Hypoxia Post-translationally Activates Iron-regulatory Protein 2*

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Iron-regulatory proteins 1 and 2 (IRP1 and IRP2) are RNA-binding proteins that post-transcriptionally regulate the expression of mRNAs that code for proteins involved in the maintenance of iron and energy homeostasis. Here we show that hypoxia differentially regulates the RNA binding activities of IRP1 and IRP2 in human 293 and in mouse Hepa-1 cells. In contrast to IRP1, where hypoxic exposure decreases IRP1 RNA binding activity, hypoxia increases IRP2 RNA binding activity. The hypoxic increase in IRP2 RNA binding activity results from increased IRP2 protein levels. Cobalt, which mimics hypoxia by activation of hypoxia-inducible factor 1 (HIF-1), also increases IRP2 protein levels; however, cobalt-induced IRP2 lacks RNA binding activity. Addition of a reductant to cobalt-treated extracts restored IRP2 RNA binding activity. Hypoxic activation of IRP2 is not because of an increase in transcriptional activation by HIF-1, because IRP2 accumulates in Hepa-1 cells lacking a functional HIF-1α subunit, nor is it because of an increase in IRP2 mRNA stability. Rather, our data indicate that hypoxia increases IRP2 levels by a post-translational mechanism involving protein stability. Differential regulation of IRP1 and IRP2 during hypoxia may regulate specific IRP target mRNAs whose expression is required for hypoxic adaptation. Furthermore, these data imply mechanistic parallels between the hypoxia-induced post-transcriptional regulation of IRP2 and HIF-1α.

Cellular hypoxia is an important component of several pathophysiological conditions, including tumorigenesis and ischemia-related disorders. In these and other hypoxic situations mammalian cells alter gene expression to counter the effects of limited O2. Hypoxia induces the transcriptional activation of a variety of genes, including erythropoietin, vascular endothelial growth factor, transferrin, tyrosine hydroxylase, and various glycolytic enzymes, all of whose products are involved in cellular adaptation to decreased O2 (reviewed in Ref. 1). Increased expression of these genes is mediated primarily by the heterodimeric transcription factor hypoxia-inducible factor 1 (HIF-1), which is composed of HIF-1α and HIF-1β subunits (1–4). Decreased O2 tension stimulates HIF-1α protein accumulation by decreasing its proteasomal degradation (5, 6).

Hypoxic stabilization of HIF-1α is blocked in the presence of H2O2, suggesting that hypoxia-induced changes in the level of this reactive oxygen species may be involved in HIF-1 activation (7). Stabilization of HIF-1α allows for heterodimerization with constitutive levels of HIF-1β, also known as aryl hydrocarbon nuclear translocator or ARNT. In turn HIF-1 binds to specific enhancer elements resulting in transcriptional activation.

The current understanding of the regulation of gene expression during hypoxia is primarily at the level of transcriptional activation by HIF-1. However, it is clear that post-transcriptional mechanisms are also employed. These mechanisms involve the induction of mRNA-binding proteins that interact with elements in 3′-untranslated regions (UTRs) as seen for the vascular endothelial growth factor (8–10) and tyrosine hydroxylase (11–13) mRNAs. For example, hypoxia induces the binding of the HuR protein to adenylate-uridylate rich elements in the vascular endothelial growth factor 3′-UTR, stabilizing this mRNA (14).

We have recently reported that hypoxia and subsequent reoxygenation post-transcriptionally regulates the activity of IRP1 (15). IRP1 and a second family member, IRP2, are iron-sensor proteins that post-transcriptionally regulate the expression of genes whose products are involved in regulating cellular iron homeostasis (reviewed in Ref. 16). IRP1 and IRP2 bind specific RNA stem-loop structures termed iron-responsive elements (IREs), which are found in either the 5′- or 3′-UTRs of specific mRNAs, including ferritin (iron storage), mitochondrial aconitase (energy metabolism), erythroid-aminolevulinate synthase (heme biosynthesis), and transferrin receptor (iron transport). IRP binding to a 5′-IRE inhibits translation of the mRNA by inhibiting the 43 S small ribosomal complex from binding to mRNA, whereas binding to a 3′-IRE protects the mRNA from degradation. The RNA binding activity of the IRPs is regulated by cellular iron: RNA binding activity decreases in iron-replete cells and increases in iron-deplete cells. In addition to iron, IRP1 activity increases in cells producing nitric oxide and in cells treated with hydrogen peroxide (17). Thus, the regulation of IRPs by iron and oxidative stress controls the expression of proteins involved in iron sequestration, uptake, and utilization, thus maintaining cellular iron homeostasis.

