Hypoxia and Mitochondrial Inhibitors Regulate Expression of Glucose Transporter-1 via Distinct Cis-acting Sequences*

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Studies of gene regulation by oxygen have recently defined the existence of a widely operative system that responds to hypoxia but not mitochondrial inhibitors and involves the induction of a DNA-binding complex termed hypoxia-inducible factor 1. This system has been implicated in the regulation of erythropoietin, certain angiogenic growth factors, and particular glycolytic isoenzymes. The glucose transporter Glut-1 is induced by both hypoxia and mitochondrial inhibitors, implying the operation of a different mechanism of oxygen sensing. To explore that possibility, we analyzed the cis-acting sequences that convey these responses. An enhancer lying 5′ to the mouse Glut-1 gene was found to convey responses both to hypoxia and to the mitochondrial inhibitors, azide and rotenone. However, detailed analysis of this enhancer demonstrated that distinct elements responded to hypoxia and the mitochondrial inhibitors. The response to hypoxia was mediated by sequences that contained a functionally critical, although atypical, hypoxia-inducible factor 1 binding site, whereas sequences lying approximately 100 nucleotides 5′ to this site, which contained a critical serum response element, conveyed responses to the mitochondrial inhibitors. Thus, rather than reflecting an entirely different mechanism of oxygen sensing, regulation of Glut-1 gene expression by hypoxia and mitochondrial inhibitors arises from the function of two different sensing systems. One of these responses to hypoxia alone and resembles that involved in erythropoietin regulation, while the other responds to mitochondrial inhibitors and involves activation of a serum response element.

Many biological processes are concerned with adaptation to the availability of oxygen, and oxygen tension is an important regulator of gene expression. A recent advance in the understanding of these processes has been the recognition that a mechanism of oxygen sensing identical or closely similar to that first recognized in the context of erythropoietin regulation is widely operative in different cell types (1–3). Production of erythropoietin, the principal humoral regulator of blood cell production, is stimulated by hypoxia in specific cells within liver and kidney. Characterization of this response has defined a number of distinctive features. In particular, the response to hypoxia is mimicked by transition metal ions such as cobalt (4)

and by iron chelating agents (5) but not by inhibitors of mitochondrial respiration (6–8). These features have led to the proposal of a specific oxygen sensing system, possibly involving the operation of a heme protein sensor (8).

Two types of evidence have been important in implicating this regulatory system in the control of genes other than erythropoietin: first, similarities in the characteristics of the inducible response; and, second, the definition of similar critical elements in the cis-acting control sequences of the different genes. For certain genes, such as several of those encoding glycolytic enzymes, the demonstration of inducible responses to hypoxia, cobalt, and iron chelating agents but not cyanide, together with the functional definition of cis-acting sequences that cross-compete with the erythropoietin 3′ enhancer for binding to the hypoxia-inducible factor 1 (HIF-1) provide firm evidence of a common regulatory mechanism (9–11). Similarly, evidence has recently implicated this mechanism in the regulation of vascular endothelial growth factor (12–15). For the genes encoding a number of other vascular growth factors, multiple similarities with the regulation of the erythropoietin gene have also been observed (14, 16, 17), but the cis-acting sequences mediating the inducible responses have not all been defined.

For other genes, inducible responses to hypoxia have been described in which there are important differences from erythropoietin regulation. One such gene is that encoding the glucose transporter, Glut-1. Both inhibition of mitochondrial respiration and cellular hypoxia are accompanied by an increase in the rate of glucose uptake, which is largely mediated by increased Glut-1 activity (18–23). This occurs by increased transporter synthesis following up-regulation of Glut-1 mRNA and by post-translational mechanisms. Since both hypoxia and mitochondrial inhibitors increase Glut-1 mRNA, it has appeared likely that induction by hypoxia is due to hypoxic compromise of mitochondrial metabolism and is therefore distinct from the mechanism underlying erythropoietin regulation. A further difference in the regulation of the two genes is seen in the responses to phorbol esters; whereas hypoxic induction of the erythropoietin gene is ablated by phorbol 12-myristate 13-acetate (PMA) (24–26), this compound induces the expression of Glut-1 (27).

