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Hypoxia acts through multiple signaling pathways to induce metallothionein transactivation by the metal-responsive transcription factor-1 (MTF-1)

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Abstract

Metal-responsive transcription factor-1 (MTF-1) is essential for the induction of genes encoding metallothionein by metals and hypoxia. Here, we studied the mechanism controlling the activation of MTF-1 by hypoxia. Hypoxia activation of *Mt* gene transcription is dependent on the presence of metal regulatory elements (MREs) in the promoter of *Mt* genes. We showed that MREa and MREd are the main elements controlling mouse *Mt-1* gene induction by hypoxia. Transfection experiments in *Mtf-1*-null cells showed that MTF-1 is essential for induction by hypoxia. Chromatin immunoprecipitation analysis showed that MTF-1 DNA-binding activity was strongly enhanced in the presence of zinc but not by hypoxia. Notably, hypoxia inducible factor- (HIF) 1α was recruited to the *Mt-1* promoter in response to hypoxia but not to zinc. MTF-1 activation was inhibited by PKC, JNK, and PI3K inhibitors and by the electron transport chain inhibitors rotenone and myxothiazol, but not by the antioxidant *N*-acetylcysteine. We showed that prolyl-hydroxylase inhibitors can activate MTF-1, but this activation requires the presence of HIF-1α. Finally, HIF-dependent transcription is enhanced in the presence of MTF-1 and induction of an MRE promoter is stimulated by HIF-1α, thus indicating cooperation between these 2 factors. However, coimmunoprecipitation experiments did not suggest direct interaction between MTF-1 and HIF-1α.

Keywords

hypoxia; metal-responsive transcription factor-1 (MTF-1); hypoxia-inducible factor-1α (HIF-1α); protein kinase; signal transduction; activation

Introduction

Metal-responsive transcription factor-1 (MTF-1) was originally isolated as the transcription factor that controls expression of the *metallothionein* (*MT*) gene in response to transition metal ions (metals) (Radtke et al. 1993). To induce transcription, MTF-1 binds to specific *cis*-acting elements termed metal-regulatory elements (MREs) that are present in multiple

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copies in the promoter of the *Mt* genes (Andrews 2001; Giedroc et al. 2001; Lichtlen and Schaffner 2001). Knockout (KO) of the *Mtf-1* gene showed that MTF-1 is essential for basal and metal-induced *Mt* gene transcription (Lichtlen and Schaffner 2001), and its ablation also sensitizes cells to the cytotoxic effects of cadmium and to killing by H_2O_2. Furthermore, the *Mtf-1* gene is essential because *Mtf-1*-null mutant mice die in utero because of liver degeneration and edema. MTF-1 regulates the expression of several genes including those encoding zinc transporter-1, c/EBP, and N-myc downstream regulated gene-1 (Wimmer et al. 2005). More recently, it has been shown that treatment of zebrafish cells with siRNA to *Mtf-1* changed the transcriptional response to zinc for over 1000 genes (Hogstrand et al. 2008). MTF-1 is also activated by oxidative stress (Dalton et al. 1996) and, notably, controls *Mt* (Murphy et al. 1999) and *Placenta growth factor* (Green et al. 2001) gene expression in response to hypoxia. Placenta growth factor is a member of the vascular endothelial growth factor (VEGF) family induced in growing tumors in response to hypoxia that stimulates blood vessel growth (Carmeliet 2005), a process called angiogenesis crucial for cancer progression.

The prototype member of the VEGF family is VEGF-A, and hypoxia induces *VEGF-A* gene expression via signaling through the central hypoxia regulator, hypoxia-inducible transcription factor-1α (HIF-1α). HIF-1α controls several genes involved in erythropoiesis, angiogenesis, glucose metabolism, cell survival, and invasion (Brahimi-Horn and Pouysségur 2006). The mechanisms controlling activation of HIF-1α in response to hypoxia are well characterized. HIF-1α forms a heterodimer with HIF-1β. The α subunit, which is barely detectable under normal oxygen conditions (normoxia), is constantly subjected to proteasomal degradation whereas the β subunit is present in the nucleus at relatively constant levels regardless of the oxygen concentration. Under normoxia, the oxygen-, 2-oxoglutarate-, and Fe(II)-dependent dioxygenase termed prolyl hydroxylase domain (PHD) protein hydroxylates HIF-1α on 2 proline residues. Then, the E3 ligase von Hippel–Lindau (pVHL) binds to prolyl-hydroxylated HIF-1α and targets it for degradation by the ubiquitin–proteasome system. Under hypoxia, HIF-1α is not hydroxylated because the hydroxylases, which require Fe(II) and O_2 for activity, are inactive and thus HIF-1α is not recognized by pVHL and accumulates. In addition, under normoxia HIF transcriptional activity is suppressed by an asparaginyl hydroxylase (FIH-1) that hydroxylates Asn803, blocking its association with coactivators (Hewitson et al. 2002). Phosphorylation and sumoylation of HIF-1α have also been reported to modulate its activity (Brahimi-Horn et al. 2005). In addition, reactive oxygen species (ROS) produced in hypoxia appear to be involved in the regulation of HIF-1α stability (Pouysségur and Mechta-Grigoriou 2006).

Recent data support an important role of MTF-1 in tumor initiation and progression to malignant growth. Using xeno-graft implantation of wild-type or *Mtf-1*-KO-mouse embryonic fibroblasts (MEF), it has been shown that loss of MTF-1 results in suppressed tumor growth (Haroon et al. 2004). A number of clinical studies documenting elevated expression of 2 MTF-1 target genes, *MT* (Cherian et al. 2003) and *Placenta Growth Factor* (Luttun et al. 2004), in a variety of aggressive human tumors, also imply a role for MTF-1 in tumor development. Interestingly, normal synthesis and stabilization of HIF-1α is highly dependent on the presence of MTF-1 (Murphy et al. 2005). In addition, HIF-1α is essential for induction of *Mt-1* by hypoxia when it appears to function as a coactivator of *Mt-1* gene

transcription by interacting with MTF-1 (Murphy et al. 2008). Thus, MTF-1 may influence malignant progression directly through control of a subset of hypoxic stress proteins such as MT and placenta growth factor, and also indirectly through its action on HIF-1α and its targets.

MTF-1 is considered a cytoplasmic zinc sensor, characterized by increased DNA binding activity in the presence of zinc (Andrews 2001; Giedroc et al. 2001). MTF-1 is mostly cytoplasmic and is translocated to the nucleus upon metal induction and under a number of stress conditions (Saydam et al. 2001). Activation of MTF-1 is dependent on the interaction of zinc with the zinc fingers of the protein, and other metals as well as ROS that can induce *Mt* apparently act by displacing zinc from the weakly bound pool, making the displaced zinc available for activation of MTF-1 (Zhang et al. 2003). In addition, we have shown that phosphorylation is involved in the activation of MTF-1 in response to metals, and is controlled by a complex kinase signal transduction pathway that includes PKC, PI3K, JNK, and a protein tyrosine kinase (LaRochelle et al. 2001*a*). However, the mechanisms governing MTF-1 activation by hypoxia are still unknown. HIF-1α is degraded in normoxia, whereas MTF-1 appears to be stable under both normoxia and hypoxia (Murphy et al. 2008), indicating that the mechanisms controlling MTF-1 under hypoxia are different from those regulating HIF-1α. The focus of this study is to define the mechanisms associated with MTF-1 activation by hypoxia, including analysis of potential signaling pathways, phosphorylation events, and nuclear translocation.

Materials and methods

Reagents

The protein kinase inhibitors GF109203X, Ly294002, and SP600125 were purchased from EMD Biosciences–Calbiochem (La Jolla, Calif.). Desferrioxamine (DFO) and dimethyloxaloylglycine (DMOG) were obtained from Frontier Scientific (Logan, Utah). The transfection reagent ExGen500 was purchased from MBI Fermentas (Burlington, Ont.), DNA modifying enzymes were obtained from New England Biolabs (Pickering, Ont.), and synthetic oligonucleotides (oligos) were from Invitrogen (Carlsbad, Calif.) or Sigma–Aldrich (St-Louis, Mo.). The polyclonal anti-MTF-1 antibody was raised in rabbit using bacterially expressed glutathione-S-transferase-MTF-1 fusion protein (LaRochelle et al. 2008), while the anti-tubulin (ab6160) and the anti-HIF-1β (ab2) antibodies were purchased from Abcam (Cambridge, Mass.). The polyclonal anti-HIF-1α antibody was provided by Jacques Pouysségur (IBDC, Centre Antoine Lacassagne, Nice, France). All other chemicals were purchased from Sigma–Aldrich.

Plasmid constructs and mutagenesis

The human MTF-1 expression plasmid pC-mMTF-1a (Radtke et al. 1993) was provided by Walter Schaffner (University of Zurich, Zurich, Switzerland), and the HIF-1α reporter plasmid 3HRE-LUC (pDE-Dtk-LUC) (Berra et al. 2003) was provided by Jacques Pouysségur. The luciferase (LUC) reporter plasmid 1843MT1-LUC contains 1843 bp of the 5′ flanking and 68 bp of the 5′ untranslated regions from the mouse *Mt-1* gene (Faraonio et al. 2000). The deletion mutants 238MT1-LUC, 150MT1-LUC, and MT1min-LUC contain

mouse *Mt-1* promoter sequence from positions −238 (relative to the transcription start point) to +68, −150 to +68, and −34 to +68 (minimal mouse *Mt-1* promoter), respectively (LaRochelle et al. 2008). Plasmid $(MREa)_6$-LUC contains 6 mouse *Mt-1* MREa elements upstream of a minimal mouse *Mt-1* promoter in MT1min-LUC (LaRochelle et al. 2001*a*). To construct plasmid $(MREd)_6$-LUC, a synthetic DNA fragment containing 6 mouse *Mt-1* MREd elements (mMREd; Table 1) in direct tandem orientation was cloned in MT1min-LUC. Similarly, to construct plasmid h$(MREa)_6$, 6 human *MT-2a* MREa elements (hMREa; Table 1) were inserted into MT1min-LUC. To construct the reporter plasmid 780MT2A-LUC, a human *MT-2a* gene DNA fragment (position −780 to +65) was excised from plasmid pUC8-hMT2A (obtained from Dean H. Hamer, NIH, Bethesda, Md.) and cloned into pGL2 basic (Promega, Madison, Wis.). The construct 293MT2A-LUC contains the human *MT-2a* promoter sequence from positions −293 to +65 in pGL2 basic.

