HIGHLIGHTED TOPIC | Oxygen Sensing in Health and Disease

Functional genomics approach to hypoxia signaling

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Seta, Karen A., and David E. Millhorn. Functional genomics approach to hypoxia signaling. J Appl Physiol 96: 765–773, 2004; 10.1152/japplphysiol.00836.2003.—Mammalian cells require a constant supply of oxygen to maintain energy balance, and sustained hypoxia can result in cell death. It is therefore not surprising that sophisticated adaptive mechanisms have evolved that enhance cell survival during hypoxia. Although the nature of the adaptive mechanisms remains unknown, reduced metabolic activity and conservation of ATP are often involved (33). However, reduced activity is not a viable option for oxygen-sensing cells (e.g., carotid body type I cells) whose role in maintaining oxygen homeostasis often requires increased activity during hypoxia. These cell types have evolved the ability to respond to reduced oxygen tension by altering protein and gene expression levels to increase oxygen delivery to hypoxic tissues and organs. To carry out these functions, oxygen-sensing cells must alter the expression patterns of potentially hundreds of genes and proteins. As a result, oxygen-sensing cells have evolved the remarkable ability to tolerate hypoxia. During the past 10 or so years, there have been a growing number of reports on hypoxia-induced transcription of specific genes that mediate such cellular functions as erythropoiesis, pulmonary ventilation and blood flow, angiogenesis, and energy metabolism. In this review, we describe a unique experimental approach that utilizes focused cDNA libraries coupled to microarray analyses to identify hypoxia-responsive signal transduction pathways and genes that confer the hypoxia-tolerant phenotype.

CELLULAR RESPONSE TO HYPOXIA: A BRIEF OVERVIEW

Exposure of pheochromocytoma (PC12) cells to reduced O2 leads to membrane depolarization and an increase in intracellular free Ca2+ (87). This membrane depolarization is mediated by the O2-sensitive potassium channel Kv1.2 (12). This depolarization and Ca2+ increase is not seen in nonexcitable, O2-sensing cell types such as HepG2 and Hep3B cells. Several critical signaling pathways regulate gene expression during hypoxia. These include the cAMP-protein kinase A (PKA) (3), Ca2+-calmodulin (3), p42/44 mitogen-activated protein kinase (MAPK) (15), stress-activated protein kinase (SAPK; p38 kinase) (16), and phosphatidylinositol 3-kinase/Akt (6) pathways.

The hypoxia-inducible factor (HIF) family of transcription factors plays a critical role in mediating the hypoxic regulation of several genes (63, 64). The functional unit of these transcription factors consists of α/β-heterodimers. HIF-1β is identical to the aryl hydrocarbon nuclear translocator and is necessary for transactivation of target genes by the α-subunits. The HIF α-subunits are basic helix-loop-helix PAS domain proteins. Both prokaryotes and eukaryotes express PAS do-
main proteins. To date, three mammalian HIF-α subunits have
been identified: HIF-1α, HIF-2α or EPAS, and HIF-3α. HIF-1α is expressed in a wide variety of cells and tissues, whereas HIF-2α and HIF-3α have a more limited distribution.

HIF-2α is the predominant HIF found in PC12 cells.

The levels of all three HIF-α subunits are rapidly increased by hypoxia as a result of protein stabilization due to the inhibition of ubiquitin-mediated proteasomal degradation (29, 34, 76). Once activated by reduced O₂, HIF-α dimerizes with HIF-1β in the nucleus and binds to the hypoxia response element (HRE), an enhancer element with the consensus sequence 5′-RCGRTG-3′, located within the regulatory region of many hypoxia-responsive genes (65, 67). The binding of the HIF-α/β complex during hypoxia regulates genes involved in a wide variety of physiological process, including angiogenesis, proliferation, cell survival and death, erythropoiesis, energy metabolism, and oxygen chemoreception. HIF-3α lacks one of the two transactivation domains found in the COOH terminus of the other HIF-α proteins and might be a negative regulator of hypoxia-inducible gene expression, possibly by competing for limited quantities of HIF-1β (31).

