Isoenzyme-specific regulation of genes involved in energy metabolism by hypoxia: similarities with the regulation of erythropoietin

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Recent studies have indicated that regulatory mechanisms underlying the oxygen-dependent expression of the haematopoietic growth factor erythropoietin are widely operative in non-erythropoietin-producing cells and are involved in the regulation of other genes. An important characteristic of this system is that the inducible response to hypoxia is mimicked by exposure to particular transition metals such as cobaltous ions, and by iron chelation. We have investigated the extent of operation of this system in the regulation of a range of genes concerned with energy metabolism. The effects of hypoxia (1 % oxygen), cobaltous ions and desferrioxamine on gene expression in tissue-culture cells was studied using RNase protection assays. Hypoxia induced the expression of glucose transporters in an isoform-specific manner; GLUT-1 and GLUT-3 were induced by hypoxia,

whereas expression of GLUT-2 was decreased. Isoenzyme-specific regulation by hypoxia was also observed for genes encoding phosphofructokinase, aldolase and lactate dehydrogenase. For all of these genes, responses to cobaltous ions and desferrioxamine correlated in both direction and magnitude with the response to hypoxia. In contrast, a reduction in mitochondrial transcripts was observed in hypoxia, but these changes were not mimicked by either cobaltous ions or desferrioxamine. These findings indicate that similarities with erythropoietin regulation extend to the oxygen-dependent regulation of genes encoding glucose transporters and glycolytic enzymes but not to the regulation of mitochondrial transcripts, and they show that in glucose metabolism regulation by this system is isoenzyme- or isoform-specific.

INTRODUCTION

Although the regulation of cellular energy metabolism in accordance with oxygen supply is mainly achieved by allosteric modifications of enzyme activity, it has long been recognized that changes in gene expression also take place [1–5]. Some insight into this process has recently been gained by the recognition that a specific mechanism of oxygen sensing and transcriptional control, defined in studies of the haematopoietic growth factor erythropoietin, also operates widely in mammalian cells [6–8] and is involved with the regulation of certain genes encoding glycolytic enzymes [9,10].

Erythropoietin can be induced by hypoxia, but not by mitochondrial inhibitors or the broad range of stimuli which induce heat shock and other cell stress responses [6,11,12]. Erythropoietin expression is also induced by particular transition metals, notably cobaltous ions, and the highly specific iron chelators, desferrioxamine (DFO) and the hydroxypyridinones [12–14]. This narrow and distinctive response pattern has led to the proposal of a specific oxygen-sensing mechanism, possibly involving a ferroprotein sensor [12,15].

Several lines of evidence have implicated the same or similar mechanisms of sensing and signal transduction in the regulation of other genes. Functional similarities in the response to agents such as cobaltous ions and iron chelators have been demonstrated for other oxygen-regulated genes [9,10,14,16,17]. For some of these genes, analysis of *cis*-acting sequences has demonstrated a common dependence of these responses on functionally critical

binding sites for an inducible transcription factor termed hypoxia-inducible factor-1 (HIF-1), which is itself inducible by cobaltous ions and iron chelators [18].

To gain further information about the extent to which this mechanism of control operates on genes involved in cellular metabolism, we have surveyed the responses of three groups of genes to hypoxia; glycolytic enzymes, glucose transporters and mitochondrial genes. Responses to hypoxia have been compared with responses to cobaltous ions and the iron-chelating agent DFO, both of which mimic hypoxic induction of erythropoietin.

Induction by hypoxia, cobalt and DFO was observed for glucose transporters and glycolytic enzymes; in each case the response was isoform- or isoenzyme-specific. In contrast, hypoxia reduced the level of particular mitochondrially encoded transcripts; however, these responses were not mimicked by cobalt or DFO, suggesting the involvement of a different regulatory mechanism.

