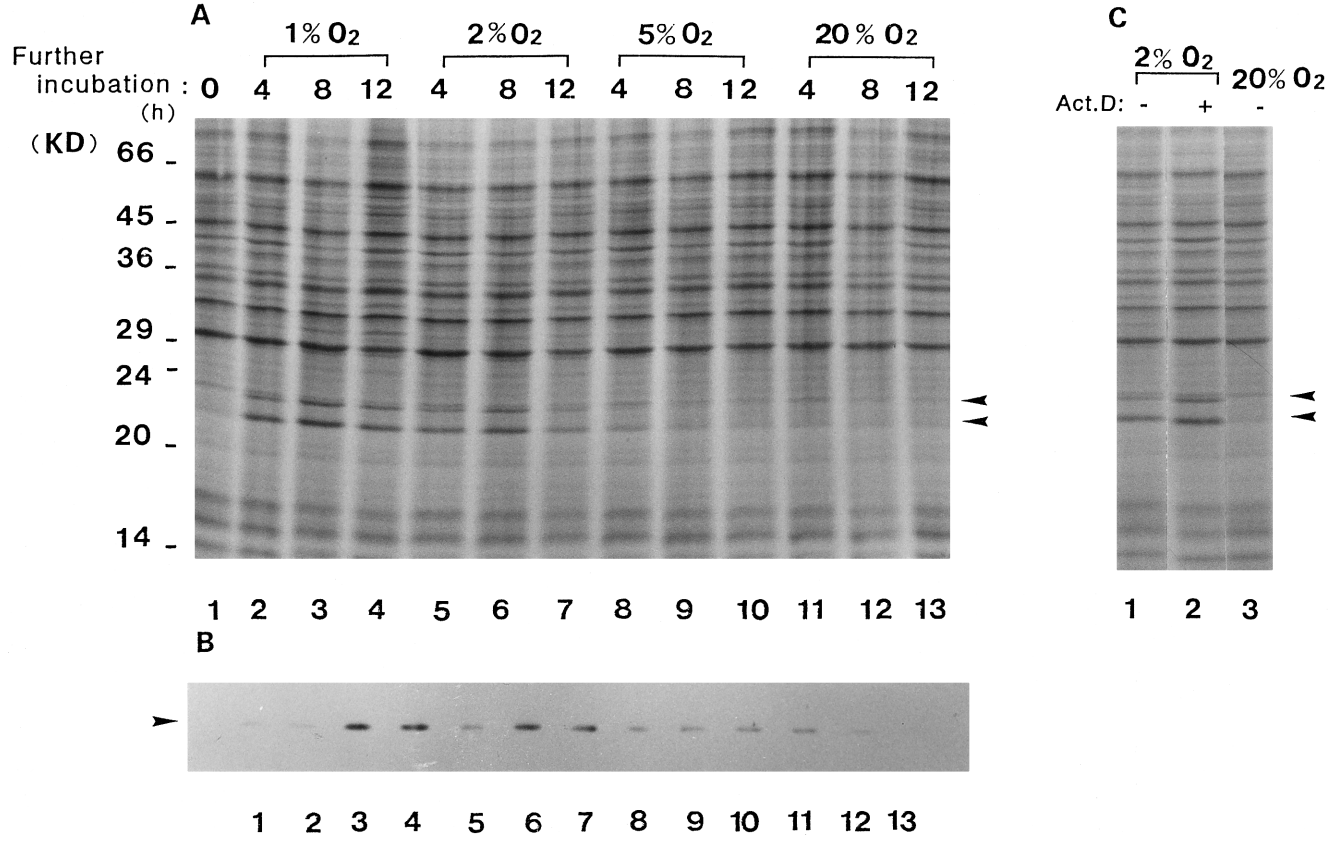

FIG. 1. Oxygen tension-dependent ferritin synthesis. A. Cells were cultured for 12 h in 20% O_2 (lane 1), incubated in 1% (lanes 2–4), 2% (lanes 5–7), 5% (lanes 8–10) or 20% (lanes 11–13) O_2 for a further 4 h (lanes 2, 5, 8, 11), 8 h (lanes 3, 6, 9, 12) or 12 h (lanes 4, 7, 10, 13). Cells were then radiolabeled for 15 min with [^{35}S]methionine cysteine mixture, and proteins were analyzed by 12. 5% SDS-polyacrylamide gel electrophoresis and autoradiography. The positions of ferritin subunits are indicated by the arrows on the right. Molecular mass markers are indicated on the left. B. The same proteins as used in A were immunoblotted and detected using a polyclonal antibody to mouse liver ferritin by the enhanced chemiluninesence. C. The cells cultured for 12 h in normoxic conditions were incubated for a further 8 h in 2% O_2 with no additives (lane 1) or 0.1 μg/ml actinomycin D (lane 2), or in 20% O_2 without actinomycin D (lane3).

described previously (19) using a ^{32}P-labeled mouse ferritin IRE probe (20) and approximately 15 μ g protein of cytosolic extract.

RESULTS

Mouse peritoneal macrophages were cultured for 12 h under normoxic conditions and then they were subjected to various oxygen tensions for times indicated and synthesis of proteins was investigated (Fig. 1A). The level of synthesis of two proteins with molecular masses of approximately 22 and 21 kDa was increased with decreasing oxygen tension. Addition of hemin or iron (ammonium iron(III) citrate) also enhanced the synthesis of these proteins (data not shown). The 22 kDa protein was recognized by a polyclonal antibody to mouse liver ferritin (Fig. 1B). In murine cells, the deduced molecular masses of the two subunits of ferritin, H and L, from cDNA clones, are 20. 9 and 20. 