The roles of the various HIF proteins in the hypoxic response are unclear. In mouse embryo fibroblasts, only HIF-1α undergoes oxygen-dependent protein degradation. HIF-2α is detected under all oxygenation conditions and is localized to the cytoplasm. Also, endogenous HIF-2α is transcriptionally inactive in hypoxia, but overexpressed HIF-2α could translocate to the nucleus and stimulate expression of hypoxia-inducible genes (54). Loss of HIF-1α, but not HIF-2α, protects embryonic stem cells from hypoxia-induced apoptosis. However, loss of either HIF-1α or HIF-2α protects these cells from hypoglycemia-induced apoptosis (9). HIF-2α has been reported to play a critical role in hematopoiesis in bone marrow (62).

Although HIF proteins mediate much of the transcriptional response to hypoxia, a variety of other factors are also involved. Hypoxia also increases levels of the p53 tumor suppressor protein (27). Agents that inhibit the accumulation of p53 by DNA-damaging agents do not prevent p53 accumulation by hypoxia, indicating that different stresses induce p53 by different mechanisms (27, 58). Although the majority of p53 is nuclear and cytoplasmic, hypoxia causes p53 accumulation in the mitochondria (60). Mitochondrial function appears to be critical for hypoxia-induced p53 accumulation (11).

NF-κβ is member of the Rel-related proteins (p50, p52, v-Rel, c-Rel, RelA/p65, and RelB; reviewed in Ref. 59). In the cytoplasm, inactive NF-κβ dimers interact with IκB. On stimulation with cytokines or stress agents, IκB becomes phosphorylated and dissociates from NF-κβ, exposing a nuclear translocation signal on NF-κβ. The p50 and p52 proteins are constitutively nuclear and lack the IκB binding site and transactivation domain. Homodimers of p50 and p52 may act as transcriptional repressors. Cytokine stimulation of NF-κβ causes serine phosphorylation and subsequent degradation of IκB (22). In contrast, stimulation of NF-κβ by hypoxia is via tyrosine phosphorylation of IκB (43) and does not always involve IκB degradation (35). Tyrosine phosphorylation of IκB prevents its phosphorylation on serine (35, 75).

Activator protein-1 (AP-1) is a transcription factor that consists of dimers of jun/jun (c-jun, junB, junD) or jun/fos [s-fos, fosB, fra-1, fra-2, ATF-2, cAMP response element binding protein (CREB)] subunits (reviewed in Refs. 47 and 81). AP-1 is induced by a variety of environmental stresses, including hypoxia. Regulation of the various subunits may be cell specific. For example, in HepG2 cells, c-Jun protein accumulates in response to hypoxia (50). We have not seen activation of the JNK pathway or c-Jun phosphorylation in PC12 cells (16), although others have reported a modest induction of c-Jun in response to hypoxia in this cell line (55). Other transcription factors regulated by hypoxia include CREB (4), GATA-1 (88) and GATA-2 (77), and forkhead protein (25, 78).

The transcription factors regulated by hypoxia work in concert to fine tune the hypoxic response. Many hypoxia-inducible genes contain promoter and enhancer elements for multiple transcription factors in their regulatory regions. For example, the tyrosine hydroxylase (TH) promoter contains AP-1, AP-2, cAMP response element, and multiple HRE sites (18, 38, 53), and all are involved in the hypoxic regulation of this gene (38, 48, 49, 51, 53). Hypoxic induction of endothelin-1 in vascular endothelial cells requires its HRE as well as AP-1, GATA-2, and nuclear factor-1 binding sites (84). IL-8 gene expression by hypoxia in human ovarian cancer cells requires cooperation of AP-1 and NF-κβ binding sites, as mutation of either abolishes induction of an IL-8 reporter construct (73, 83). In addition, these transcription factors regulate the expression and activity of each other. HIF-1α and p53 interact in vivo (1). p53 promotes murine double minute2-mediated ubiquitination and proteasomal degradation of the HIF-1α subunit of HIF-1 (57), whereas HIF-1α promotes stabilization of p53 (51). HIF-1α also interacts with Jab1 (Jun activation domain-binding protein-1), a coactivator of AP-1, which promotes HIF-1α stability at least in part by interfering with the binding of HIF-1α to p53 (2). In addition, NF-κβ binds to the p53 promoter and regulates p53 expression (82). It is obvious that a thorough understanding of how cells respond to hypoxia will require an understanding of how the many signaling pathways and transcription factors involved in the hypoxic response are coordinately regulated.