MATERIALS AND METHODS

Cell culture

HT 1080, HepG2, HeLa and 293 cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, U.K.). HepG2, HeLa and 293 cells were grown in minimal essential medium with Earle's salts supplemented with 10 % (v/v) fetal-calf serum, glutamine (2 mM), penicillin (50 units/ml) and streptomycin sulphate (50 µg/ml). HT 1080 cells were grown in similar medium supplemented with non-essential amino acids.

Abbreviations used: ALD, aldolase; DFO, desferrioxamine; HIF-1, hypoxia-inducible factor-1; PFK, phosphofructokinase; LDH, lactate dehydrogenase; NRF-1, nuclear respiratory factor-1; HCS, human somatic cytochrome c; ND 1, NADH dehydrogenase subunit 1; ATPase 6, ATPase subunit 6; Cox III, cytochrome c oxidase subunit III; mtDNA, mitochondrial DNA.

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Table 1 Location and length of riboprobes

Riboprobes were generated for genes of interest by PCR amplification using primers based on the EMBL accession numbers listed below. When the genomic structure of the gene was known, the location of riboprobes within the gene is given. When only a cDNA sequence was available, the location is listed as cDNA. Locations of riboprobes for mitochondrial genes are given using the numbering system described by Anderson et al. [21].

Riboprobe	5' end	3' end	Protected length (nt)	GenBank/EMBL accession no.	Location
GLUT-1	1063	1198	136	K03195	cDNA
GLUT-2	682 (L09675)	408 (L09676)	195	L09675/76	Exon 2–exon 3
GLUT-3	2710	2830	121	M20681	cDNA
GLUT-4	3720	3847	85	M91463	Intron 2–exon 3
PFK-L	362	518	157	X15573	Exon 4–exon 5
PFK-M	251	363	113	M26066	Exon 5–exon 6
PFK-C	752	918	167	D25328	cDNA
ALD-A	609	738	130	M11560	cDNA
ALD-B	370	548	179	X01098	cDNA
ALD-C	1702	1868	167	X05196	Exon 3–intron 3
LDH-A	275	367	93	X02152	Exon 2–exon 3
LDH-B	225	348	123	Y00711	Exon 3–exon 4
NRF-1	803	955	153	L22454	cDNA
HCS	1447	1667	172	M22877	Intron 2–exon 3
16 S rRNA	2242	2301	60	[21]	
ND 1	3863	3952	90	[21]	
ATPase 6	8903	9019	117	[21]	
Cox III	9640	9795	156	[21]	

Fetal-calf serum and culture media were purchased from Gibco (Paisley, Scotland, U.K.). Subconfluent flasks of cells were exposed in parallel to normoxia (5% CO₂/95% air), hypoxia (1% O₂/5% CO₂/94% N₂) in a Napco 7301 incubator (Precision Scientific, Chicago, U.S.A.), cobalt (100 µM), or DFO (100 µM) for 16 h in fresh medium prior to harvesting using trypsin/EDTA. Cobalt chloride and desferrioxamine mesylate (Sigma, Poole, Dorset, U.K.) were dissolved in water before addition to the culture medium.

Riboprobes

Riboprobes to genes involved in glucose transport, glycolysis, and mitochondrial respiration were generated by PCR (Table 1). Sequences for genes of interest were obtained from the GenBank/EMBL accession numbers listed in Table 1, and primers were designed with 18 bp of complementary sequence. Riboprobes to genes for which the genomic structure is known were designed to cross exon–intron boundaries or exon–exon boundaries. The 5' ends of primers were engineered with restriction sites to facilitate cloning; forward primers contained an *Eco*RI site and 3' primers contained a *Bam*HI site. PCR products were cloned into the polylinker of pSP72 and confirmed by dideoxy sequencing. Plasmids were linearized with *Bgl*II, and ³²P-labelled riboprobes were transcribed using SP6 RNA polymerase.