6 kDa, respectively. However, the H subunit had a faster electrophoretic mobility than that of the L subunit in a denaturing gel (21). Therefore, we concluded that these two protein bands (apparently 22 kDa and 21 kDa) are ferritin L and H subunits, respectively. Actinomycin D did not alter the enhanced synthesis of ferritin under 2% O_2 (Fig. 1C). This suggests that enhanced ferritin synthesis by low oxygen is regulated at a posttranscriptional level.

To determine the involvement of the IRE/IRP system in the regulation of ferritin synthesis by oxygen tension, we analyzed the IRE-binding activity of IRP-1 derived from the macrophages incubated in various oxygen tensions by gel retardation assays in the absence or presence of 2-mercaptoethanol, which is known to fully activate IRP-1. As shown in Fig. 2, IRP-1 derived from cells exposed to low oxygen tension (1-5%) for 4 h decreased IRE binding activity, whereas high IRE binding activity was observed in IRP-1 taken from cells incubated in normoxic conditions.

Ferritin synthesis was enhanced by incubation of cells at 2% O_2 for 4 h, which returned to control levels when cells were transferred back to normoxic conditions for 4 h (Fig. 3A). When these cells were returned to hypoxic conditions for a further 4 h, the synthesis of ferritin was again enhanced. These results indicate that the synthesis of ferritin was switched on and off by oxygen tension. The alteration of the IRE-binding activity of IRP-1 was investigated when cells were moved between hypoxic and normoxic conditions (Fig. 3B). When cells were in normoxic conditions, the IRE-binding activity of IRP-1 was relatively high whereas the binding activity decreased in cells incubated under hypoxic conditions. These results are consistent with the synthesis of ferritin (Fig. 3A) and suggest that the IRE-binding activity of IRP-1 is reversibly altered by oxygen tension.