Here we report that, despite these characteristics, there are also similarities between the regulation of Glut-1 and erythropoietin. Induction of Glut-1 mRNA by hypoxia could be mimicked by cobalt and the iron chelator, desferrioxamine (DFO). To characterize further the response of Glut-1 to hypoxia and mitochondrial inhibitors, we analyzed the regulatory sequences that convey these responses. The mouse Glut-1 5′

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1 The abbreviations used are: HIF-1, hypoxia-inducible factor 1; PMA, phorbol 12-myristate 13-acetate; kb, kilobase pair(s); SRE, serum response element; DFO, desferrioxamine; bp, base pair(s); WT, wild-type Mut, mutant.
enhancer, lying at −3.3 to −2.7 kb from the transcription initiation site, was found to possess hypoxically inducible activity and also to be responsive to azide, rotenone, cobalt, DFO, and PMA. Deletional and mutational analysis demonstrated that distinct cis-acting sequences within this enhancer conveyed responses to hypoxia and to the mitochondrial inhibitors. Hypoxic stimulation was mediated by a HIF-1 binding site, the operation of which was unaffected by PMA. Responses to mitochondrial inhibitors were mediated by a serum response element. In experiments using the Glut-1 promoter with a growth hormone reporter, 50 μg of the test plasmid was used with 3 μg of the control plasmid pBSw. For transfections with TKGH plasmids, 50 μg of the test plasmid was used with 25 μg of pBSw. After transfection, each pool of transfected cells was divided so that control and treated plates were incubated in parallel for 14--16 h. Exposure to hypoxia or pharmacological agents was commenced 1 h after transfection.

RNA Preparation and Analysis—RNA was extracted using a modified acid guanidinium thiocyanate/phenol/chloroform extraction method (RNAzol B, Cinna/Biobrex Laboratories, Houston, TX). RNase protection assays and quantitation on a flat-bed scintillation counter (model 1205 Betaplate; Pharmacia-Wallac OY, Turku, Finland) were performed as described previously (29). To assay endogenous gene expression, 10 μg of RNA was hybridized with a riboprobe to the Glut-1 gene, which protects a 136-bp fragment (nucleotides 1063–1198, GenBank accession number K03195). As a control for recovery and gel loading, a small quantity of antisense RNA generated by in vitro transcription of a 200-bp fragment of the α-globin gene including a splice site was used as a control. As a positive control, a smaller quantity of antisense RNA generated by in vitro transcription of a 200-bp fragment of the mouse phosphoglycerate kinase-1 gene was used as a positive control. Hybridization, together with a sense probe that protected 260 bp of that transcript. To measure the expression of transfected plasmids, 5 μg of RNA from transfected cells was assayed by double hybridization with probes that protected 120 bp of GH mRNA and either 132 or 97 bp of α-globin mRNA, depending on whether the α-globin gene was the reporter for the test or control plasmid.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays—Nuclear extract was prepared using a modification of the protocol described by Dignam et al. (30). Oligonucleotides derived from the mouse erythropoietin 3′ enhancer, mouse phosphoglycerate kinase-1 5′ enhancer, or the Glut-1 5′ enhancer were used as probes and competitors. The oligonucleotide sequence for the Epo wild-type (Epo WT) was 5′-GGCAACTGCTGCTGCTGACTGCG-3′. Epo mutant (Epo Mut) was 5′-GCCCTAATGTCTGCTGCATGGC-3′. PGK wild-type (PGK WT) was 5′-ATTGTGCACTGCTGACAGCGC-3′. Glut-1 wild-type (Glut WT) was 5′-CAGCAGCTGCTGCTGACTGCG-3′. Each of these oligonucleotides and complementary 24-mers was purified by polyacrylamide gel electrophoresis. Probes were labeled using [γ-32P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase and annealed with 4-fold molar excess of the unlabeled complementary strand; equimolar concentrations of complementary strands were annealed for use as competitors. HepG2 nuclear extract (5 μg) was incubated in a 20-μl binding reaction mixture containing 50 mM KCl, 1 mM MgCl2, 0.5 mM EDTA, 5 mM dithiothreitol, 5% (v/v) glycerol, and sonicated poly-d (dC) (250 and 50 ng, respectively, for the Epo WT and Glut-1 WT probes) for 10 min at room temperature. HepG2 nuclear extract (250 ng) and specific competitors were added. After 10 min of further incubation, reactions were loaded on a 5% polyacrylamide gel and electrophoresed (12.5 V/cm) at 4°C in 0.3 × TBE (30 mM Tris, 30 mM boric acid, 0.06 mM EDTA, pH 7.3, at 20°C). RESULTS