IRP1 is a dual-function protein, because iron converts it from its apo-RNA binding form to its [4Fe-4S] cytosolic aconitase form (18). Unlike IRP1, IRP2 does not have detectable aconitase activity. Rather, in iron-replete conditions, IRP2 is targeted for rapid degradation by the proteasome (19, 20). Degradation of IRP2 is mediated by an iron-dependent oxidation mechanism that requires a unique 73 amino acid domain containing three essential cysteine residues (19–21).

Our continuing studies on IRP regulation during changing O2 tension revealed an overall increase in total IRP RNA binding activity when human 293 cells and mouse Hepa-1 cells were exposed to hypoxia. Because human IRPs co-migrate during bandshift analysis, it was not readily apparent whether IRP1 or IRP2 was responsible for the increase in total activity. We

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† The abbreviations used are: HIF-1, hypoxia-inducible factor-1; IRP, iron-regulatory protein; IRE, iron-responsive element; UTR, untranslated region; DTT, dithiothreitol; ProA, pronasterone A.
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MATERIALS AND METHODS

Cell Culture—Rat FTO2B hepatoma, human 293 embryonic kidney, and human HeLa cervical cancer cells were all maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). Ecr-293 cells were grown in the same medium containing 400 μg/ml zeocin (Invitrogen). HeLa-1 c15 (wildtype HIF-1β) and HeLa-1 c4 (mutant HIF-1β) (kindly provided by Dr. Oliver Hankinson, UCLA) were grown similarly except in minimal essential medium with Earle’s salts (Life Technologies, Inc.). All cells were grown at 37 °C in a 5% CO₂ humidified incubator. Cells were plated at a density of 1.2 × 10⁶ (FTO2B) and 2.5 × 10⁵ (293, HeLa, and Hepa-1) respectively, in 35-mm culture plates 1 day before normoxic or hypoxic exposure. Hypoxia was achieved by flushing a Modular Incubator (Billups-Rothenburg, Del Mar, CA) for 20 min at a flow of 2 p.s.i with 1% O₂, 5% CO₂, and a balance of nitrogen. 1 h before normoxia/hypoxia, the medium was replaced with 1.2 ml of fresh medium. Normoxic control cells were treated identically except with 21% O₂. The experiment was performed in human 293 cells is elevated following hypoxic exposure. Hypoxia decreased IRP1 RNA binding activity in rat FTO2B hepatoma cells as previously reported (15). Rodent IRP1 and IRP2 separate during bandshift analysis unlike human IRPs. The hypoxia-induced increase in total IRP activity from hypoxically treated cells compared with normoxic controls was more notable after 18 h of hypoxia (Fig. 1B). Addition of hemin decreased total IRP RNA binding activity in both normoxic and hypoxic cells, demonstrating that the IRP1 and IRP2 response to iron is not impaired during hypoxia.

Cytosolic Extract Preparation and RNA Bandshift Analysis—Cells were washed once with cold phosphate-buffered saline and cytosolic extracts were prepared by addition of 125 μl of lysis buffer (20 mM Hepes, pH 7.6, 25 mM KCl, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride) to the 35-mm dish and incubated at room temperature for 3 min. Cell debris was removed by centrifugation at 15,000 × g for 30 min at 4 °C. Protein concentration was determined by the Coomassie assay method (Pierce). RNA bandshifts were performed as described previously (24). Quantification of IRP-RNA complexes was determined by PhosphorImager analysis. When “supershift” assays were performed, antisense that specifically recognizes the unique 73 amino acid insert of IRP2 was added for 20 min before electrophoresis (25). Dithiothreitol (DTT) was used at 10 mM as shown. Because the nonshifted complexes represent IRP1, it can be seen that IRP1 RNA binding activity decreased as a result of hypoxia (compare lanes 6–8 with 10–12 in Fig. 2A), which is similar to what is seen in FTO2B cells (Fig. 1A). Furthermore,
consistent with our earlier studies, the hypoxic decrease in IRP1 RNA binding activity was not because of decreased protein levels because IRP1 protein levels did not change following a 20 h hypoxic exposure (Fig. 2B) (15). These data indicate that IRP1 and IRP2 are both regulated during hypoxia but in opposite directions.