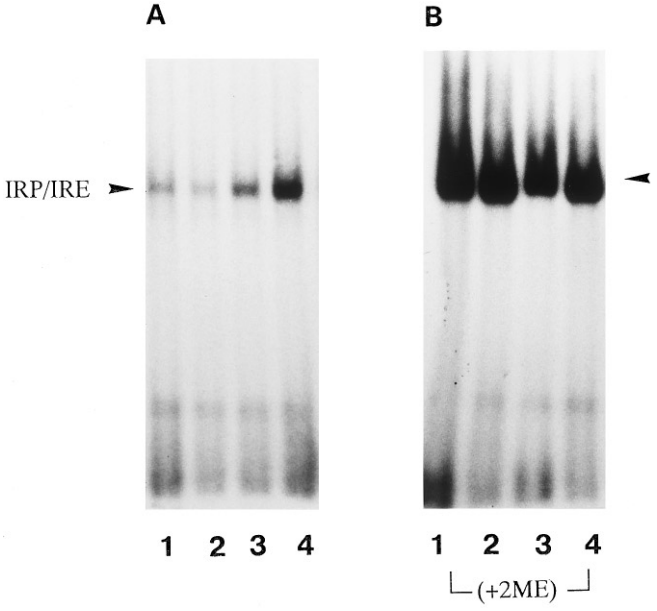

FIG. 2. Oxygen tension-dependent modulation of IRP activity. The cytosolic extracts derived from cells cultured in 20% O_2 and incubated for a further 4 h in 1% (lane 1), 2% (lane 2), 5% (lane 3), or 20% (lane 4) were analyzed by the gel-retardation assay in the absence (A) or presence (B) of 2% 2-mercaptoethanol.

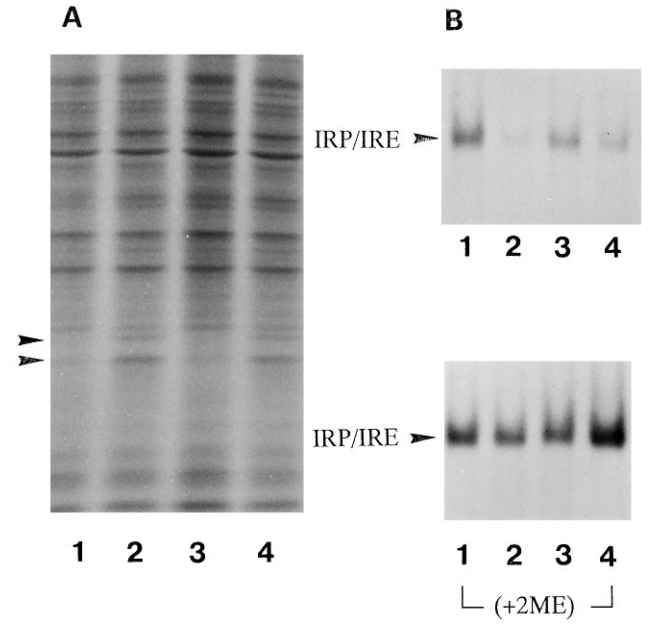

FIG. 3. Switching of ferritin synthesis and the IRE binding activity of IRP by oxygen tensions. A. Cells were incubated for 24 h in 20% O_2 (lane 1), for 20 h in 20% O_2 and 4 h in 2% O_2 (lane 2), for 16 h in 20% O_2, 4 h in 2% O_2 and 4 h 20% O_2 (lane 3), or for 12 h in 20% O_2, for 4 h in 2% O_2 , 4 h in 20% O_2 and 4 h in 2% O_2 (lane 4). The cell proteins were analyzed as described in the legend in Fig. 1. B. The cell extracts derived from the cells treated as described above were analyzed by the gel retardation assay in the absence (top panel) or presence of 2% 2-mercaptoethanol (bottom panel).