RNA preparation and analysis

RNA was prepared using a modified acid/guanidinium thiocyanate/phenol/chloroform extraction method (RNAzol B; Cinna/Biotech Laboratories, TX, U.S.A.) and dissolved in hybridization buffer [80% formamide, 40 mM piperazine-*N,N'*-bis(2-ethanesulphonic acid), 400 mM NaCl and 1 mM EDTA, pH 8]. The quantity of RNA analysed varied from 1 µg for mitochondrially encoded genes to 100 µg for genes encoding the less highly expressed glycolytic isoenzymes. As an external standard to control for sample processing and gel loading, a small quantity of antisense RNA was generated by *in vitro*

transcription of a fragment of the rat endothelin-1 gene and added to the RNA sample along with a sense probe which protects 260 bp of the external standard. For RNase protection assays, RNA was hybridized overnight at 55 °C with 5 × 10⁵ c.p.m. of radiolabelled probe in a volume of 50 µl. RNase digestion was performed at 25 °C for 30 min in a volume of 400 µl, and was terminated by the addition of proteinase K (1 mg/ml)/3% SDS and a further 30 min incubation at 37 °C. Following phenol–chloroform and chloroform extractions, RNA was ethanol-precipitated, dissolved in 80% formamide, and electrophoresed on a denaturing 8% (w/v) polyacrylamide gel. Each gel also included a control lane in which the relevant probe was hybridized to tRNA alone.

Gels were quantified by aligning autoradiographs with the dried gel and excising gel portions corresponding to protected bands. The protected bands were counted using a flat-bed liquid-scintillation counter (model 1205 Betaplate; Pharmacia-Wallac OY, Turku, Finland). Each sample was counted for 3600 s to generate a raw count. Background counts were subtracted, and the test sample count was divided by the external control count in each lane. In each experiment, the test exposure was made with a parallel normoxic control sample. Induction was calculated as the ratio of mRNA expression in the test sample over the normoxic control sample.

Nuclear extract preparation and electrophoretic mobility shift assay

Nuclear extracts were prepared from HepG2 cells as described previously [9]. The sequence of the Epo WT oligonucleotides were 5'-GCCCTACGTGCTGCCTCGCATGGC-3' and a complementary 24-mer, and the sequence of the nuclear respiratory factor-1 (NRF-1) oligonucleotides were 5'-TGCTAGCCCGCA-TGCGCGCGCACCTT-3' and a complementary 26-mer [19]. Oligonucleotides were purified by PAGE, and complementary oligonucleotides were annealed for use as competitors. Probes were labelled with [γ -³²P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase and annealed to a 4-fold molar excess of the

complementary strand. 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 250 ng of poly(dI-dC) for the Epo WT probe or 400 ng of calf thymus DNA for the NRF-1 probe. After a 5 min incubation at room temperature, the probe (approximately 0.1 ng) and specific competitors were added, and the binding reaction was incubated for a further 10 min before being 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).

Mitochondrial DNA

To assay mitochondrial DNA content in 20 % and 1 % oxygen, HepG2 cells were harvested after 16 h exposure to normoxia or hypoxia. Mitochondrial DNA, relative to nuclear DNA, was measured as described previously [20]. Briefly, DNA was extracted, and samples (250–600 ng) were dot-blotted on a positively charged nylon membrane. Membranes were probed with a mitochondrial probe, mt 3, corresponding to base pairs 2578–4122 of the mitochondrial genome [21], labelled with α-³⁵S-labelled dCTP and a nuclear probe, nucleotides 252–1283 of the arginosuccinate synthetase gene, labelled with [α-³²P]dCTP. Filters were washed in 2 × SSC/0.1 % SDS at 61 °C for 20 min and at 63 °C for a further 20 min (SSC: 0.15 M NaCl/0.015 M sodium citrate). Each spot was counted for 3600 s on a flatbed scintillation counter (Betaplate Wallac OY, Turku, Finland).