Point mutations were introduced in 2 nucleotides of the MREc core sequence or in 3 nucleotides of the MREa and MREd sequences by PCR site-directed mutagenesis (Cormack, B.1997) using specific oligos (ΔMREa, ΔMREc, and ΔMREd; Table 1) and 1843MT1-LUC plasmid DNA as the template, thus generating plasmids with mutation(s) in the MREa (ΔMREa-LUC), MREd (ΔMREd-LUC), MREa and MREd (ΔMREa/d-LUC), or MREa, MREc, and MREd (ΔMREa/c/d-LUC) sites. For all mutants, correct insertions and mutations were confirmed by sequencing.

Cell culture and transfection

Mouse L and NIH 3T3 cells were obtained from Dean H. Hamer and M. Lebel (Centre de recherche, Hôtel Dieu de Québec, Que.), respectively, and C_2C_{12} myoblasts were purchased from the American Type Culture Collection (Manassas, Va.). The human HepG2 and HEK293 cells were from A. Anderson and J. Landry (Centre de recherche, Hôtel Dieu de Québec), respectively. *Mtf-1*-null mutant cells (dko7) (Radtke et al. 1995) were provided by W. Schaffner, and *Hif-1α*-null and wild-type MEF cells (Ryan et al. 2000) were obtained from S. Lee (University of Ottawa, Ont.) (originally from Randall S. Johnson, University of California, San Diego, Calif.). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Where indicated, the antioxidant or the protein kinase, hydroxylase, and mitochondrial electron transport inhibitors were added 45 min prior to the direct addition of metals or exposure to hypoxia, as indicated in figure legends, and were maintained during the remainder of the incubation period. For metal induction, $ZnCl_2$ (final concentration 100 μmol/L), $CdCl_2$ (2.5 μmol/L), $CoCl_2$ (0.3 mmol/L), or $NiCl_2$ (0.5 mmol/L) were added directly to the medium and the cells were harvested 3 or 16 h later, as indicated in the figure legends. Inhibitors were dissolved in sterile water or DMSO as a 1000× stock solution and stored at −80 °C. To induce hypoxia, cells were incubated in 1% oxygen for 16 h in a sealed anaerobic workstation (Ruskin Technology). The oxygen concentration was fixed with a gas mixture containing 5% carbon dioxide and 95% nitrogen. All solutions were preincubated in the hypoxic chamber before addition to cells.

Cells were transfected with the different plasmids using ExGen500 in accordance with the manufacturer's instructions. Briefly, cells were seeded in 12 well plates (3×10^4 cells/well) 24 h prior to transfection, exposed to the DNA precipitate for 16 h, and then the mixture was

replaced by normal growth medium. Following 6 h under normoxia, cells were serum-starved (0.1% fetal bovine serum) and treated or not with inhibitors and metals or exposed to hypoxia. In some experiments, cells were cotransfected with the CMV-MTF-1 expression vectors pC-mMTF-1a. The plasmid pTK-rLUC (Promega) was used as an internal standard to monitor transfection efficiency. The total amount of DNA added to the cells was adjusted to 2–4 μg per dish with salmon sperm DNA. LUC activities were determined with a Dual-LUC assay kit (Promega) according to the recommendations of the manufacturer. The transcriptional activity of the reporter plasmids was evaluated in triplicate in at least 3 independent transfections. In each experiment, cells were examined under the microscope to make sure that their appearance and morphology were normal as compared with the control cells.

Northern analysis, electrophoretic mobility shift assays, and chromatin immunoprecipitation, coimmunoprecipitation, and immunoblotting

Extraction of total RNA with guanidium isothiocyanate and Northern analyses of *Mt-1* mRNA were performed as previously described (Moffatt and Séguin 1998) the cDNA insert for the mouse *Mt-1* or an oligo (18S; Table 1) corresponding to a conserved region of the ribosomal 18S RNA using as probes. Northern analyses were quantified using a PhosphorImager 860 and ImageQuant 4.2 software (Molecular Dynamics, Sunnyvale, Calif.).

Nuclear extracts were prepared and electrophoretic mobility shift assays (EMSA) were performed as described previously (LaRochelle et al. 2001*b*). MTF-1 and HIF-1α chromatin immunoprecipitation (ChIP) assays were performed using the ChIP-it Express kit from Active Motif (Carlsbad, Calif.) (LaRochelle et al. 2008). The chromatin was immunoprecipitated with anti-MTF-1 or anti-HIF-1α antibodies or normal rabbit serum. ChIP samples were subjected to PCR using primers specific for the mouse *Mt-1* (LaRochelle et al. 2008) or *Glut-1* promoter (GLUT-1; Table 1). For immunobloting experiments, cells were washed in cold PBS and recovered by scraping in 1.5× Laemmli buffer (90 mmol/L Tris–HCl, pH 6.8, 15% glycerol, and 3% SDS). Nuclei and lysates from hypoxic cells were prepared in the hypoxic chamber using buffers pre-equilibrated under hypoxia. Lysates were heated to 95 °C for 8 min, clarified by centrifugation at 15 000*g* for 10 min, and normalized for protein content. Proteins were resolved on 10% SDS polyacrylamide gels and transferred onto a PVDF Immobilon-P membrane (Millipore, Mississauga, Ont.). The membrane was blocked in 5% nonfat milk in TN (50 mmol/L Tris–HCl, pH 7.4, and 150 mmol/L NaCl), incubated with anti-MTF-1 (1/600), anti-HIF-1α (1/2000), anti-HIF-1β (1/4000), or anti-tubulin (1/5000) antibodies, washed in TNT (TN + 0.1% Triton X-100), and incubated with HRP-conjugated anti-IgG secondary antibodies. Proteins were visualized using a Super Signal West Dura detection kit (Thermo Scientific, Rockford, Ill.). For coimmunoprecipitation, 3 μL of anti-MTF-1 or anti-HIF-1α antibody was first coupled to 30 μL of 50% (*v*/*v*) Protein A Sepharose for 90 min at 4 °C in PBS. The beads were centrifuged (15 000*g* for 2 min), mixed with 200 μg of pre-cleared whole-cell protein extract and a mix of protease inhibitors (Complete, Mini, EDTA-free protease inhibitor cocktail; Roche Applied Sciences), and incubated in the presence of 0.1% BSA at 4 °C for 2 h or overnight. The whole-cell protein extract was prepared in 50 mmol/L Tris–HCl, pH 7.5, 100 mmol/L

NaCl, 1.25 mmol/L $MgCl_2$, 1% NP-40, 50 mmol/L NaF, and 0.2 mmol/L Na_3VO_4 and pre-cleared by mixing with Protein A Sepharose and incubated at 4 °C for 1 h. Precipitates were washed 5 times with 50 mmol/L Tris–HCl, pH 7.5, 150 mmol/L NaCl, and 1 mmol/L EDTA containing the mix of protease inhibitors before Western blotting for HIF-1α, HIF-1β, and MTF-1. In an alternate procedure, coimmunoprecipitation was performed as described previously (Murphy et al. 2008) to confirm our results.

Data are expressed as means ± SD. Statistical analysis was performed using the Student's 2-tailed *t* test (Figs. 1B, 2C, 3, 4, 5, and 6). In Figs. 1A and 2B, a 1-sample 1-sided *t* test was used to test whether the observed ratios of the measurements to the basal values were equal to or greater than one on average (*, $p < 0.05$; **, $p < 0.01$).

Results

Induction of MTs by hypoxia

Transcription of human *MT-2a* and mouse *Mt-1* genes is induced by hypoxia in C_2C_{12} mouse myoblast cells and in Ha-*ras*-transformed NIH 3T3 cells via MREs and MTF-1 (Murphy et al. 1999). To extend these observations to other cell types, we undertook a series of Northern analyses using RNA from various cell lines. Human HepG2 and HEK293 cells, mouse C_2C_{12}, L, and NIH 3T3 cells, MEFs with targeted deletions of both *Mtf-1* alleles (*Mtf-1*$^{-/-}$ cells) and wild-type MEF cells were exposed to hypoxia or metals for 16 h before harvesting RNA for Northern analysis with a mouse *Mt-1* cDNA probe.

In these different cell types the *Mt* gene was induced 2- to 3-fold in response to hypoxia and approximately 7-fold in response to zinc compared with control cells in normoxia and not exposed to metals (Fig. 1A). Figures 1B and 1C show representative results obtained with L cells and with wild-type and *Mtf-1* null MEF cells. In *Mtf-1*$^{-/-}$ cells, induction of *Mt* by cadmium was completely inhibited, whereas induction by hypoxia was reduced by 70%. These results show that the *Mt* gene is inducible by hypoxia in different cell types and confirm that MTF-1 is essential for this induction (Murphy et al. 1999).