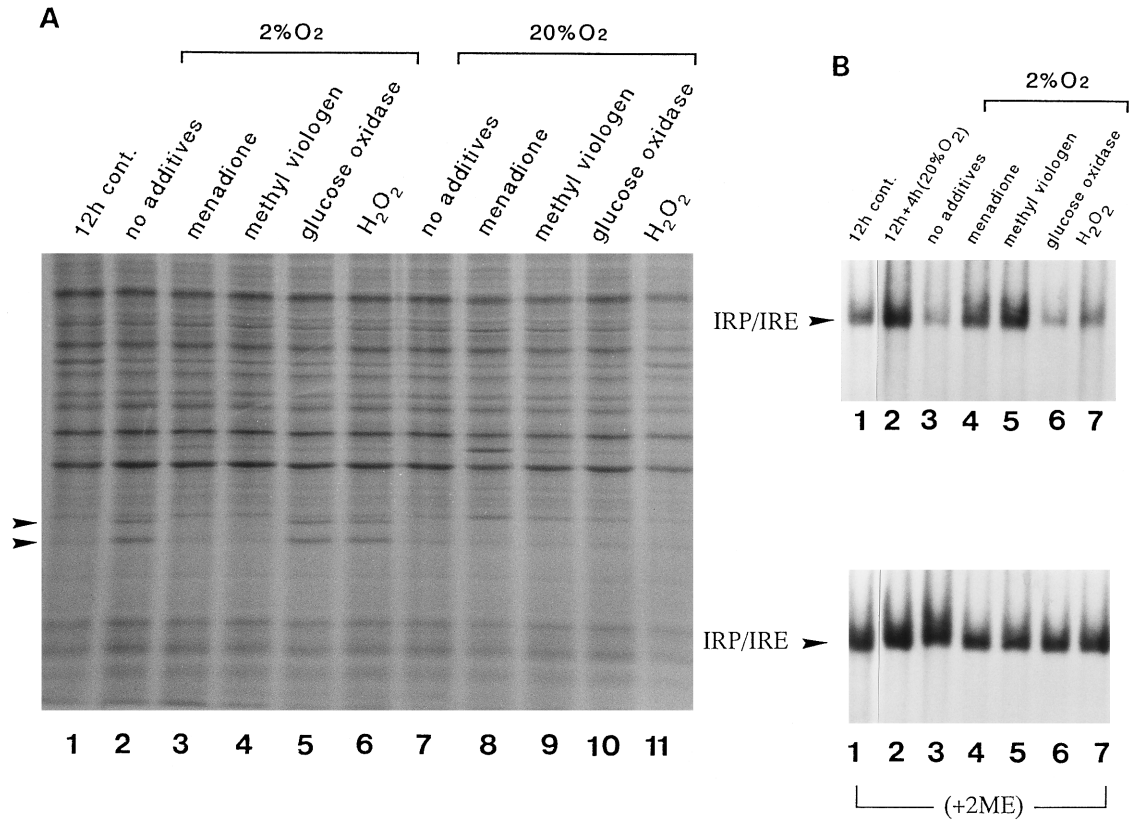

FIG. 4. Effects of oxidative stress on the enhancement of ferritin synthesis by hypoxia. A. The cells were cultured for 12 h in 20% O_2 (lane 1) then incubated for a further 4 h with no additives (lanes 2, 7), 5 μM menadione (lanes 3, 8), 200 μM methyl viologen (lanes 4, 9), 50 mU/ml glucose oxidase (lane 5), 5 mU/ml glucose oxidase (lane 10), or 50 μM H_2O_2 (lanes 6, 11) in 2% O_2 (lanes 2–6) or in 20% O_2 (lanes 7–11). Cell proteins were analyzed as described in the legend in Fig. 1. B. The cell extracts derived from the cells cultured for 12 h in 20% O_2 (lane 1) and incubated for a further 4 h in 20% O_2 with no additives (lane 2), or in 2% O_2 with no additives (lane 3), 5 μM menadione (lane 4), 200 μM methyl viologen (lane 5), 50 mU/ml glucose oxidase (lane 6), or 50 μM H_2O_2 (lane 7) were analyzed by the gel retardation assay in the absence (top panel) or presence of 2% 2-mercaptoethanol (bottom panel).

Various oxidative stress agents were added to the macrophages cultured under 2% O_2 and the synthesis of ferritin was investigated (Fig. 4A). The enhanced synthesis of ferritin by hypoxic conditions was blocked by addition of menadione or methyl viologen, which produces O_2^- in the cell (22, 23). However, the addition of glucose oxidase or H_2O_2, which generates H_2O_2 (24), had no effects on the enhanced synthesis of ferritin by hypoxia. These results suggest that O_2^- is involved in the reduced synthesis of ferritin by oxygen.

The IRE-binding activity of IRP-1 derived from the cells exposed to these agents was also investigated (Fig. 4B). Cells were incubated for 4 h in a 2% O_2 atmosphere with these stress agents and the isolated cytosols were used for gel retardation assays. The IRP-1 derived from cells exposed to H_2O_2 or glucose oxidase retained a low IRE binding activity, whereas the IRP-1 derived from cells exposed to the O_2^--producing agents recovered their binding activity. These results are consistent with the observed reduction in ferritin synthesis by O_2^- (Fig. 4A).