The hypoxic increase in IRP2 RNA binding activity could result from activation of IRP2 or from increasing IRP2 protein levels. Therefore, we measured IRP2 protein levels following 4, 7, and 19 h of hypoxic exposures. The immunoblot in Fig. 2C demonstrates that hypoxia increases IRP2 protein levels. Quantification of the bands in Fig. 2C revealed a 1.5-, 2.9- and 3.9-fold increase in IRP2 protein levels at 4, 7, and 19 h of hypoxia, respectively. The increase in IRP2 protein levels reflected a similar fold increase in IRP2 RNA binding activity as determined by PhosphorImager analysis (Fig. 2D). Therefore, we conclude that the increase in IRP2 RNA binding activity during hypoxia is because of an increase in IRP2 protein levels.

IRP2 Hypoxic Induction Is Mediated by a Post-transcriptional Mechanism—To determine whether the hypoxic increase in IRP2 protein is the result of increased IRP2 mRNA, mRNA levels were measured by Northern blot analysis. Total RNA was isolated from 18 h normoxic and hypoxic human 293 cells and hybridized with a radiolabeled IRP2 probe. Fig. 3A shows that IRP2 mRNA levels are not elevated during hypoxia. In fact, IRP2 mRNA is slightly decreased, perhaps because of a general hypoxic down-regulation of RNA synthesis (27). These results indicate that the increase in IRP2 protein during hypoxia is not because of increased transcription of the IRP2 gene nor to increased stability of the IRP2 mRNA.

Although we could eliminate the direct transcriptional activation of the IRP2 gene during hypoxia, it was possible that an HIF-1 target gene was required for hypoxic induction of IRP2. To analyze this possibility, a mouse hepatoma cell line harboring a mutated HIF-1β subunit that is nonfunctional (28, 29) was used to determine whether hypoxic induction of IRP2 was dependent on HIF-1 activation. Hepa-1 c4 c7 cells, which contain a mutated HIF-1β and wild-type Hepa-1 c1c7 were exposed to normoxia or hypoxia for 18 h, and IRP1 and IRP2 RNA binding activities were measured. Fig. 3B shows that IRP2 binding activity increased in both cell lines to a similar extent as seen for 293 cells. Furthermore, IRP1 RNA binding activity decreased in Hepa-1 c4c7 cells similar to hypoxic rat FTO2B and human 293 cells. These data indicate that hypoxic activation of IRP2 is not downstream of HIF-1.

Hypoxia Stabilizes IRP2—Because hypoxic induction of IRP2 does not result from increased mRNA levels, we next examined whether IRP2 is stabilized during hypoxia. EcR-293 cells were transiently transfected with the pIND(sp1)IRP2 vector and allowed to recover for 24 h before IRP2 expression was induced with ProA. Following 20 h of induction, the cells were then either harvested or the medium was replaced with medium lacking ProA and exposed to normoxia or hypoxia for 12 h. Immunoblot analysis demonstrates that recombinant IRP2 is induced in the presence of ProA (Fig. 4, lanes 1 and 2). When expression of IRP2 is shut off by removal of ProA, the levels of

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**FIG. 2.** Hypoxia increases IRP2 RNA binding by increasing IRP2 protein levels concomitant with a decrease IRP1 RNA binding. A, human 293 cells (lanes 5-12) were exposed to normoxia (N) or hypoxia (H) for 18 h followed by supershift analysis using 12 μg of cytosolic extract and anti-IRP2 antiserum as described under “Materials and Methods.” Either no antiserum or the indicated amount of IRP2 antiserum was added to the extracts after the RNA-protein complexes were allowed to form. Cytosolic extracts (12 μg) from FTO2B cells (lanes 1-4) were included as controls to ensure that the amount of IRP2 antiserum used did not shift IRP1. Rat IRP1 and IRP2 (rIRP1 and rIRP2), co-migrating human IRPs (hIRP1/2) and supershifted IRP2 are indicated. B, 293 cells were exposed to 20 h of normoxia (N) or hypoxia (H), and IRP1 levels were examined by immunoblot analysis. C, determination of IRP2 protein levels by immunoblot analysis followed by LumImage analysis (Boehringer) for the indicated times of hypoxic exposure was performed (see text). D, IRP2 supershift analysis of extracts in A. PhosphorImager quantification was then performed.
IRP2 decay after 12 h of normoxia, but remain elevated following 12 h of hypoxia (Fig. 4, lanes 3 and 4). These data demonstrate that the increase in IRP2 protein during hypoxia is because of protein stabilization. Furthermore, stabilization of IRP2 appears specific, because IRP1 protein levels were not significantly altered following 20 h of hypoxia (Fig. 2B) (15).