Identification of response elements involved in induction by hypoxia

The mouse *Mt-1* promoter contains 5 metal response elements (MREa through MREe), 2 Sp1 binding sites, 2 USF binding sites, an antioxidant response element (ARE) that overlaps the more downstream USF site (Andrews 2000), and 2 NF-1 binding sites (Fig. 2A) (LaRochelle et al. 2008). Experiments with synthetic MRE sequences showed that the different MREs have different efficiencies in stimulating transcription. MREd is the strongest, MREa and MREc are 50% to 80% weaker, MREb is very weak, and MREe is apparently nonfunctional (Labbé et al. 1991; Stuart et al. 1985). In a previous study (Murphy et al. 1999), it was found that MREc and MREd of mouse *Mt-1* can confer induction by hypoxia to a minimal *Mt-1* promoter. However, although isolated MREc can bind MTF-1 in vitro and confer metal induction when inserted in front of a minimal promoter, we have shown that in the context of the intact promoter, MREc overlaps a functional NF-1 site and is not a bona fide MTF-1 binding site (LaRochelle et al. 2008).

Therefore, in a second set of experiments we set out to determine the relative importance of the different MREs in the context of the entire mouse *Mt-1* promoter during activation by hypoxia using transient transfection of L and C_2C_{12} cells. We first analyzed the induction obtained with reporter plasmids containing different lengths of the mouse *Mt-1* promoter sequence (Fig. 2A). The reference plasmid MT1-1843-LUC containing *Mt-1* promoter sequences up to nucleotide −1843 was induced approximately 5-fold by hypoxia in L cells (Fig. 2B). Deletion of *Mt-1* promoter sequences between −1843 and −150 did not substantially modify hypoxia-induced expression (Fig. 2B). These results show that the DNA sequences involved in activation of the mouse *Mt-1* gene in response to hypoxia are downstream of position −150. In contrast, the capacity of the promoter to be induced by metals, which is approximately 7-fold in the reference plasmid, increased to 20-fold when base pairs −238 through −150 were deleted from the promoter (Fig. 2B). The increase in induction by zinc observed here with the −150 mutant can be explained by a decrease in constitutive transcriptional activity, which produces the effect of increasing the ratio of metal-induced to basal activity. Similar results were obtained with C_2C_{12} cells, in which the plasmids containing promoter sequences up to −1843 or −150 were both induced 4- to 5-fold by hypoxia (data not shown).

The MREa and MREd of mouse *Mt-1* both conferred induction by hypoxia and by zinc to the minimal promoter in L (Fig. 2B) and C_2C_{12} (Murphy et al. 1999) cells (data not shown), showing that these MREs are capable of inducing transcription in response to hypoxia. To confirm their central role in the induction of the mouse *Mt-1* gene in response to hypoxia and to evaluate the role of the other MREs, particularly MREc, in the context of the intact promoter, we generated point mutations by site-directed mutagenesis to inactivate MREa (ΔMREa) or MREd (ΔMREd), as well as a double mutant in which both MREa and MREd are inactivated (ΔMREa/d), and a triple mutant targeting MREa, MREc, and MREd (ΔMREa/c/d). These plasmids were then transfected into different cell types. The mutations in MREc modify the consensus sequence TGCRCNC required for MTF-1 binding and transcriptional activation (Andrews 2001), but leave the adjacent NF-1 site intact (LaRochelle et al. 2008). In these experiments, L cells transfected with the control plasmid displayed an ~5-fold induction in response to hypoxia (Fig. 2C). Mutations in MREa or MREd inhibited the response to hypoxia nearly 2-fold; mutation of MREd resulted in a drop of more than 50% in the basal activity, which contributes to maintenance of the induction level at greater than 3-fold. However, the overall activity of the promoter upon induction is 3.5-fold lower than that of the control plasmid MT1-1843-LUC. In the case of the double mutant ΔMREa/d, the basal level was the same as with ΔMREd, but the level induced by hypoxia was reduced to 2-fold above the basal level and the overall activity upon induction was reduced <20% of that of the control plasmid. For the triple mutant ΔMREa/c/d, the transcriptional activity of the *Mt-1* promoter was not more affected than the double mutant ΔMREa/d, either at the basal level or in the response to hypoxia, suggesting that in the native context of the intact *Mt-1* promoter, MREc is not involved in transcriptional induction by hypoxia. These mutants were also tested in HEK293, HepG2, NIH 3T3, and C_2C_{12} cells, and similar results were obtained (data not shown). In sum, these results suggest that MREa and MREd are the 2 main regulatory elements controlling induction of the mouse *Mt-1* gene by hypoxia. Other sequences and transcription factors besides these MREs and MTF-1

appear to play a role as well, since the triple mutant promoter remains weakly inducible by hypoxia. For example, AP1 and Sp1, both factors binding to the *Mt-1* promoter, have been reported to cooperate with other transcription factors such as HIF-1α and NF-1, which also bind to the *Mt-1* promoter, to complement the activation of hypoxia-sensitive genes (Cummins and Taylor 2005). MREb could also be involved in the residual induction of the triple mutant.

For comparison, the plasmids containing the human *MT-2A* promoter sequences up to nucleotides −846 or −293 were induced ~2.5-fold in response to hypoxia in L cells (data not shown), which is slightly weaker than the induction obtained with the mouse promoter. The MREa alone of human *MT-2A* inserted in front of a minimal mouse *Mt-1* promoter conferred a 2-fold induction (data not shown).

MTF-1 stability, localization, and DNA binding

Activity of HIF-1α is controlled at the level of protein stability, and under normoxia the protein is degraded by proteasomes. To determine whether MTF-1 is stabilized by a similar mechanism under hypoxia, we performed immunoblot analyses with a polyclonal anti-MTF-1 antibody to evaluate the level of MTF-1 protein in normoxia and hypoxia in lysates of L cells. Figure 7 shows that total MTF-1 levels were similar in normoxia and hypoxia, in contrast to those of HIF-1α whose concentration is greatly increased in hypoxia as well as in response to $NiCl_2$, $CoCl_2$, or ethyl 3,4-dihydroxy-bezoate (EDHB), 3 PHD inhibitors used as hypoxia-mimetic agents. It is noteworthy that zinc, and to a lesser extent cobalt and EDHB, increased MTF-1 levels. Neither MTF-1 nor HIF-1α was detected in *Mtf-1*-null cells grown under normoxia. These results suggest that the mechanisms controlling MTF-1 activation in response to hypoxia are different from those controlling HIF-1α.

A fundamental aspect of the mechanism of MTF-1 activation in response to metals is an increase in its binding to DNA in response to zinc ions (Heuchel et al. 1994). To determine whether this binding is also increased in hypoxia, EMSA analyses were performed using extracts of C_2C_{12} cells grown in the presence or absence of zinc or under hypoxia. MTF-1 protein from control extracts of cells grown under normoxia was capable of forming a complex with the MRE probe that was easily detectable by EMSA. While induction of cells by zinc increased DNA binding of MTF-1 by 3- to 4-fold, exposure of cells to hypoxia only had a small effect compared with the binding observed in control cells (Fig. 8A). These results are comparable with the effect of cadmium, which does not affect the DNA binding of MTF-1 (Bittel et al. 1998; LaRochelle et al. 2001b). In addition, in extracts of control cells as well as those from cells exposed to hypoxia, MTF-1 protein is present in both the cytosol and the nucleus, whereas in extracts prepared from zinc-treated cells, almost 100% of the MTF-1 protein was present in the nuclear fraction (Fig. 8B). Altogether, these results do not support the contention that hypoxia induces translocation of MTF-1 to the nucleus.

To confirm these results, we complemented these experiments with the in vivo method of ChIP. MTF-1 was bound to the mouse *Mt-1* promoter in uninduced control cells, and this binding was strongly enhanced in the presence of zinc (Fig. 8C). However, as we observed in vitro, DNA binding of MTF-1 was not affected in cells exposed to hypoxia. Notably, hypoxia but not zinc induced the recruitment of HIF-1α to the *Mt-1* promoter as well as to

the *Glut-1* promoter used as a positive control (Fig. 8C), as reported by others (Murphy et al. 2008). These results indicate that MTF-1 activation in response to hypoxia is not associated with a detectable increase in its binding to DNA, but rather occurs by some other mechanism.

PKC, JNK, and PI3K protein kinases are involved in MTF-1 activation by hypoxia

To address the role of individual MAPK pathways and of PKC and PI3K signaling cascades in MTF-1 activation by hypoxia, we examined the effects of PD98059 (an ERK pathway inhibitor), SB203580 (a p38 inhibitor), SP600125 (a JNK inhibitor), GF109203X (a PKC inhibitor), and LY294002 (a PI3K inhibitor) on the transcriptional activity of a transfected MRE-LUC promoter construct. C_2C_{12} cells were transfected with the $(MREd)_6$-LUC reporter plasmid in the presence of protein kinase inhibitors. We chose this plas-mid because it has a higher level of constitutive transcriptional activity than the $(MREa)_6$-LUC plasmid (data not shown) (Culotta and Hamer 1989; Stuart et al. 1985) while retaining a comparable fold induction, thus allowing evaluation of the effects of different factors on both the basal and induced transcriptional activities. Additionally, this type of plasmid allows specific evaluation of MTF-1 activity under different experimental conditions since the MREd response element is strictly specific for MTF-1. Compared with normoxic control cells, the reporter gene was induced ~3-fold in response to zinc and ~2.5-fold in response to hypoxia (Fig. 3). Treatment of cells with 10 μmol/L GF109203X or LY294002 or with 25 μmol/L SP600125 completely inhibited MTF-1 activation in response to hypoxia (Fig. 3). The response to zinc was also completely inhibited by LY294002 and SP600125, whereas at the tested concentrations of 5 and 10 μmol/L, GF109203X inhibited the response by ~60%. PD98059 and SB203580 had no effect on either basal or hypoxia-induced transcriptional activity of the MRE reporter plasmid, and none of the inhibitors had any effect on the basal activity (data not shown). Similarly, LUC expression from the TK-renilla-LUC internal control plasmid was not affected by any of the kinase inhibitors, indicating that the inhibition observed was not due to nonspecific effects on RNA polymerase II or general transcription factors. These results suggest that hypoxia-mediated MTF-1 activation involves complex signal transduction that includes the conventional PKC, PI3K, and JNK pathways, but that the ERK and the p38 pathways do not appear to be essential.