Regulation of Glut-1 mRNA—The induction of Glut-1 mRNA was measured after exposure to an atmosphere of 1% oxygen for 16 h in three cell types: HepG2 (hepatoma), HT1080 (fibrosarcoma), and HeLa (cervical carcinoma). In comparison with a parallel incubation of cells under 21% oxygen (normoxia), hypoxia induced Glut-1 mRNA 3–5-fold. Results for each of the cell types are given in Table I.

To test if, despite the reported induction of Glut-1 mRNA by mitochondrial inhibitors, there might be similarities with erythropoietin regulation, we determined whether Glut-1 mRNA could be induced by cobalt or DFO. HepG2 cells were exposed to cobalt chloride (100 μM) or to DFO (100 μM) for 16 h. Both of these agents induced Glut-1 mRNA to a similar degree to that observed following hypoxia (Table II).

Location of cis-Acting Sequences That Convey Inducible Responses to Hypoxia—Three sequences with known transcriptional effects in the mouse Glut-1 gene (28) were tested for inducible responses to hypoxia by transient transfection of
Hypoxic induction of Glut-1 mRNA by cobalt and DFO. Exposure of HepG2 cells to cobalt and DFO was shown to mimic hypoxia in the induction of Glut-1 mRNA. Induction was calculated as the ratio of Glut-1 mRNA under the test condition to Glut-1 mRNA in a parallel untreated culture. Values are given as the mean ± S.E.; n = number of experiments.

Table I

| Plasmid  | Glut-1 sequence | hypoxic induction |
|----------|-----------------|------------------|
| pG5GH    | -1036 to +53    | 1.1 ± 0.1        |
| pG5'E(1-610)α | -3.3 to -2.7 kb | 4.1 ± 0.8        |
| pG5Eα    | +16.7 to +18 kb  | 1.1 ± 0.1        |

Fig. 1. Localization of hypoxically inducible cis-acting sequences to the Glut-1' enhancer. Sequences with known transcriptional activity from the mouse Glut-1 gene (28) were cloned into reporter constructs as described under "Materials and Methods." The Glut-1' enhancer was capable of conferring hypoxic inducibility on the reporter gene, but the intronic enhancer and promoter were not hypoxically inducible. In each transfection, expression was related to that of a co-transfected control plasmid; induction was calculated as the ratio of hypoxic to normoxic expression. The figures shown are the mean values for three independent experiments ± S.E.

HepG2 cells. Results are summarized in Fig. 1. The 1.03-kb Glut-1 promoter showed no response to hypoxia. Whereas both the 5' enhancer (-2.7 to -3.3 kb) and the intronic enhancer (+16.7 to +18.0 kb) demonstrated similar constitutive activity, hypoxically inducible activity was entirely confined to the 5' enhancer. In hypoxic cells, the activity of the 5' enhancer was increased 4.1 ± 0.8-fold, whereas that of the intronic enhancer was unchanged.

Location of an Hypoxically Responsive Element within the Glut-1 5' Enhancer—In order to localize the cis-acting sequence critical for hypoxically inducible activity of the Glut-1 5' enhancer, two series of deletions were made: one from the 5' end of the enhancer and the other from the 3' end of the enhancer. The constitutive and hypoxically inducible levels of activity for each deletion are shown in Fig. 2. Deletion of 3' sequences demonstrated a sharp reduction in hypoxically inducible activity, but not constitutive activity, when nucleotides between 262 and 300 were removed. Deletion of 5' sequences demonstrated a more progressive reduction in both constitutive and hypoxically inducible activity. Deletion to nucleotide 228 reduced constitutive activity to 24 ± 1% of the activity of the full-length enhancer, but an inducible response was retained. Further deletion to nucleotide 288 abolished inducible activity. Thus, a sequence of 27 bp, corresponding to nucleotides 262-288, was determined to be necessary for hypoxically inducible activity.