RESULTS

Regulation by hypoxia of genes encoding different glycolytic isoenzymes

To characterize the pattern of regulation by hypoxia among genes encoding glycolytic enzymes, messenger RNA levels for each of the major isoenzymes of three glycolytic enzymes, phosphofructokinase (PFK), aldolase (ALD) and lactate dehydrogenase (LDH), were measured using RNase protection assays. The basal level of expression of these genes was first assayed under normoxic culture conditions (subconfluent cells in an atmosphere of 21 % O₂) in four cell lines; HT 1080 (fibrosarcoma), HeLa (cervical carcinoma), 293 (embryonal kidney) and HepG2 (hepatoma). Results are given in Table 2. With the

exception of aldolase B, there was detectable expression of all the genes, though the level of expression varied greatly. The pattern of isoenzyme expression was similar in the different cell lines irrespective of the tissue of origin.

Responses to hypoxia were measured in parallel cultures exposed to 21 % oxygen or 1 % oxygen for 16 h. Similar responses were seen in the four cell lines. More detailed studies of each gene were performed using HT 1080 and HepG2 cells. The results of these studies are summarized in Table 3. For each enzyme, at least one isoenzyme demonstrated up-regulation of its mRNA by hypoxia. However, comparison of hypoxic induction among different isoenzymes showed differential regulation, with the response to hypoxia being restricted to genes encoding particular isoenzymes. Thus for LDH, the LDH-A isoenzyme was up-regulated by hypoxia but the LDH-B isoenzyme was not (Figure 1). For PFK, both L and C isoenzymes were up-regulated by hypoxia, whereas PFK-M showed no up-regulation.

To characterize this pattern of response further, cells were exposed to cobaltous ions (100 µM) and the iron-chelating agent DFO (100 µM) for 16 h. In each case, induction of gene expression was similar using these stimuli to that observed following hypoxic exposure. Gene expression for LDH-A, PFK-L, PFK-C, aldolase A and aldolase C was induced by hypoxia, cobaltous ions and DFO; and for each gene there was a correlation between the level of induction by hypoxia and by the other two stimuli. In contrast, for LDH-B and PFK-M there was no response to any of the stimuli.

Regulation of genes encoding different glucose transporter isoforms

To determine the responses to hypoxia among a different group of genes involved in energy metabolism, RNA levels were assayed for four of the isoforms of the facilitative glucose transporter family, GLUT-1, GLUT-2, GLUT-3 and GLUT-4. GLUT-1 and GLUT-3 mRNA were expressed in each of the four cell lines, whereas GLUT-4 was not expressed in any of these cell types. GLUT-2 mRNA was only expressed in HepG2 cells. For determination of the inducible response to hypoxia, RNA was prepared from parallel cultures of HepG2 and HT 1080 cells exposed to 21 % (normoxia) and 1 % oxygen (hypoxia) for 16 h. The results are shown in Table 4. Hypoxic induction of both GLUT-1 and GLUT-3 mRNA was observed; induction being most marked for GLUT-3. In contrast, the level of GLUT-2 mRNA in HepG2 cells was reduced by approximately 3-fold during the hypoxic incubation. GLUT-4 mRNA remained below the limit of detection.

When cells were exposed to cobaltous ions and DFO, inducible responses to both stimuli were observed, which again closely resembled those obtained by hypoxic exposure. These stimuli induced the expression of both GLUT-1 and GLUT-3 to a similar level as hypoxia, but again reduced expression of GLUT-2 by approximately 3-fold.

Regulation of genes involved in mitochondrial metabolism

To pursue the possibility that similar hypoxically inducible responses might operate even more widely among genes involved in energy metabolism, we looked for evidence of the operation of this system on genes involved in mitochondrial respiration using HepG2 cells prepared under conditions identical to those in the studies of glycolytic and glucose transporter genes. We first examined the nuclear-encoded mitochondrial genes encoding NRF-1 and human somatic cytochrome *c* (HCS). NRF-1 has recently been characterized as a major regulator of nuclear genes encoding components of the mitochondrial electron transport

Table 2 Levels of expression of glycolytic isoenzymes

Expression of genes encoding isoforms of three glycolytic enzymes was measured by an RNase protection assay on at least two separate experiments. As a standard on each gel, 10 µg of HepG2 RNA was probed with a riboprobe to LDH-A. The level of expression of each gene in four tissue-culture cell lines is expressed relative to the expression of LDH-A in HepG2 cells which is arbitrarily set to a value of 100. A representative set of experiments is shown. Abbreviation: ND, not detected.