Role of oxidative stress in MTF-1 activation by hypoxia

There is extensive data suggesting that mitochondrial ROS production and mitochondrial O_2 consumption in hypoxic cells play a role in HIF-1α activation and constitute important elements of the mechanism controlling HIF-1α activation in hypoxia but not in anoxia (Pouysségur and Mechta-Grigoriou 2006). Moreover, MTF-1 is activated by oxidative stress (Andrews 2001). Therefore, to determine whether ROS and mitochondrial activity play a role in MTF-1 activation in response to hypoxia, we transfected L cells with the $(MREd)_6$-LUC reporter plasmid, grew these cells in media containing rotenone or myxothiazol, inhibitors of mitochondrial electron transport, and then exposed them to hypoxia for 16 h. As a control, we used the 3HRE-LUC reporter plasmid specific for HIF-1α. Rotenone and myxothiazol inhibited HIF-1α activation by 70% and 80%, respectively (Fig. 4A), and induction of the $(MREd)_6$-LUC plasmid was also inhibited although to a lesser extent (30% and 40%, respectively) (Fig. 4B). However, the antioxidant *N*-acetyl-cysteine did not affect

induction of HIF-1α or MTF-1 reporters in response to hypoxia (data not shown). These results indicate that mitochondria O_2 consumption but not ROS is involved in activation of HIF-1α by hypoxia and show that mitochondria O_2 consumption also plays a role in activation of MTF-1.

Possible involvement of hydroxylases in the signaling pathway controlling MTF-1 activation in response to hypoxia

In normoxia, HIF-1α is hydroxylated by a family of hydroxylases leading to its degradation by proteasomes. To evaluate whether hydroxylases are involved in MTF-1 activation by hypoxia, C_2C_{12} and L cells were transfected with the $(MREd)_6$-LUC plasmid and treated with DMOG, described as a nonspecific PHD inhibitor (Wright et al. 2003), or EDHB a specific inhibitor of PHDs that competitively binds the ascorbate and 2-oxoglutarate binding sites of the enzyme (Majamaa et al. 1986) and stabilizes HIF-1α under normoxia. As a control, cells were transfected with the 3HRE-LUC reporter plasmid. Both inhibitors were very effective in activating HIF-1α, and DMOG did so even more effectively than hypoxia in C_2C_{12} cells (Figs. 5A and 5B, *3HRE-LUC*). Surprisingly, both PHD inhibitors also induced expression of the MRE reporter, albeit a little less than 2–3-fold for DMOG but more than 5-fold for EDHB (*$(MREd)_6$-LUC*; Figs. 5A and 5B). Figure 5A shows that, like HIF-1α, MTF-1 is induced by other hydroxylase inhibitors such as DFO, an iron chelator, and $CoCl_2$ and $NiCl_2$, which are sometimes used to mimic hypoxia in inducing the HRE promoter. These results suggest that a hydroxylase may be involved upstream of MTF-1 in the signaling pathway that controls its activation in response to hypoxia.

Interaction between the MTF-1 and HIF-1α signaling pathways

Our results with hydroxylase inhibitors (Fig. 5) as well as recent studies by other laboratories (Murphy et al. 2005, 2008) indicate that MTF-1 contributes to activation of HIF-1α in response to hypoxia. To characterize possible interactions between MTF-1 and HIF-1α, we performed transient transfections in cells null for MTF-1 or HIF-1α. Figure 6B (right panel) shows that in the absence of MTF-1, the MRE promoter is strongly inhibited both before and after exposure of the cells to metals or hypoxia. Cotransfection of an MTF-1 expression vector in *Mtf-1*-null mutant cells restored both basal and metal- and hypoxia-induced transcription of the MRE reporter plasmid. In *Mtf-1*-null cells, the HRE promoter is induced ~60-fold in response to hypoxia, and the maximal activation is ~5-fold greater when MTF-1 is present. Similarly, while the MREd promoter is induced a little over 2-fold in cells containing HIF-1α protein, this induction does not occur when HIF-1α is absent (Figs. 6A and 6C). In addition, HIF-1α is required for induction of the MRE promoter or complete activation of the *Mt-1* promoter in response to the hydroxylase inhibitor EDHB (Fig. 6C). These results show that MTF-1 and HIF-1α each stimulate the transcriptional activity of the other in response to hypoxia and suggest that they may interact physically as reported previously (Murphy et al. 2008). However, we have been unable to demonstrate an interaction using 2 coimmunoprecipitation approaches; as expected, HIF-1β coprecipitated with HIF-1α in cells exposed to cobalt or nickel or exposed to hypoxia (Fig. 9), but HIF-1α did not coprecipitate with MTF-1 nor did MTF-1 with HIF-1α.

Discussion

Response elements conferring induction of MTs by hypoxia

In this study, we investigated the mechanisms by which MTF-1 is activated in response to hypoxia. In transient transfection of L and C_2C_{12} cells, the different mouse *Mt-1* promoters used here were induced 3- to 5-fold above the basal level in hypoxia, which is similar to what was reported in another study (Murphy et al. 1999). However, the human promoters are induced only 2–3-fold in L cells, which is nearly 5 times lower than the effect observed in C_2C_{12} cells (Murphy et al. 1999) but similar to that reported in human adipocytes (Wang et al. 2008). Similarly, the human MREa promoter only reaches a 2-fold induction in L cells compared with almost 4-fold in C_2C_{12} cells (Murphy et al. 1999), possibly because of cell type differences. We also evaluated the relative strength of the different MREs in the context of the entire promoter. We show here that the transcriptional response of the *Mt-1* promoter in response to hypoxia is dependent on MREa and MREd.

MTF-1 activation in response to hypoxia

An important contribution of this study relates to the mechanisms by which MTF-1 is activated in hypoxia. MTF-1 activation by zinc leads to translocation of the protein from the cytosol to the nucleus and a marked increase in its binding to DNA (Daniels and Andrews 2003), but we have not been able to observe detectable translocation into the nucleus under hypoxia by Western analysis (Fig. 8B) although some nuclear translocation under hypoxia was previously detected in human cancer cells using immunofluorescence (Cramer et al. 2005). With regard to binding of MTF-1 to DNA, an in vitro study showed that binding to an MRE oligo increases significantly after 2 h of exposure to hypoxia and reaches a maximum after 4 h (Murphy et al. 1999) although this increase is much less than that induced by zinc. Another study conducted in vivo using ChIP showed that MTF-1 is recruited to the mouse *Mt-1* promoter, but this recruitment is less pronounced than after exposure to zinc (Murphy et al. 2008). However, we have not seen a significant increase in DNA binding of MTF-1 either in vitro or in vivo. The reasons for these different findings are not clear and will require more studies. It is possible that MTF-1 functions in a cell-specific manner or that the assay used in the present study was not sensitive enough to detect an increase in DNA binding.

ChIP assays show the interaction of some MTF-1 with the *Mt-1* promoter in uninduced cells (Fig. 8C) (Daniels and Andrews 2003; LaRochelle et al. 2008; Marr et al. 2006), and it is thus possible that MRE-bound MTF-1 undergoes further activation in response to hypoxia and induces transcription.

The results of our binding studies indicate that posttranslational modifications may play a role in MTF-1 activation by hypoxia. To determine whether phosphorylation plays a role in MTF-1 activation by hypoxia, we used different protein kinase inhibitors and observed that this activation is controlled by a complex signaling pathway that includes the same kinases, namely PKC, PI3K, and JNK, that are used by the cell in response to metals but that as for metals, the p38 and ERK pathways do not appear to be essential. Our results suggest that phosphorylation is essential for its transactivation function. As in the case of metals, we

propose here that MTF-1 phosphorylation in response to hypoxia is a primary event leading to an increase of its activity. Hypoxia could trigger a phosphorylation cascade leading to a kinase that phosphorylates preexisting nuclear MTF-1 on regulatory sites necessary for its transcriptional activity. This model does not exclude participation of other molecular components, such as hydroxylases (see below), in MTF-1 activation by hypoxia. While phosphorylation is essential for MTF-1 activation by hypoxia in C_2C_{12} cells, further experiments using other cell lines will be required to confirm this model.