The Glut-1 5' Enhancer Conveys Responses to Multiple Stimuli—To characterize the inducible mechanisms underlying the response of the Glut-1 gene to hypoxia and mitochondrial inhibitors, the responsiveness of the 5' enhancer to these and related stimuli was tested. The majority of experiments were performed in HepG2 cells transfected with pG5'E(1-326)α, containing bases 1-326 of the 5' enhancer, since this sequence conveyed equivalent or greater hypoxically inducible activity than pG5'E(1-610)α. Cells were exposed to chemicals for 16 h, and in each case results were compared with parallel pools of unstimulated cells from the same transfection. The stimuli applied, azide (1 mM), cobaltous chloride (100 μM), DFO (100 μM), and rotenone (0.1 μM/ml), all induced expression of pG5'E(1-326)α. Results are summarized in Table III.

Distinct cis-acting Sequences Are Critical for Responses to Hypoxia and Mitochondrial Inhibitors—To compare the responses to hypoxia and mitochondrial inhibitors, cells transfected with plasmids containing critical deletions were tested for responsiveness to azide, rotenone, cobaltous chloride, and DFO (Fig. 3). Inducible responses to cobaltous chloride and DFO were observed for pG5'E(1-326)α; as with hypoxia, these responses were preserved for 5' deleted plasmid, pG5'E(228-610)α, but abolished by the 3' deleted plasmid, pG5'E(1-262)α. In contrast, the effect of deletions on responsiveness to azide and rotenone was the reverse. The 5' deletion to nucleotide 228 abolished responsiveness to both of these stimuli, whereas the 3' deletion to nucleotide 262 had little effect on the responses. These experiments indicated that the responses to hypoxia and mitochondrial inhibitors were dependent on different cis-acting sequences, most probably indicating that they operate via different sensing and signal transduction pathways.

Definition of Critical Sites for Inducible Responses by Site-directed Mutagenesis—Site-directed mutagenesis was used to define critical elements within these sequences. In the 27-bp region implicated by deletional analysis as necessary for hypoxic induction, an area of homology was identified with hypoxically inducible sequences from other genes. Within this sequence, a 4-bp mutation was made at the site that conformed most closely to the HIF-1 binding site consensus, pG5'E(ΔSRE)α. This mutation completely abolished inducible responses to hypoxia and cobalt but had no effect on induction by azide.

Failure of plasmid pG5'E(228-610)α to respond to azide or rotenone indicated that the sequence lying 5' to nucleotide 228 was responsible for this effect. We therefore tested pG5'E(108-610)α and found preserved responses to azide (data not shown). Since the sequence between nucleotides 108 and 228 contains a conserved serum response element, plasmid pG5'E(ΔSRE)α was constructed with a four-nucleotide mutation at this site. This mutation abolished the response to azide but had little effect the response to hypoxia or cobalt (Fig. 4).

The SRE Conveys Responses to Mitochondrial Inhibitors—Since the SRE was necessary for responses to azide, we tested whether an SRE could, by itself, convey responses to mitochondrial inhibitors. An oligonucleotide corresponding to the SRE in the c-fos gene promoter was inserted as a trimer adjacent to the minimal promoter in pTKGH (see "Materials and Methods"). The minimal promoter alone was unresponsive to these stimuli, but the c-fos SRE was induced 3.1 ± 0.1-fold (n = 3) by azide and 2.7 ± 0.2-fold (n = 3) by rotenone.