Iron inactivates IRE-binding activity of IRP-1, resulting in enhanced synthesis of ferritin. We therefore investigated the effect of oxygen tension on the stimulation of ferritin synthesis by iron. As shown in Fig. 5A, the synthesis of the ferritin L subunit is enhanced by iron as ammonium iron(III) citrate in a concentration-dependent manner under both hypoxia and normoxia. However, in a 2% O_2 atmosphere, the synthesis of the L subunit was enhanced by low concentrations of iron compared with the synthesis of the subunit in a 20% O_2 atmosphere. This suggests that the synthesis of the L subunit was more sensitive to iron concentrations in cells incubated at 2% O_2 than those at 20% O_2. The synthesis of the H subunit was also enhanced by iron in a concentration dependent manner in 20% O_2 atmosphere. However, it seemed to be enhanced maximally only under hypoxia.

Enhancement of ferritin synthesis by hypoxia was abolished when cells were cultured for the initial 12 h with iron chelators, desferioxamine or o-phenanthrolin (data not shown). These results indicate that intracel-

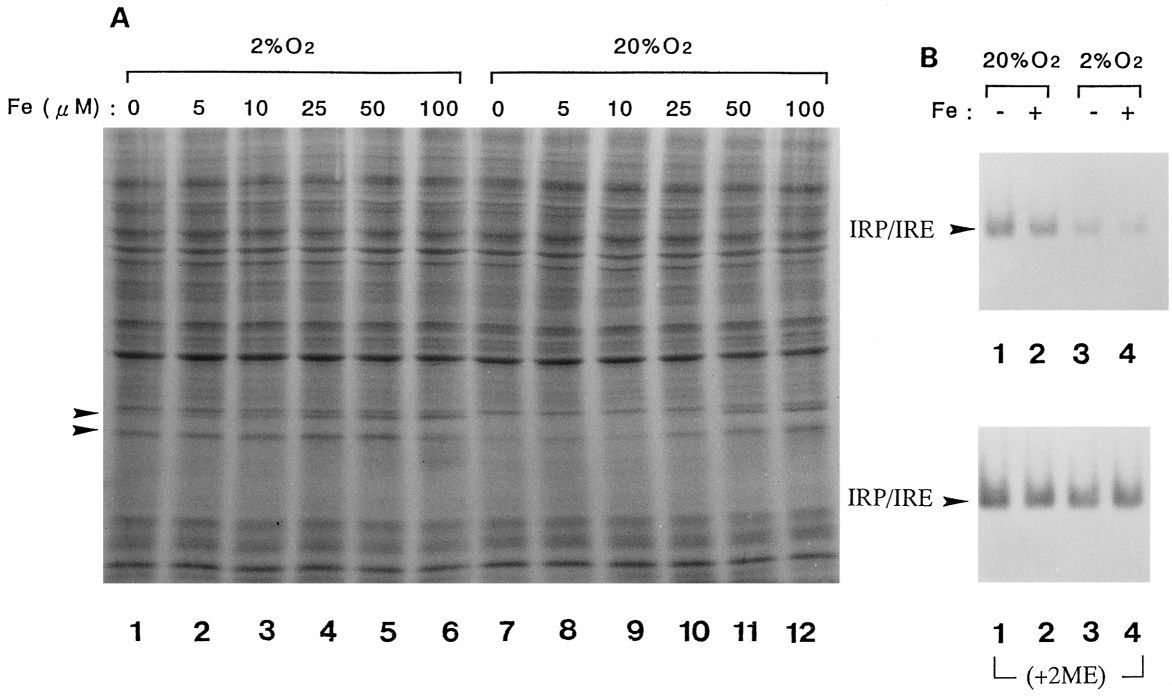

FIG. 5. Effects of oxygen tension on ferritin synthesis by iron. A. Cells were cultured for 12 h in 20% O_2 then incubated for a further 4 h with no additives (lanes 1, 7) 5 μM (lanes 2, 8), 10 μM (lanes 3, 9), 25 μM (lanes 4, 10), 50 μM (lanes 5, 11), or 100 μM ammonium iron (III) citrate (lanes 6, 12) in 2% O_2 (lanes 2–6) or 20% O_2 (lanes 7–12). The cell proteins were analyzed as described in the legend in Fig. 1. B. The cell extracts derived from the cells cultured for 12 h in 20% O_2 and incubated for further 4 h with no additives (lanes 1, 3) or 10 μM ammonium iron (III) citrate (lanes 2, 4) in 20% O_2 (lanes 1, 2) or in 2% O_2 (lanes 3, 4) analyzed by the gel retardation assay in the absence (top panel) or presence of 2% 2-mercaptoethanol (bottom panel).

lular chelatable iron pools are required to enhance ferritin synthesis by hypoxia.

The IRE-binding activity of IRP-1 was investigated in cells cultured with 10 μ M iron (ammonium iron(III) citrate) in 20% or 2% O_2 atmosphere (Fig. 5B). The binding activity of IRP-1 derived from cells incubated with 10 μ M iron in 2% O_2 was significantly lower than those from the cells incubated in 20% O_2.

DISCUSSION

The present study demonstrates that synthesis of ferritin in peritoneal macrophages is modulated by physiological alterations in oxygen tension. Qi, et al. also demonstrated that hypoxia specifically and reversibly induced the synthesis of ferritin in rat oligodendrocytes and human oligodendroglioma cells (15, 16). These findings are consistent with our data presented here. We have further demonstrated that the reversible regulation of ferritin synthesis is mediated by changes in the IRE-binding activity of IRP-1 under different oxygen tensions in macrophages.