The role of ROS

The dioxygenases responsible for initiating the degradation of HIF-1α through hydroxylation, PHD1, PHD2, and PHD3, use 2-oxoglutarate as a cosubstrate (Brahimi-Horn and Pouysségur 2006). In addition, for maximal activity these PHDs require Fe(II) and ascorbate as cofactors. Under hypoxia, defined as a decrease of oxygen level to no lower than 1%, it has been suggested that the production of ROS induces formation of Fe(III) that cannot be used as a cofactor by PHDs and should thereby effectively inhibit PHDs, resulting in accumulation of HIF-1α (Pouysségur and Mechta-Grigoriou 2006). However, it has recently been shown that ROS do not regulate PHD activity directly (Chua et al. 2010). Instead, mitochondria could modulate the cellular response to hypoxia through altered respiratory activity, likely by regulating the availability of cellular oxygen (Doege et al. 2005; Hagen et al. 2003). We show here that the mitochondrial electron transport inhibitors rotenone and myxothiazol inhibit HIF-1α activation in hypoxia by more than 75% and, interestingly, also inhibit MTF-1 activation by ~35%. However, the antioxidant *N*-acetyl-cysteine had no effect on activation of either HIF-1α or MTF-1 in hypoxia. These results suggest that mitochondrial O_2 consumption plays a role in MTF-1 activation by hypoxia and suggest overlap between the signaling pathways controlling hypoxia-induced activation of MTF-1 and HIF-1α.

Several studies suggest that during MTF-1 activation by oxidative stress, ROS production causes the release of zinc from MTs and other cellular metalloproteins and that this free zinc may play a central role in MTF-1 activation by ROS (Bi et al. 2004; Dalton et al. 1996; Lichtlen and Schaffner 2001). It is then possible that ROS generated in hypoxia may cause an increase in free intracellular zinc and that this may activate MTF-1. Studies using zinc chelators are needed to resolve this question. Further, the inhibitory effect of ROS inhibitors on MTF-1 activity may involve HIF-1α, which enhances MTF-1 activity (see below).

The role of hydroxylases

An interesting aspect of our results is the activation of MTF-1 by hydroxylase inhibitors. While protein degradation by a mechanism involving hydroxylases does not seem to play a part in MTF-1 activation by hypoxia, we show that hydroxylase inhibition by different known inhibitors such as EDHB, DMOG, $NiCl_2$, and $CoCl_2$ stimulates MTF-1 activity up to 5-fold in C_2C_{12} cells (Figs. 5A and 5B), suggesting that hydroxylases act somewhere in the signaling pathway controlling MTF-1 activity or in its mechanism of action. Based on these results, we cannot exclude the possibility that MTF-1 may be directly hydroxylated, since it contains 1 potential hydroxylation site (LPATAP) located in the proline-rich domain. However, we favor a more indirect model taking into account, among other things, the time

course of MTF-1 activation under hypoxia that is slower and weaker than induction of HIF-1α (Fig. 5 and data not shown). Different scenarios could explain the mechanism by which hydroxylases influence MTF-1 activation.

First, it is possible that a hydroxylation event inhibits the activity of one of the kinases involved in the activation of MTF-1 in response to hypoxia, freeing the target kinase from its inhibition, thus leading to activation of the MTF-1 signaling pathway. A number of transcription factors are activated in response to hypoxia (Cummins and Taylor 2005) including NF-κB, where prolyl-hydroxylase-1 inhibits IKKβ, a protein kinase that positively regulates NF-κB activity by phosphorylating and thus inhibiting IκBα protein, the natural inhibitor of NF-κB (Cummins et al. 2006).

Second, MTF-1 activation by hydroxylase inhibitors could be due to HIF-1α. Indeed, we have shown that HIF-1α amplifies MTF-1 activity (Fig. 6), while others have shown that HIF-1α contributes to induction of the mouse *Mt-1* gene by hypoxia (Murphy et al. 2008). It is therefore possible that HIF-1α protein, whose concentration increases considerably in the presence of the hydroxylase inhibitors used in this study, may be responsible for the increase in MTF-1 activity through an as yet undiscovered mechanism.

Third, it is also possible that DFO and EDHB as well as the other hydroxylase inhibitors activate MTF-1 by a mechanism that is completely independent of hydroxylases. Indeed, it is useful to recall that these agents provoke oxidative stress and increase ROS production by 50%–75% in cultured rabbit cardiomyocytes (Philipp et al. 2006). It is therefore possible that EDHB causes an increase in intracellular ROS that then act as second messengers in the signaling cascade controlling MTF-1 activity or that act more indirectly by causing the release of zinc from MTs and other cellular zinc-binding proteins. This released zinc could activate MTF-1 or activate kinases in the MTF-1 signaling pathway. Further experiments are needed to test these hypotheses.

Overlap between the HIF-1α and MTF-1 signaling pathways

Our results confirm observations made in previous studies of the surprising overlap between the MTF-1 and HIF-1α signaling pathways (Murphy et al. 2005, 2008). We show that MTF-1 contributes to HIF-1α activity and conversely, HIF-1α enhances MTF-1 activity. Our results show that in the absence of HIF-1α, induction of the $(MREd)_6$-LUC promoter is strongly inhibited whereas in the absence of MTF-1, induction of the 3HRE-LUC promoter is reduced but still very strong.

The mechanism by which MTF-1 and HIF-1α interact in response to hypoxia remains unclear. Coimmunoprecipitation experiments have shown that MTF-1 and HIF-1α form a complex that is recruited to the promoter in vivo in response to hypoxia, suggesting that HIF-1α functions as a coactivator of *Mt-1* gene transcription by interacting with MTF-1 (Murphy et al. 2008). However, while we showed that HIF-1α is recruited to the mouse *Mt-1* promoter in response to hypoxia (Fig. 8), we have not been able to demonstrate physical interactions between the 2 proteins using 2 different coimmunoprecipitation approaches (Fig. 9). The reason for this is not clear, and further experiments will be required to determine whether a direct interaction occurs between these 2 transcription factors or

whether any signaling molecules mediate this stimulatory action indirectly. It is possible that HIF-1α binds directly to the *Mt-1* promoter under hypoxia without directly interacting with MTF-1 since 2 putative hypoxia response elements (RCGTG), partially overlapping the USF/ARE sites, are present on the promoter between nucleotide positions −90 and −100. In vivo kinetics of binding of MTF-1 and HIF-1α to the *Mt* promoter in response to hypoxia showed that while MTF-1 DNA binding is not dependent on HIF-1α, the presence of MTF-1 is necessary for hypoxia-induced HIF-1α recruitment to the promoter (Murphy et al. 2008). MTF-1 is also essential for the induction of binding of NF1 to DNA in response to metals (LaRochelle et al. 2008), and thus it is possible, as suggested to explain metal-induced MTF-1-dependent NF1 binding to the *Mt* promoter, that MTF-1 controls hypoxia-mediated *Mt* gene induction in part through the modification of chromatin structure and the subsequent recruitment of HIF-1α and other transcription factors to the promoter. However, the precise interactions between the MTF-1 and HIF-1α signaling pathways remain to be defined and represent an important challenge for the future.

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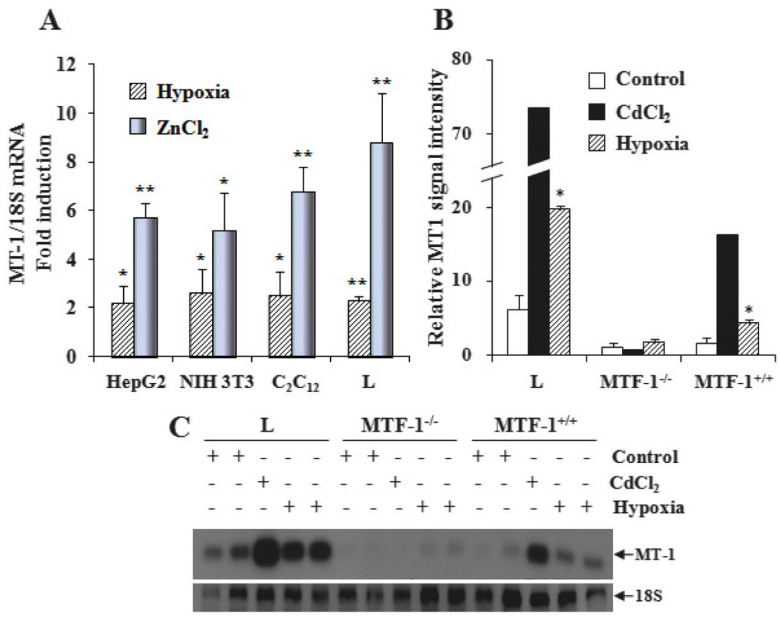

Fig. 1.

Northern blot analysis of *Mt-1* mRNA in different cell lines exposed to metals or hypoxia. (A) Human HepG2 and mouse NIH 3T3, C_2C_{12}, and L cells were untreated, treated with 100 μmol/L $ZnCl_2$, or exposed to hypoxia (pO_2 = 1%) for 16 h before isolation of total RNA. The RNA was electrophoresed, transferred to Hybond-N membranes, and hybridized with a ^{32}P-labeled cDNA encoding mouse *Mt-1*. Membranes were stripped and rehybridized with an oligo corresponding to 18S ribosomal RNA. Northern data were quantified by phosphoimager analysis and the *Mt-1*:18S ratio was calculated. Results are presented as fold induction over basal levels in nontreated cells kept in normoxia and represent the mean ± SD from 3 different experiments. (B) Mouse L cells and wild-type and *Mtf-1*-null mouse embryonic fibroblasts (MEFs) (MTF-$1^{+/+}$ and MTF-$1^{-/-}$) were untreated, treated with 2.5 μmol/L $CdCl_2$, or exposed to hypoxia (pO_2 = 1%) for 16 h, as indicated, before isolation of total RNA as described in (A). Data are representative of 3 separate experiments. (C) Northern blot from representative samples in Fig. 1B. Significant differences between control and treated samples are based on the 1-sample 1-sided (A) or Student's *t* test (B). *, $P < 0.05$; and **, $P < 0.01$.