Enzyme	Expression of gene (%)			
	HT 1080	HeLa	293	HepG2
PFK-L	15	35	26	20
PFK-M	11	15	15	9
PFK-C	48	33	31	1
ALD-A	339	122	189	320
ALD-B	ND	ND	ND	ND
ALD-C	11	13	13	17
LDH-A	61	300	63	100
LDH-B	7	9	7	6

Table 3 Regulation of glycolytic isoenzymes by hypoxia, cobalt and DFO in HT 1080 cells

For each experiment, regulation was calculated as the ratio of mRNA in the test sample relative to mRNA in a parallel control sample. Values are given as the mean $\pm$ S.E.M. of n independent experiments. Hypoxic regulation was observed for PFK-L, PFK-C, ALD-A, ALD-C and LDH-A, but not for PFK-C or LDH-B. For each gene, response to cobalt and DFO mimicked the effect of hypoxic stimulation. A two-tailed paired Student's t -test was performed on corrected counts for each pair of test and control samples, and significant P values are given ($*P < 0.05$). Induction of mRNA by hypoxia and by cobalt was correlated with a Spearman's rank correlation coefficient of 0.86 ($P < 0.05$) and by hypoxia and DFO with a coefficient of 0.96 ($P < 0.05$).

Gene	Regulation (mRNA in test sample/mRNA in control)		
	Hypoxia	Cobalt	DFO
PFK-L	$2.1 \pm 0.19^*$ ($n = 6$)	$1.6 \pm 0.15^*$ ($n = 5$)	$2.3 \pm 0.21^*$ ($n = 5$)
PFK-M	1.0 ± 0.13 ($n = 8$)	1.1 ± 0.19 ($n = 5$)	1.2 ± 0.20 ($n = 5$)
PFK-C	$1.5 \pm 0.10^*$ ($n = 6$)	$1.5 \pm 0.06^*$ ($n = 6$)	$1.6 \pm 0.09^*$ ($n = 6$)
ALD-A	$1.7 \pm 0.31^*$ ($n = 4$)	$2.1 \pm 0.16^*$ ($n = 4$)	$2.4 \pm 0.28^*$ ($n = 4$)
ALD-C	$4.6 \pm 0.31^*$ ($n = 4$)	$5.7 \pm 0.78^*$ ($n = 4$)	$9.3 \pm 1.9^*$ ($n = 4$)
LDH-A	$3.8 \pm 0.35^*$ ($n = 7$)	$2.0 \pm 0.23^*$ ($n = 5$)	$3.2 \pm 0.55^*$ ($n = 5$)
LDH-B	0.94 ± 0.05 ($n = 6$)	1.2 ± 0.26 ($n = 4$)	1.0 ± 0.18 ($n = 4$)

Figure 1 RNase protection assay demonstrating differential regulation of LDH-A and LDH-B mRNA

RNA was prepared from HT 1080 cells cultured for 16 h in normoxia (N), hypoxia (H), 100 μ M cobalt (Co), or 100 μ M desferrioxamine (DFO). Expression of LDH-A was induced by hypoxia, cobalt and DFO, but LDH-B was not affected by any of the stimuli. The lane labelled control shows the external standard provided by the addition of a small quantity of antisense RNA generated by *in vitro* transcription of the rat endothelin-1 gene along with a sense probe which protects 260 bp of the external standard.