Our data suggest that O_2^- is involved in the changes in IRP-1 activity by oxygen tension (Fig. 4). Gardner and Fridovich reported that aconitase of E. coli is inactivated by O_2^- in a dose dependent manner and suggested that this inactivation is involved in oxidative attack on the prosthetic Fe-S cluster (25, 26). IRP-1 shows the aconitase activity when it contains intact Fe-S cluster (27). Recently, O_2^- has been shown to inactivate the aconitase activity of human IRP (28). Therefore, under low oxygen conditions, the Fe-S cluster of IRP-1 may be intact, resulting in the increase of ferritin synthesis. When oxygen tension rises, production of O_2^- in cells inevitably increases and the Fe-S cluster may be attacked. This process probably involves conversion of IRP-1 from the inactive form to the active form in the cell, causing the repression of ferritin synthesis.

Recently, it has been shown that NO augments IRP-1 binding activity to IRE in mouse peritoneal macrophages and macrophage cell lines (7, 8). Under the experimental conditions used here, production of NO is hardly detectable (29). Therefore, it is likely that the pathway of IRP-1 modulation by oxygen is independent of that of NO.

Pantopoulos, et al. have demonstrated that H_2O_2 activates IRP-1 and decreases the synthesis of ferritin in mouse B6 fibroblasts cultured under normoxia (10,30). IRP-1 may be activated and the synthesis of ferritin

decreased by H_2O_2 also in the macrophages incubated under normoxia. However, since the synthesis of ferritin is very low in these cells under normoxia, a further reduction of ferritin synthesis could not be detectable. On the other hand, as shown in Fig. 4, H_2O_2 had little effect on the enhanced synthesis of ferritin and the activation of IRP-1 when the cells were exposed to H_2O_2 under hypoxia. Pantopoulos, et al. have suggested that the activation of IRP-1 by H_2O_2 does not result from a direct oxidant attack on the Fe-S cluster of IRP-1 but instead involves additional cellular activities such as phosphorylation. These activities may be influenced by the changes of oxygen tensions in such cells like fibroblasts.

The factor(s) which sense the alteration of oxygen tension have not been isolated although such a sensor is thought to be a heme protein (31). When the iron concentration was low, the binding activity of IRP-1 changed drastically with changes in oxygen tension. These results suggest that intracellular iron plays an important role to sense changes in oxygen tension. The observations shown in this study suggest mechanisms by which the cells sense changes of oxygen tension.

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