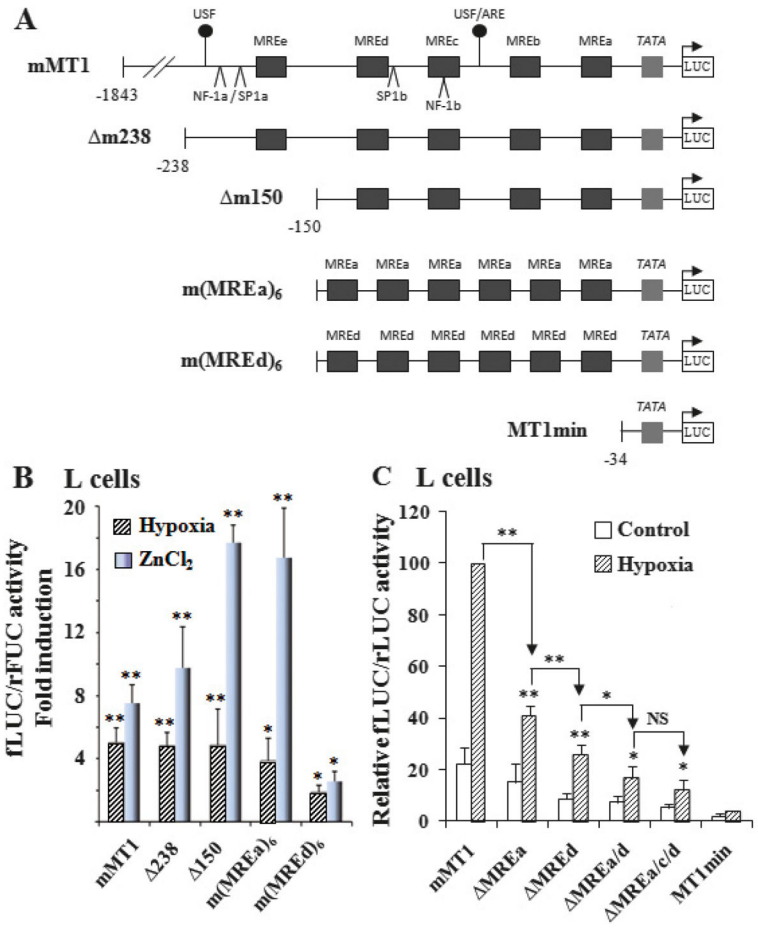

Fig. 2.

Mapping of the mouse *Mt-1* promoter elements involved in the transcriptional response to hypoxia. (A) Schematic representation of the proximal promoter, the deletion mutants, and of the *Mt-1*-derived LUC constructs used in this analysis. Arrangement of the 5 metal regulatory elements (MREs) (solid rectangles), the E-box 1 (USF), the USF/ARE elements, the binding sites for the transcription factors Sp1 and NF1, and the TATA box. The numbers at the left end of each construct refer to the positions relative to the transcription start point. (B) Mouse L cells were transfected with the indicated plasmid construct and pTK-rLUC as an internal standard, serum starved, and treated or not with 100 μmol/L $ZnCl_2$ or exposed to hypoxia (pO_2 = 1%) for 16 h. Cell extracts were prepared and LUC activity was measured with a dual LUC kit. Reporter inductions were calculated as fold induction over basal levels. (C) L cells were transfected as described in Fig. 2B with a plasmid mixture including the reporter wild-type MT1-LUC or the MREa mutant reporter plasmids ΔMREa-LUC (ΔMREa), ΔMREd-LUC (ΔMREd), ΔMREa/d-LUC (ΔMREa/d), or ΔMREa/c/d-LUC (ΔMREa/c/d), and pTK-rLUC as an internal standard. Results are expressed as the percent of firefly LUC (fLUC) activity relative to the level directed by the renilla LUC (rLUC) construct. In (B) and (C), the data represent the mean ± SD of 3 experiments performed in triplicate. Significant differences between control and treated samples or between the

different mutants are based on the 1-sample 1-sided (B) or the Student's *t* test (C). *, $P < 0.05$; **, $P < 0.01$; and NS, non-significant.

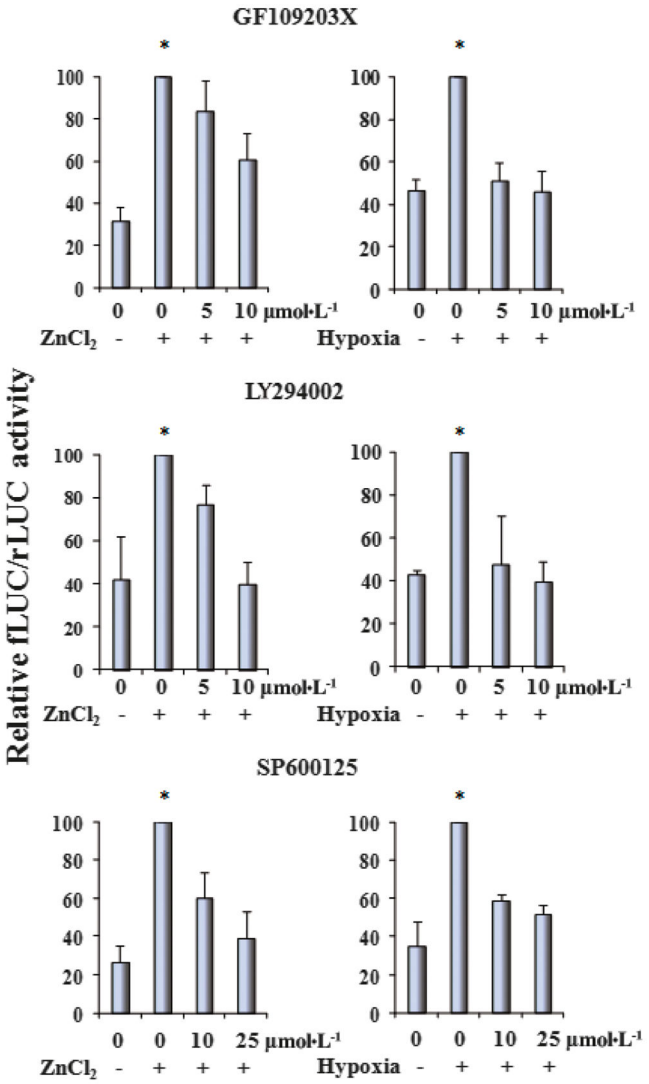

Fig. 3.

Effect of GF109203X, LY294002, and SP600125 on $(MREd)_6$-LUC expression. C_2C_{12} cells transfected with a mixture of $(MREa)_6$-LUC and pTK-rLUC were serum starved and preincubated for 45 min with the vehicle alone (0) or increasing concentrations of GF109203X (0 to 10 µmol/L), LY294002 (0 to 10 µmol/L), or SP600125 (0 to 25 µmol/L) as indicated, and then untreated, treated with 50 µmol/L $ZnCl_2$, or exposed to hypoxia (pO_2 = 1%) for 16 h before measurement of LUC activity as described in Fig. 2B. Results represent the mean ± SD of 3 independent experiments performed in triplicate. Significant differences between control and treated samples are based on the Student's *t* test. *, $P < 0.05$.

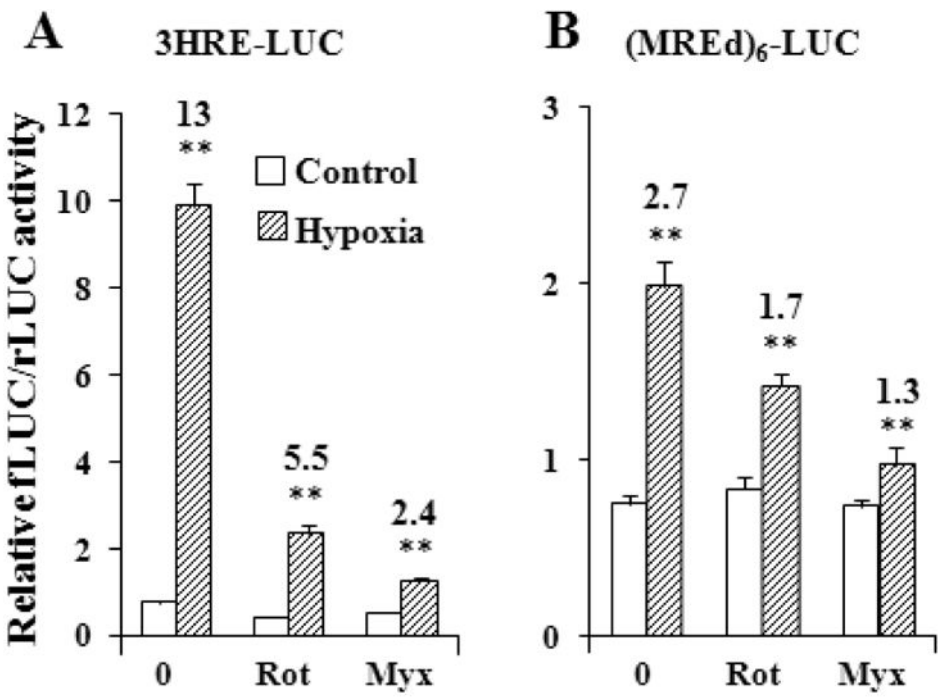

Fig. 4.