Cobalt Increases IRP2 Protein During Normoxia—Addition of cobalt to normoxic cells mimics hypoxia by the activation of expression of several HIF-1 target genes. This is because of the cobalt-induced increase in the HIF-1α protein and subsequent HIF-1 DNA binding (6, 22). HIF-1α protein stabilization has been suggested to be downstream of a heme protein that functions as an O2 sensor (1, 3). It is thought that cobalt, by displacing the heme iron, inactivates the O2 sensor. A more recent report suggests that the mitochondria may be the upstream O2 sensor for HIF-1 activation (42). Treatment of 293 cells with cobalt increased IRP2 protein levels in both a dose- and time-dependent manner (Fig. 5A and B). Although 100 μM CoCl2 increased IRP2 protein levels, maximum stimulation was achieved at 1 mM, which is ~10-fold greater than that required for inducing HIF-1 activation in HeLa cells (22). The requirement for a higher dose of cobalt in 293 cells is presumably because of differences in cobalt uptake, because IRP2 was induced in HeLa cells treated with 100 μM CoCl2 for 8 h (Fig. 5C).

To determine whether the cobalt-induced increase in IRP2 protein levels reflects an increase in IRP2 RNA binding activity, 293 cells were subjected to normoxia, 1 mM CoCl2, hypoxia, or hypoxia in the presence of 1 mM CoCl2 for 8 h followed by supershift and immunoblot analyses. Fig. 6A shows that in contrast to hypoxia where IRP2 RNA binding activity increased, cobalt treatment resulted in an inhibition of IRP2 activity (Fig. 6A, compare lanes 5 and 6), even though both conditions increased IRP2 protein levels (Fig. 6C). Furthermore, cobalt treatment also inhibited the increase in IRP2 RNA binding activity by cobalt could be reversed by a reductant, 10 mM DTT was added to the binding reactions before supershift analysis. DTT restored IRP2 RNA binding activity in both hypoxia- and hypoxia/CoCl2-treated extracts (Fig. 6B). Treatment with DTT also increased IRP1 RNA binding activity in all extracts as
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Previously we demonstrated that hypoxia decreased IRP1 RNA binding activity in rat hepatoma cells and primary cardiac myocytes (15). In that study, IRP2 RNA binding activity increased slightly after prolonged hypoxic exposure (18 to 21 h). We also observed an increase in IRP2 activity in FTO2B cells during anoxia.2 These results prompted us to carry out the experiments reported here, which investigated IRP2 regulation during hypoxia in other cell types, including 293, Hepa-1, and HeLa cells. Both 293 and Hepa-1 cells displayed significant hypoxia-inducible activation of IRP2 RNA binding activity. The regulation of IRP2 by hypoxia appears to be characteristic of many cell types with different cells varying in their magnitude of IRP2 response.

IRP1 and IRP2 RNA binding activities responded to hypoxia differently: hypoxia decreased IRP1 and increased IRP2 RNA binding activities. The decrease in IRP1 RNA binding activity was paralleled by a ~35% increase in its cytosolic aconitase activity with no change in IRP1 protein levels (data not shown). These results suggest hypoxia stabilizes the [4Fe-4S] aconitase active form of IRP1 at the expense of its RNA binding activity (15). Unlike IRP1, IRP2 regulation by hypoxia occurs by accumulation of the IRP2 protein. Therefore, hypoxic regulation of IRP1 and IRP2 RNA binding activities occurs by different mechanisms.

What are the functional consequences of the differential regulation of IRP1 and IRP2 during hypoxia? One notion is that IRP1 and IRP2 regulate different cellular IRE-mRNAs. Studies using in vitro synthesized IREs (33, 34) showed that IRP1 and IRP2 can preferentially bind to specific IRE-like structures. In addition, a recent study showed that the range of IRE binding of IRP1 is more extensive than IRP2. IRP1 binds with high affinity to different IREs, including those of the ferritin, transferrin receptor, and erythroid-aminolevulinate synthase mRNAs, whereas high affinity IRP2 binding is restricted to ferritin IREs (35). The regulation of IRP1 and IRP2 activities in an opposing manner during hypoxia may lead to the specific regulation of different IRE-mRNAs whose expression/repression may be required for adaptation during the hypoxia.