chain [19,22]. HCS is subject to regulation by NRF-1, and was chosen as an example of a nuclear-encoded mitochondrial enzyme [19]. Expression of NRF-1 mRNA was assayed by RNase protection assay in normoxic (21 % oxygen) and hypoxic (1 % oxygen) cultures of HepG2 cells, and in cells exposed to cobalt (100 μ M) and DFO (100 μ M). No change in the mRNA expression was observed under any of these conditions. Since regulation of NRF-1 activity might occur by post-translational mechanisms, we assayed NRF-1 DNA binding activity by electrophoretic mobility shift assay. Figure 2 shows that the binding activity was similar in nuclear extracts prepared from HepG2 cells exposed to 21 % oxygen or 1 % oxygen for 12 h. In keeping with these results, expression of HCS mRNA was unchanged by hypoxia or the other stimuli.

Since other mitochondrial regulatory mechanisms might be subject to control by hypoxia, we assayed for hypoxically induced changes in mitochondrial DNA (mtDNA) and in the mitochondrially encoded transcripts for mitochondrial 16 S rRNA, ATPase subunit 6 (ATPase 6), NADH dehydrogenase subunit 1 (ND 1), and cytochrome *c* oxidase subunit III (Cox III). No significant change in mtDNA was observed. In normoxia, mtDNA relative to nuclear DNA was 113 ± 3.8 ($n = 9$) in arbitrary units ($\pm$ S.E.M.); in parallel hypoxic samples, mtDNA relative to nuclear DNA was 106 ± 4.1 ($n = 9$). Exposure to hypoxic conditions did, however, produce a substantial decline in mitochondrial mRNA transcripts. After 16 h of hypoxia, Cox III, ND 1 and ATPase 6 mRNAs were reduced 2–3-fold, whereas the level of mitochondrial 16 S rRNA was unchanged (Table 5).

Table 4 Regulation of glucose transporters in response to hypoxia, cobalt and DFO

For each experiment, regulation was calculated as the ratio of mRNA in the test sample relative to mRNA in a parallel control sample. Values are given as the mean $\pm$ S.E.M. of n independent experiments. Hypoxia increased expression of GLUT-1 and GLUT-3, but decreased expression of GLUT-2. Cobalt and DFO regulated the isoforms in the same pattern.

Gene (cell type)	Regulation (mRNA in test sample/mRNA in control)		
	Hypoxia	Cobalt	DFO
Glut-1 (HT 1080)	3.7 ± 0.7 ($n = 6$)	1.9 ± 0.2 ($n = 5$)	3.0 ± 0.4 ($n = 5$)
Glut-1 (HepG2)	3.5 ± 0.6 ($n = 4$)	2.8 ± 0.9 ($n = 4$)	2.8 ± 0.7 ($n = 4$)
Glut-2 (HepG2)	0.41 ± 0.08 ($n = 5$)	0.46 ± 0.11 ($n = 4$)	0.60 ± 0.07 ($n = 4$)
Glut-3 (HepG2)	17.9 ± 2.8 ($n = 4$)	8.2 ± 1.4 ($n = 4$)	9.9 ± 1.7 ($n = 4$)

Figure 2 Effect of hypoxia, cobalt and DFO on nuclear-encoded genes involved in respiration

(A) NRF-1 expression was measured in RNA samples prepared from HepG2 cells exposed to normoxia, hypoxia, cobalt and DFO. Values are expressed as the ratio of mRNA in the test sample to mRNA in the control sample. NRF-1 was not affected by any of the stimuli. (B) To test whether NRF-1 binding activity is regulated by hypoxia, electrophoretic mobility shift assays were performed using an oligonucleotide probe based on the NRF-1-binding site and nuclear extracts prepared from normoxic and hypoxic HepG2 cells. Binding of a constitutive species to the Epo WT probe demonstrates that the normoxic and hypoxic extracts have equivalent concentrations of DNA-binding proteins, and binding of the hypoxically inducible species (HIF-1) demonstrates that there are hypoxically induced DNA-binding activities in the hypoxic nuclear extract. Binding to the NRF-1 probe, however, was equal in normoxic and hypoxic nuclear extracts. Competition with a 200-fold excess of unlabelled, double-stranded oligonucleotide was used to demonstrate specificity of binding activities. (C) Response to hypoxia was also measured for HCS mRNA, a nuclear gene which is regulated by NRF-1 and forms part of the mitochondrial respiratory chain. Neither hypoxia nor cobalt or DFO affected expression of HCS. n indicates the number of independent experiments.