Effect of 2 inhibitors (rotenone and myxothiazol) of mitochondrial electron transport on $(MREd)_6$-LUC expression. L cells transfected with a mixture of pTK-rLUC and (A) 3HRE-LUC or (B) $(MREa)_6$-LUC were serum starved and then treated or not (0) with rotenone (Rot, 100 ng/mL) or myxothiazol (Myx, 50 ng/mL) as indicated, and then exposed or not (control) to hypoxia (pO_2 = 1%) for 16 h before measurement of LUC activity as described in Fig. 2B. Numbers above the columns indicate fold induction over basal levels in control cells. Results represent the mean ± SD of 3 independent experiments performed in triplicate. Significant differences between control and treated samples are based on the Student's *t* test. **, $P < 0.01$.

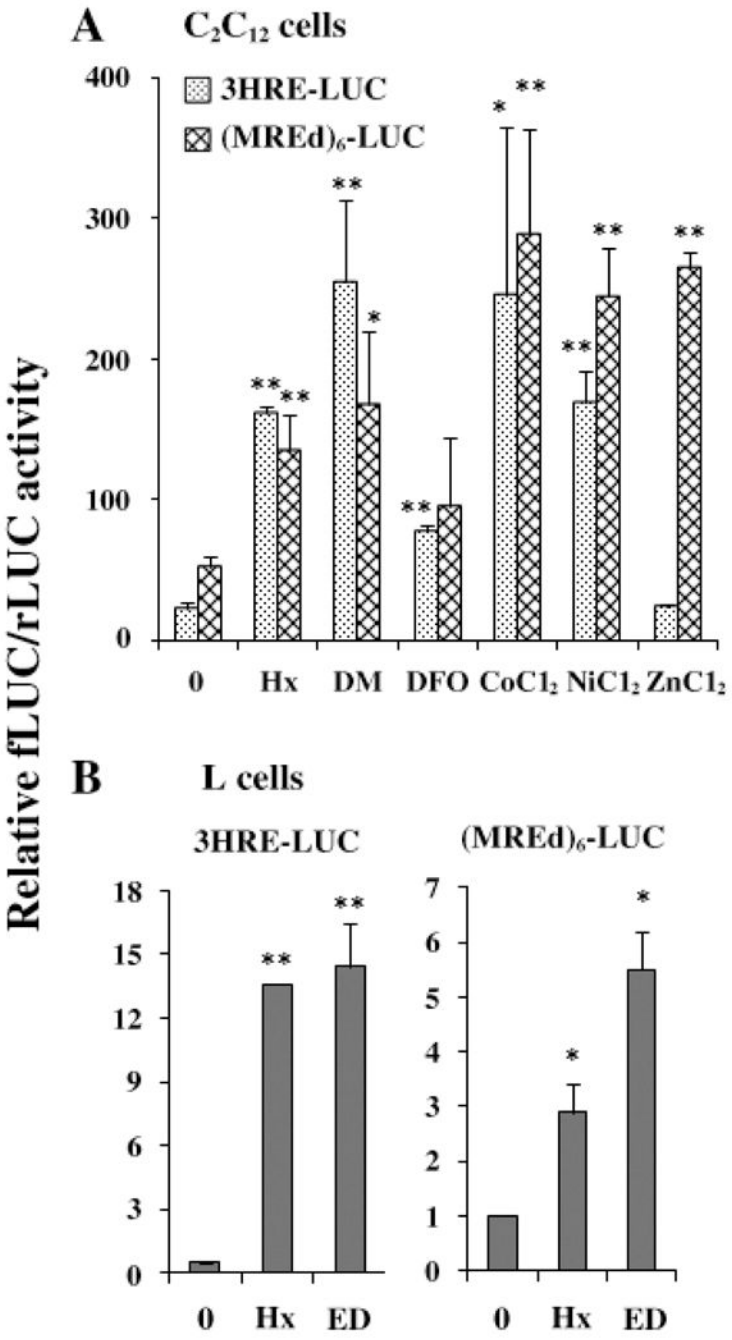

Fig. 5.

Effect of DMOG, EDHB, and other hypoxia-mimetic agents on $(MREd)_6$-LUC expression. (A) C_2C_{12} cells transfected with a mixture of pTK-rLUC and 3HRE-LUC or $(MREd)_6$-LUC were serum starved and then untreated (0), treated with DMOG (DM, 1 mmol/L), DFO (100 µmol/L), $CoCl_2$ (300 µmol/L), $NiCl_2$ (500 µmol/L), $ZnCl_2$ (100 µmol/L), or exposed to hypoxia (Hx, pO_2 = 1%), as indicated, for 16 h before measurement of LUC activity as described in Fig. 2B. (B) L cells transfected with a mixture of pTK-rLUC and 3HRE-LUC or $(MREd)_6$-LUC, as indicated, were serum starved and then untreated (0), treated with

EDHB (ED; 500 μmol/L), or exposed to hypoxia (Hx, pO_2 = 1%), as indicated, for 16 h before measurement of LUC activity as described in Fig. 2B. Results represent the mean ± SD of 3 independent experiments performed in triplicate. Significant differences between control and treated samples are based on the Student's *t* test. *, $P < 0.05$; and **, $P < 0.01$.

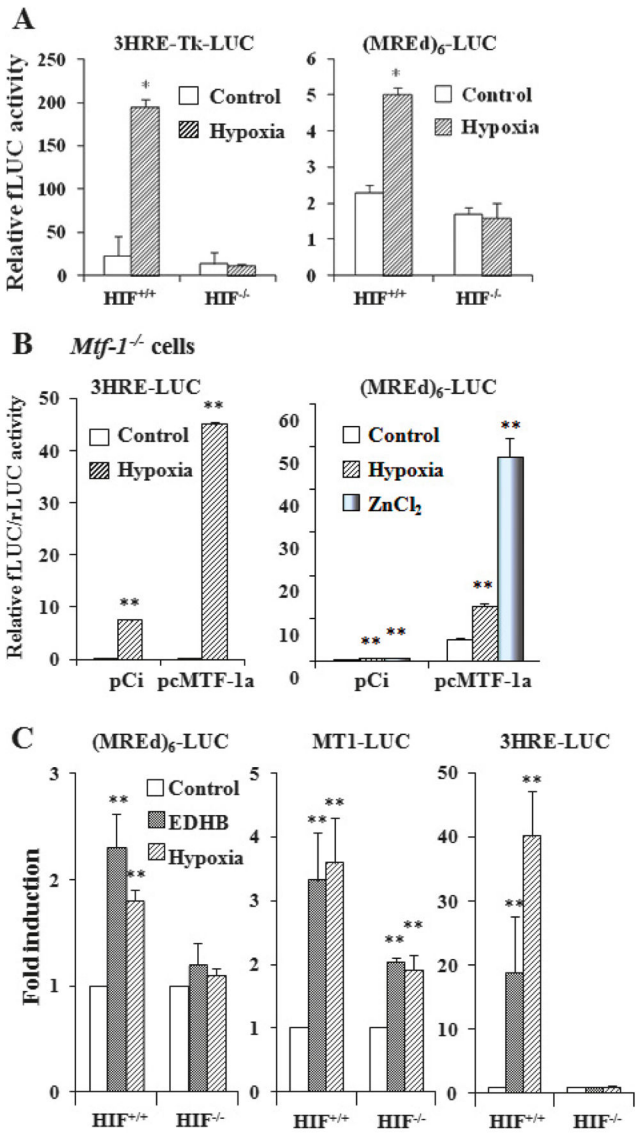

Fig. 6.

Effect of MTF-1 or HIF-1α loss on the activity of 3HRE-LUC and $(MREd)_6$-LUC expression. (A) *Hif-1*α-null and wild-type mouse embryonic fibroblasts (MEFs) were transfected with a mixture of 3HRE-LUC (left panels) or $(MREd)_6$-LUC (right panels) and pTK-rLUC and then unexposed (control) or exposed to hypoxia (pO_2 = 1%) for 16 h before measurement of LUC activity as described in Fig. 2B. Results represent the mean ± SD of 3 independent experiments performed in triplicate. (B) *Mtf-1*-null mutant MEFs were transfected with a plasmid mixture containing 3HRE-LUC (left panel), $(MREd)_6$-LUC (right panel), pTK-LUC, and 100 ng of the human MTF-1 expression plasmid (pcMTF-1a) or the empty vector (pCi) as indicated below the graphs. Cells were cultured for 24 h and then treated or not (control) with 50 μmol/L $ZnCl_2$ or exposed to hypoxia (pO_2 = 1%) for 16 h, as indicated, before measurement of LUC activity as described in Fig. 2B. Data represent the mean ± SD of 3 independent experiments performed in triplicate. (C) *Hif-1*α-null and wild-type MEFs were transfected with a mixture of $(MREd)_6$-LUC, MT1-LUC, or 3HRE-LUC,

as indicated, and pTK-rLUC, and then treated or not (control) with 500 μmol/L EDHB or exposed to hypoxia (pO_2 = 1%) for 16 h before measurement of LUC activity as described in Fig. 2B. Results represent the mean ± SD of 3 independent experiments performed in triplicate. Significant differences between control and treated samples are based on the Student's *t* test. *, $P < 0.05$; and **, $P < 0.01$.

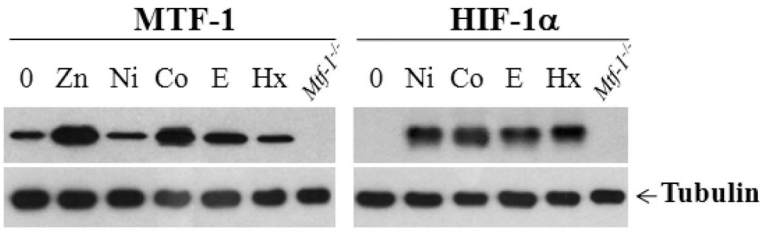

Fig. 7.