Hypoxic activation of IRP2 is not a result of an increase in mRNA levels because of increased transcription or increased mRNA stability. Activation is also not dependent on HIF-1, because full IRP2 activation occurs in Hepa-1 c4 cells that do not contain a functional HIF-1β. Furthermore, hypoxic accumulation of IRP2 is not because of impaired proteasomal function, because hemin-treated cells resulted in IRP2 degradation during hypoxia. Rather, our data suggest a model in which the IRP2 protein is stabilized during hypoxia. This model is consistent with the mechanism of iron-dependent regulation of IRP2 (19, 20, 25). IRP2 is regulated by iron by a mechanism involving metal-catalyzed oxidation of the protein followed by ubiquitination and degradation via the proteasome (21). This process requires the 73-amino acid degradation domain that potentially coordinates iron via three conserved cysteines, which are required for iron-mediated degradation (20). Iron coordination may provide a “localized” Fenton reaction leading to IRP2 oxidation. Any model for the regulation of IRPs during hypoxia must depart from one that relies solely on changes in iron levels, because during hypoxia RNA binding activity of IRP1 decreased, whereas IRP2 activity increased. One possible mechanism for hypoxic regulation of IRP2 is that the generation of $H_2O_2$, possibly from a heme-containing $O_2$ sensor, acts as an IRP2 degradation signal. A decrease in $H_2O_2$ output from

![Diagram of IRP2 regulation](image)

FIG. 6. Cobalt induces IRP2 lacking RNA binding activity. A, human 293 cells were exposed for 8 h to normoxia (N, lanes 1 and 5), 1 mM CoCl$_2$ (Co, lanes 2 and 6), hypoxia (H, lanes 3 and 7), or both 1 mM CoCl$_2$ and hypoxia (Co + H, lanes 4 and 8). Cytosolic protein (12 μg) was then subjected to bandshift analysis with (lanes 5–8) or without (lanes 1–4) anti-IRP2 antiserum (1.5 μg). B, supershift analysis was carried out as in A except that 10 mM DTT was present in the binding reactions. C, immunoblot analysis using IRP2 antiserum was performed on the same extracts.

previously shown (30). Because cobalt, hypoxia, and a combination of both increased IRP2 protein levels to a similar extent (Fig. 6C), their modes of action are not synergistic. These results suggest that cobalt affects IRP2 by two distinct mechanisms: first, by mimicking hypoxia, cobalt increases IRP2 protein levels, and second, through a mechanism unrelated to hypoxia whereby cobalt inhibits IRP2 RNA binding activity.

**DISCUSSION**

Mammalian cells employ adaptive responses when exposed to hypoxia (1, 3). One such response is the regulated and specific alteration in gene expression imposed by hypoxia-induced transcriptional activation. To date HIF-1 is the most thoroughly characterized transcriptional regulator of gene expression during hypoxia (reviewed in Refs. 31 and 32), whereas considerably less is known about post-transcriptional mechanisms (9, 11, 12, 14). In this study we report that IRP2, in addition to IRP1, is post-translationally regulated by hypoxia. These data suggest that both proteins are components of a hypoxic-regulatory pathway involved in post-transcriptional gene regulation.

2 E. S. Hanson and E. A. Leibold, unpublished observation.
such a sensor during hypoxia would lead to a decrease in IRP2 oxidation and consequently an increase in IRP2 stability. In this scenario, hypoxic regulation of IRP2 would be dependent on the relative cytosolic levels of both H$_2$O$_2$ and Fe$^{2+}$. A similar mechanism could potentially be involved in hypoxic HIF-1α protein stabilization.

Several important parallels can be drawn between hypoxic activation of IRP2 and HIF-1α. First, both IRP2 and HIF-1α (5–7) proteins accumulate during hypoxia by a post-translational mechanism involving increased protein stability. Second, cobalt, which mimics hypoxia, stimulated the activation of both IRP2 and HIF-1α (6, 22, 37), perhaps by inactivating a heme-containing O$_2$ sensor (38). Third, iron chelation elevates the protein levels of both IRP2 (19, 23) and HIF-1α (37, 39). Fourth, the activation of IRP2 and HIF-1α by hypoxia and cobalt occurs in different cell types, suggesting that both proteins are part of a global mechanism of gene regulation during hypoxia. Collectively, these data suggest that the regulation of IRP2 and HIF-1α during hypoxia occurs, at least in part, by similar mechanisms.

An unexpected finding was that cobalt, in addition to increasing IRP2 protein levels, inactivated IRP2 RNA binding activity. Because IRP1 activity was not decreased, this suggested that the effect of cobalt was specific for IRP2. IRP2 RNA binding activity could be restored by the addition of DTT, suggesting that cobalt either directly or indirectly resulted in the oxidation of critical cysteines required for IRP2 RNA binding. Cobalt has been shown to cause a reduction of cellular glutathione and oxidize thiols in lung cells (40). These results are consistent with our previous studies showing that inactivation of IRP2 RNA binding activity by oxidants in vitro could be reversed by treatment with high concentrations of reductants (41). Similar redox control of DNA binding activity of HIF-1α has also been reported (7). Whether IRP2 RNA binding activity can be modulated by redox in vivo remains to be determined.

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