The Response to Hypoxia Is Conveyed by a HIF-1 Binding
Element—We next compared the functional and DNA-binding properties of the region surrounding the ΔHIF mutation with those of known HIF-1 binding sites using an oligonucleotide consisting of nucleotides 273–296. In electrophoretic mobility shift assays, this double-stranded oligonucleotide bound both inducible and constitutive species with mobilities similar to those observed for previously described HIF-1 binding sites (Fig. 5A). However, when compared with other functional HIF-1 binding sites, such as that in the PGK 5′ enhancer, the Glut-1 oligonucleotide was a less effective competitor for the binding of HIF-1 to the site within the erythropoietin 3′ enhancer (Fig. 5B). To determine the functional activity of this oligonucleotide, it was inserted as a trimmer 5′ to the thymidine kinase/human growth hormone fusion gene, pTKGH. After transfection into HepG2 cells a hypoxically inducible response was observed, the reporter gene expression being induced 3.2 ± 0.3-fold (n = 4) by hypoxia.

Responses to Hypoxia Are Unaffected by PMA—The ability of phorbol esters such as PMA to abrogate the induction of erythropoietin expression by hypoxia has suggested that these compounds interact with the mechanism of oxygen sensing and signal transduction underlying erythropoietin regulation. Since PMA is known to activate Glut-1 expression, we tested whether the 5′ enhancer was induced by PMA by exposing cells to 100 nM PMA for 16 h immediately after transfection with Plasmid pG5E(1–610)α. An inducible response was observed, as shown in Table IV. Thus, the response of this enhancer to both PMA and hypoxia provided an opportunity to test interactions between these stimuli.

Plasmid pG5E(1–326)α responded to both PMA and hypoxia, and the combination produced an augmented response. Plasmid pG5E(228–610)α, in which the SRE and two AP-1 sites are deleted, showed a strikingly reduced response to PMA but retained the ability to respond to hypoxia. The response to PMA and hypoxia was similarly reduced, suggesting that it arose from an interaction between the HIF-1 complex and sequences lying 5′ to nucleotide 228. Though some responsiveness to PMA was retained, very similar results were obtained with the plasmid pG5E(ΔSRE E(1–610)α, indicating that the SRE contributed to the response to PMA. These results also suggested that PMA did not affect the hypoxically inducible response conveyed by the HIF-1 binding site itself.

**DISCUSSION**

One of the major metabolic consequences of hypoxia is compromise of mitochondrial metabolism, and limitation of mitochondrial electron transport is associated with many biochemical changes that could potentially mediate oxygen-dependent responses. Therefore, in considering mechanisms of oxygen sensing, an important characteristic is whether mitochondrial inhibitors have a similar action to hypoxia. In the case of erythropoietin regulation, this is not the case, indicating that the mechanism of oxygen sensing is not dependent on the consequences of hypoxic limitation of mitochondrial metabolism (6–8). However, activation by mitochondrial inhibitors is

### Table III

Induction of the Glut-1 5′ enhancer by multiple stimuli

| Stimulus | Induction |
|----------|-----------|
| Hypoxia | 6.5 ± 0.6 | n = 12 |
| Cobalt | 3.4 ± 0.7 | n = 9 |
| DFO | 5.9 ± 0.3 | n = 6 |
| Azide | 3.5 ± 0.6 | n = 8 |
| Rotenone | 3.6 ± 0.4 | n = 9 |

Fig. 2. Deletional analysis of the Glut-1 5′ enhancer. Hypoxically inducible cis-acting sequences within the Glut-1 5′ enhancer were localized using sequential deletions from the 5′ and 3′ ends of the Glut-1 5′ enhancer. Nucleotides within the enhancer contained in each deleted construct are given in the plasmid name. Activity of the sequence in normoxic cells is related to that of pG5E(1–610)α, which was arbitrarily designated 100. Induction was calculated as the ratio of hypoxic to normoxic expression. The bars show the mean value for at least three independent experiments ± S.E.
seen in a number of other systems that respond to hypoxia, suggesting that these responses represent the operation of different types of sensing mechanism (18, 31–33).

In this study, we have analyzed the transcriptional regulation of the glucose transporter Glut-1 as an example of such a response. In keeping with the induction of Glut-1 mRNA by hypoxia and a number of different mitochondrial inhibitors, we found that an enhancer element lying 5' to the Glut-1 gene could be activated by hypoxia, azide, and rotenone. When the structure of this enhancer was examined in detail, distinct cis-acting sequences were found to convey responses to hypoxia and the mitochondrial inhibitors. Thus, rather than reflecting the operation of a fundamentally different system, these properties reflect the integration of two different inducible responses, one of which is activated by mitochondrial inhibitors but not exposure to 1% oxygen, and the other of which resembles erythropoietin regulation in responding to hypoxia but not mitochondrial inhibitors. Further similarities of this hypoxically inducible response to erythropoietin regulation were demonstrated by induction of Glut-1 mRNA by both cobaltous ions and DFO, and by the definition of the critical cis-acting sequence for these responses as a HIF-1 binding site.