Immunoblot analysis of HIF-1α and MTF-1 induction in response to hypoxia. L cells were unexposed (0, control) or exposed to $ZnCl_2$ (Zn, 100 μmol/L), $NiCl_2$ (Ni, 500 μmol/L), $CoCl_2$ (Co, 300 μmol/L), EDHB (E, 500 μmol/L), or hypoxia (Hx, pO_2 = 1%) for 16 h and whole-cell lysates were prepared and analyzed by immunoblotting with antibodies against HIF-1α and MTF-1, as well as tubulin, which was used as the loading control. The results shown are representative of 3 independent experiments. *Mtf-1*$^{-/-}$, extracts prepared from control *Mtf-1*-null cells.

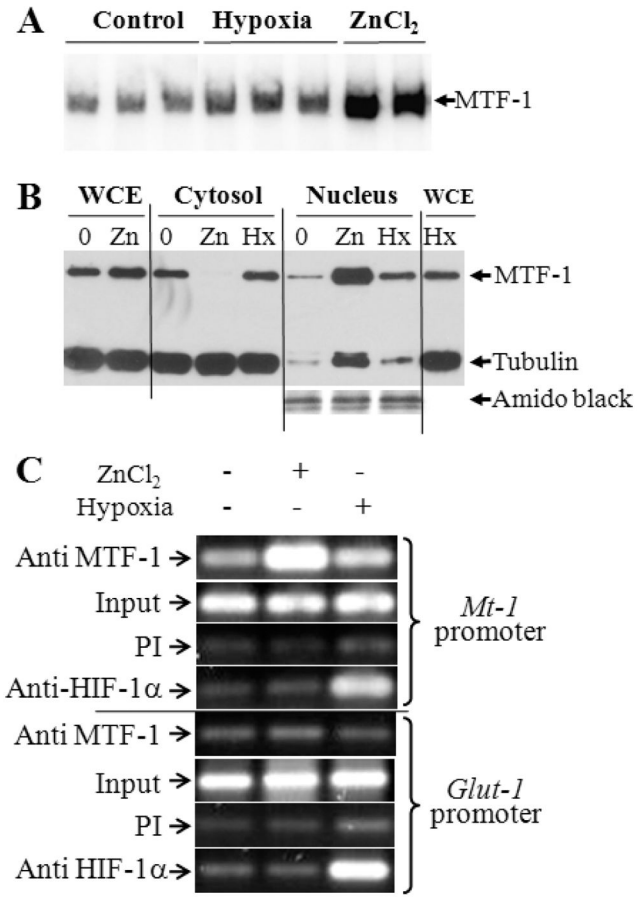

Fig. 8.

Effects of zinc and hypoxia on nuclear localization and DNA binding of MTF-1. (A) C_2C_{12} cells were untreated (control) with 100 μmol/L $ZnCl_2$, or exposed to hypoxia (pO_2 = 1%) for 16 h, as indicated. Extracts were analyzed by electrophoretic mobility shift assays (EMSA) using labeled MRE-s oligos. Only the region of the gel containing the specific MTF-1 complexes is shown. (B) Immunoblotting detection of MTF-1 in whole-cell (WCE), nuclear, and cytosolic extracts. Extracts were prepared from L cells that were untreated (0), treated with 100 μmol/L $ZnCl_2$ (Zn), or exposed to hypoxia (Hx) (pO_2 = 1%), as indicated, and analyzed by immunoblotting with antibodies against MTF-1 and tubulin. Because tubulin is not an appropriate loading control for nuclear extracts, a particular band detected on the blot by staining with amido black is shown. The results shown in panels A and B are representative of 3 independent experiments. (C) ChIP assays were performed using chromatin isolated from L cells that were untreated or treatedwith 100 μmol/L $ZnCl_2$ for 3 h or exposed to hypoxia for 16 h, prior to formaldehyde crosslinking. Immunoprecipitation of crosslinked chromatin was done with MTF-1 (anti-MTF-1) or HIF-1α (anti-HIF1α) antibodies or a pre-immune normal rabbit serum (PI), as indicated. DNA from both the IP input (prior to immunoprecipitation) and the IP-bound fractions was amplified by PCR with primer pairs for the mouse *Mt-1* or *Glut-1* promoter. Input, amplification of DNA prior to immunoprecipitation. The input sample contained 0.4% of the supernatant used for immunoprecipitation of crosslinked MTF-1. The PCR products were analyzed by agarose

gel electrophoresis. These ChIP assays were performed 3 times using 3 different chromatin preparations with similar results.

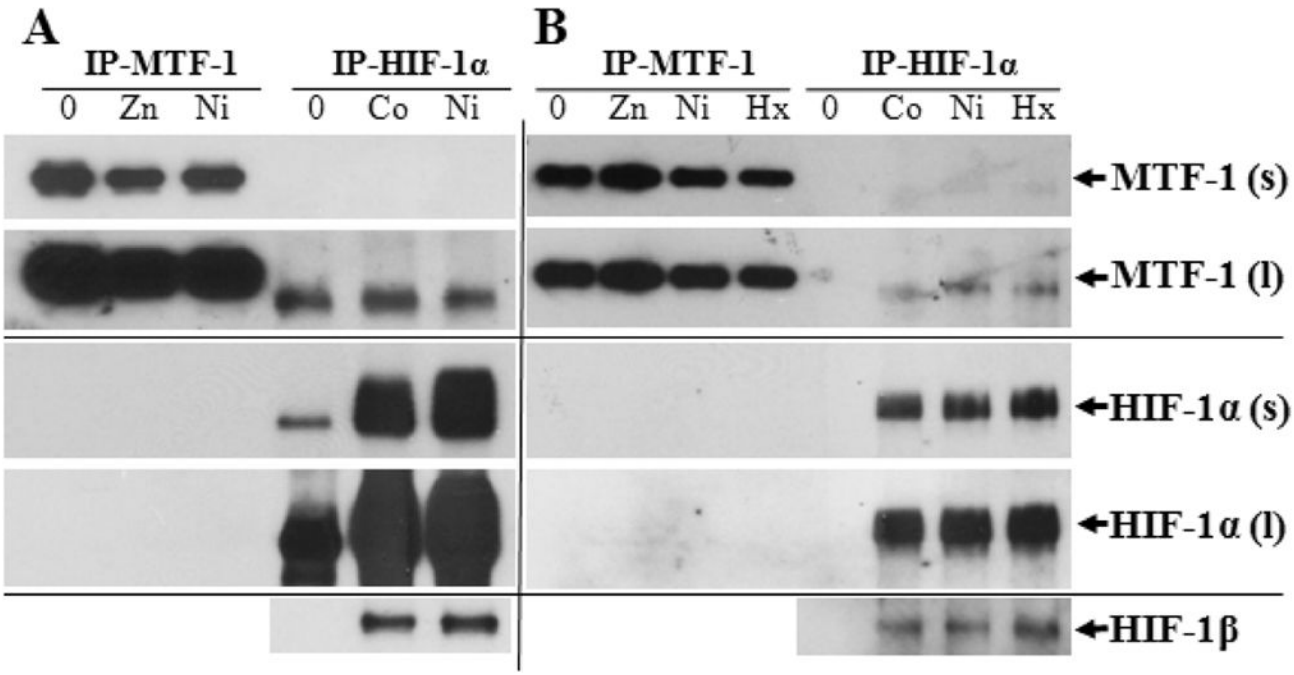

Fig. 9.

Coimmunoprecipitation of mouse MTF-1, HIF-1α, and HIF-1β. L cells were untreated (0), or treated with $ZnCl_2$ (Zn, 100 μmol/L), $NiCl_2$ (Ni, 500 μmol/L), $CoCl_2$ (Co, 300 μmol/L) for 3 h, or were incubated under hypoxia (Hx, pO_2 = 1%) for 16 h. Whole-cell extracts were prepared and immunoprecipitated (IP), as described in experimental procedures (A) or according to Murphy et al. (2008) (B), using antibodies against MTF-1 or HIF-1α followed by Western blot analysis of the immunoprecipitates for MTF-1, HIF-1α, and HIF-1β, as indicated. Films were exposed for a short (s) or a long (l) period of time; on long exposure, a nonspecific band migrating with an Mr slightly smaller than that of MTF-1 was seen in HIF-1α immunoprecipitates (panel MTF-1-l, lanes IP-HIF-1α). The protein associated with this non-specific band was also immunoprecipitated in one of the control extracts (IP-HIF-1α, lane 0). Exposures were as follows: (A) s = 1 s, l = 1 min, and HIF-1β = 3 min; and (B) s = 3 min, l = 10 min, and HIF-1β = 10 min.

Table 1

Sequences of the synthetic oligos used in this study.

Oligo name	Sequence
mMREd	5′-TCCAGGGAGCTC**TGCACTC**CGCCCGAAAAGT-3′
hMREa	5′-GGGGCTTT**TGCACTC**GTCCCGGCTC-3′
ΔMREa	5′-CTGGGTGCAAACCCTT**aatGCCC**GGACTCGTCC-3′
ΔMREc	5′-CTCCGCCCGAAAAG**atCGCTC**GGCTCTGCC-3′
ΔMREd	5′-CCAGGGAGCTC**aatACTC**CGCCCGAAAAG-3′
GLUT-1	5′-GGGCTGTCTTACTCACTCTT-3′
	5′-CTCTTCCTGGGTTGTGTTCA-3′
18S	5′-CTTCCTCTAGATAGTCAAGTTCGACCGTCT-3′

Note: Nucleotides in bold designate the core metal regulatory elements sequence and those in lower case letters correspond to mutations introduced in a given MRE.