After 48 h of hypoxia, Cox III mRNA levels were severely reduced, 16 S rRNA levels were not significantly changed, and induction of aldolase A mRNA remained elevated. The ratio of mRNA expression in hypoxia to normoxia was 0.1 for Cox III, 0.9 for 16 S rRNA and 2.6 for aldolase A. In contrast with the genes encoding enzymes involved in glucose metabolism, exposure of cells to cobaltous ions or DFO did not mimic this response.

DISCUSSION

Recent evidence for a common system of oxygen sensing underlying the regulation of genes encoding the haematopoietic growth factor, erythropoietin and particular glycolytic genes, has raised the question as to the extent of involvement of this system among genes concerned with energy metabolism [9,10]. In this report we have surveyed genes involved with glucose transport, glycolysis and mitochondrial function using the distinctive pattern of response to hypoxia, cobaltous ions and iron chelation to define genes which are responsive to this regulatory mechanism. We observed responses to these agents for several genes encoding glucose transporters and glycolytic enzymes, and showed that in each case responsiveness was isoform- or isoenzyme-specific. In contrast, we obtained no evidence for this pattern of response among genes concerned with mitochondrial biogenesis and function.

Although the precise mechanism by which cobaltous ions and DFO stimulate gene expression is not known, several lines of evidence indicate that their operation is closely connected with the mechanism of oxygen sensing or hypoxic signal transduction in this system. First, in studies of the transcriptional activation of a number of genes by hypoxia, cobalt and DFO, identical *cis*-acting sequences convey responses to the different agents [6,9,13]. Secondly, all three stimuli induce the expression and the DNA-binding activity of HIF-1, the hypoxically inducible transcription factor which is critical for the operation of these sequences [18]. Thirdly, when responses to these agents were compared between genes encoding erythropoietin and a group of hypoxically regulated angiogenic growth factors, a close correlation between responses to hypoxia, cobalt and DFO was observed [14].

In the present study, we found a similar correlation between responses to hypoxia and responses to cobalt and DFO among genes encoding glucose transporters and glycolytic enzymes. Genes such as GLUT-1 and GLUT-3 were induced by all three stimuli; genes such as LDH-B and PFK-M, which showed no response to hypoxia, were not induced by cobalt or DFO; the GLUT-2 gene, for which expression was reduced by hypoxia, was also repressed by cobalt and DFO. These findings strongly suggest that all the responses are dependent on the same or a closely similar mechanism of oxygen sensing, and they extend the groups of genes known to be regulated in this way to include those encoding glucose transporters. In support of this we have found that, for one of these genes (GLUT-1), hypoxic regulation is conveyed by a critical HIF-1-binding site in an enhancer lying 5' to the gene. Interestingly, induction of GLUT-1 by mitochondrial inhibitors [23], which has previously suggested a different mechanism from the cyanide-insensitive regulation of erythropoietin and HIF-1, was found to be dependent on a functionally distinct *cis*-acting sequence which was activated independently by mitochondrial inhibitors [24].