Similar evidence has been assembled to link the regulation of erythropoietin and genes encoding a number of glycolytic enzymes to this common oxygen-sensing mechanism (9–11). The current experiments extend these observations to a different, although functionally related, type of gene, and provide further
evidence for the importance of this oxygen sensing mechanism, and HIF-1 or related species, in the control of cellular metabolism. Comparison of the critical cis-acting sequences at the HIF-1 binding site from these different genes raises several points. First, although sequence conservation at the HIF-1 binding site is clear, the Glut-1 site differs from the previously defined consensus (10), and the cross-competition with the erythropoietin enhancer was more complete for the constitutive species than HIF-1 itself. Affinity purification of HIF-1 using oligonucleotides derived from the human erythropoietin enhancer has recently enabled the cloning of cDNAs encoding two DNA binding subunits, HIF-1α and HIF-1β, both of which are members of the basic helix-loop-helix-PAS group of transcription factors, and form a heterodimer (34). It is possible that the complex binding at the Glut-1 site differs in one or more components of the heterodimer. Second, it is interesting that all of the functional HIF-1 binding sites defined to date appear to bind both inducible and constitutive species (9, 11, 15, 35), suggesting the possibility of a functional relevance for the constitutive species. Third, sequence conservation exists not only at the HIF-1 binding site but also at a precisely distanced adjacent site shown to be of functional relevance in other genes regulated by HIF-1 (Fig. 6), suggesting that the factors that bind at this adjacent site are important for the operation of HIF-1 (11, 35, 36).

Whereas activation of the Glut-1 5′ enhancer by rotenone and azide was unaffected by mutation or deletion of the HIF-1 binding site, the response to these agents was dependent on the presence of an intact SRE lying 102 nucleotides 5′ to this site. Moreover, oligonucleotides derived from the SRE in the c-fos promoter were sufficient to convey responses to these stimuli. Although the mechanisms of sensing and signal transduction underlying these responses were not analyzed in detail, these results indicate that induction by mitochondrial inhibitors involves the activation of factors that bind at this site.

Although the primary aim of this work was to analyze responses to hypoxia and mitochondrial inhibitors, activation of the Glut-1 5′ enhancer by both PMA and hypoxia provided an opportunity to study the action of PMA on the hypoxically inducible response. Following the observation that in hematoma cells PMA severely reduces hypoxically inducible erythropoietin production, it has been argued that some consequence of exposure to PMA (for instance either activation or depletion of protein kinase C) has a critical action on the mechanism of oxygen sensing (24–26). Surprisingly, however, other genes such as vascular endothelial growth factor, whose induction by hypoxia otherwise resembles that of erythropoietin, are also induced by PMA (12, 37). Our studies indicate that the increased activation of the Glut 5′ enhancer by PMA and hypoxia arose from a positive integration of effects on different cis-acting sequences, with the operation of the HIF-1 binding site being substantially unaffected by PMA. This is not a specific property of the Glut-1 HIF-1 site, since under conditions that

**TABLE IV**

|          | PM A | Hypoxia | PMA + hypoxia |
|----------|------|---------|---------------|
| p5′E(1–326)WT | 3.1 ± 0.6 | 6.5 ± 0.6 | 17.4 ± 1.8   |
| p5′E(228–610)WT | 1.4 ± 0.1 | 4.2 ± 0.7 | 6.5 ± 1.2   |
| p5′E(dsRE)WT   | 1.8 ± 0.1 | 3.9 ± 0.2 | 6.2 ± 0.2   |

![Fig. 6. Comparison of functionally defined HIF-1 sites from different genes that have been subject to detailed mutational analysis. Nucleotides that have been shown to be functionally critical by mutational analysis are underlined.](image)

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