Reports of co-ordinate and reciprocal regulation by oxygen of genes encoding glycolytic and mitochondrial enzymes [4], and the recent isolation of genes such as NRF-1 [19,22] which are involved in the co-ordination of mitochondrial gene expression, led us to test whether the pattern of response to hypoxia, cobalt and DFO was also seen in the regulation of these genes. In keeping with previous reports [4,25,26], we found that the levels of the mitochondrially encoded transcripts for Cox III, ND 1 and ATPase 6 were reduced by hypoxia. However, we found no evidence for oxygen-dependent expression or DNA-binding activity of the regulatory gene NRF-1, nor did we see a similar pattern of response to cobalt and DFO among the mitochondrial genes whose expression was reduced by hypoxia. These findings suggest the operation of a different mechanism of oxygen-

Table 5 Regulation of mitochondrially encoded genes by hypoxia, cobalt and DFO in HepG2 cells

Induction was calculated as the ratio of mRNA in the test to that in control samples and is given as the mean $\pm$ S.E.M. of n independent experiments. In response to hypoxia, the expression of ALD-A is increased, the expression of mitochondrially encoded mRNAs (Cox III, ATPase 6, and ND 1) was decreased, and expression of mitochondrially encoded 16 S rRNA was not changed. For the glycolytic enzyme, ALD-A, the effects of cobalt and DFO mimic exposure to hypoxia. However, for the mitochondrial mRNAs, cobalt and DFO do not mimic the effects of hypoxia. The mRNA levels in the test conditions were compared with control levels using a two-tailed paired Student's *t*-test and significant *P* values ($*P < 0.05$) are given.

	Regulation (mRNA in test sample/mRNA in control)		
	Hypoxia	Cobalt	DFO
ALD-A	2.7 $\pm$ 0.41 ($n = 3$)	3.3 $\pm$ 1.0 ($n = 3$)	3.2 $\pm$ 0.86 ($n = 3$)
Cox III	0.33 $\pm$ 0.04* ($n = 4$)	0.94 $\pm$ 0.20 ($n = 4$)	0.91 $\pm$ 0.03 ($n = 4$)
ATPase 6	0.41 $\pm$ 0.01* ($n = 4$)	1.1 $\pm$ 0.13 ($n = 4$)	0.68 $\pm$ 0.18 ($n = 4$)
ND 1	0.53 $\pm$ 0.10* ($n = 4$)	1.2 $\pm$ 0.21 ($n = 4$)	0.81 $\pm$ 0.17 ($n = 4$)
16 S rRNA	1.1 $\pm$ 0.23 ($n = 4$)	1.3 $\pm$ 0.25 ($n = 4$)	1.1 $\pm$ 0.19 ($n = 4$)

dependent gene expression, which is not dependent on the action of HIF-1 or NRF-1.

For each of the glycolytic genes, regulation by hypoxia was found to be isoenzyme-specific. Isoenzyme expression is altered in transformed tissue-culture cell lines, and it is of interest that the isoenzymes showing up-regulation in malignant cells [27] such as PFK-L [28], aldolase A and C [29], LDH-A [30], and GLUT-1 and GLUT-3 [31] are also inducible by hypoxia; though, from comparison of Tables 2 and 3, it is clear that no simple relationship exists between hypoxic inducibility and the total level of expression.

In previous work, we have shown that the regulation of LDH-A by oxygen was dependent on an HIF-1-binding site in the promoter [32]. The operation of this regulatory system on the LDH-A gene, but not the LDH-B gene, therefore provides a likely explanation for shifts in LDH isoenzyme expression which have been reported following hypoxic stimulation in animal experiments [33] and clinical conditions such as myocardial ischaemia [34]. Isoform-specific regulation of glucose transporters is also consistent with the previously reported effects of hypoxia on glucose uptake, which include a decrease in the apparent K_m for glucose, as well as an increase in apparent V_{max} [23]. GLUT-3 shows the largest induction by hypoxia and has the lowest apparent K_m for glucose [35].

In summary, this study has provided further evidence for the involvement of a highly conserved mechanism of oxygen-regulated gene expression among genes concerned with glucose metabolism, but no evidence for its involvement in the regulation of genes concerned with mitochondrial respiration. Activation of this system by hypoxia induces isoenzyme- and isoform-specific changes in gene expression with the potential to enhance glycolytic capacity in hypoxia.

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