A MODEL SYSTEM

Gaining a deeper knowledge of how oxygen-sensing cells transduce reduced O₂ tension into signals that alter gene expression has proved difficult due to the low abundance of these cells in intact tissues. To address this problem, clonal cell lines that respond to hypoxia in a fashion similar to oxygen-sensing cells in vivo have been established as model systems. One such cell line is the PC12 cell line. Oxygen-sensing capabilities have been documented in both rat (13, 87) and mouse (23) pheochromocytoma cell lines. There is now a considerable body of evidence that PC12 cells are an attractive model for carotid body type 1 cells (reviewed in Ref. 8). PC12 cells closely resemble type I cells morphologically and phenotypically (28). Both carotid body type I cells and PC12 cells depolarize rapidly (within seconds) during hypoxia via inhibition of an O₂-sensitive outward K⁺ current (13, 19, 46, 86). This depolarization is followed by an increase in intracellular Ca²⁺ (45, 80). Both cell types synthesize and release dopamine in response to acute hypoxia (17, 18, 24, 26, 72). Carotid body type I cells and PC12 cells have similar longer-term responses to hypoxia (within hours), including induction of TH (17, 18) c-fos and junB (53) mRNAs. This cell line is a useful system in which to study the
molecular and cellular basis of O₂ chemosensitivity and the mechanisms by which O₂-responsive genes are regulated by hypoxia.

Using this model system, our laboratory has identified a number of genes that are regulated by hypoxia (reviewed in Refs. 14 and 70). The adenosine A₂A receptor is upregulated by hypoxia (39, 42). In contrast, the N-methyl-D-aspartate receptor (41), PKA, and Ca²⁺/calmodulin-dependent protein kinase II (41) are downregulated. The transcription factors CREB (4, 5) and HIF-2α (also known as EPAS1) (15) are activated. Our laboratory has also implicated several signal transduction pathways in hypoxia-induced gene regulation in PC12 cells. The p42/p44 MAPK and p38β/α/SAPK pathways are activated by hypoxia (16). HIF-2α trans-activation of a transfected HRE-reporter construct depends on MAPK activation but is independent of Ras and requires Ca²⁺/calmodulin (15). In contrast, regulation of MAPK phosphatase-1 (MKP-1), which dephosphorylates MAPKs and SAPKs, is Ca²⁺ independent and is partially mediated by p38 SAPK but not by MAPK (69). The increase in adenosine A₂A receptor levels requires Ca²⁺ via protein kinase C rather than through calmodulin (42).

Table 1. Frequency of hypoxia-induced genes isolated in the SSH library

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Ref.</th>
<th>No. of Copies</th>
<th>No. of Fragments</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>JunB</td>
<td>53, 55</td>
<td>14</td>
<td>2</td>
<td>5.2%</td>
</tr>
<tr>
<td>TH</td>
<td>18</td>
<td>5</td>
<td>7</td>
<td>1.9%</td>
</tr>
<tr>
<td>VEGF</td>
<td>74</td>
<td>3</td>
<td>4</td>
<td>1.1%</td>
</tr>
<tr>
<td>Hexokinase II</td>
<td>52</td>
<td>2</td>
<td>1</td>
<td>0.7%</td>
</tr>
<tr>
<td>Bnip3</td>
<td>75a</td>
<td>2</td>
<td>2</td>
<td>0.7%</td>
</tr>
<tr>
<td>A₂ adenosine receptor</td>
<td>39</td>
<td>1</td>
<td>1</td>
<td>0.4%</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>66</td>
<td>1</td>
<td>1</td>
<td>0.4%</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor</td>
<td>24a</td>
<td>1</td>
<td>3</td>
<td>0.4%</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>66</td>
<td>1</td>
<td>3</td>
<td>0.4%</td>
</tr>
</tbody>
</table>

TH, tyrosine hydroxylase; SSH, subtractive suppression hybridization.

Fig. 1. Overview of the signal transduction pathways activated by hypoxia. ATF-2, activating transcription factor-2; CREB, cAMP response element binding protein; EPAS-1, endothelial PAS protein-1; FKHR, forkhead transcription factor; GRB2, growth-factor-receptor-bound protein 2; GSK3, glycogen synthase kinase-3; HIF, hypoxia-inducible factor; LRG, leucine-rich glycoprotein; MEK, MAPK kinase; MEKK, MAPK kinase kinase; MKP-1, MAPK phosphatase-1; PKA, 3-phosphoinositide-dependent protein kinase 1; PI3-kinase, phosphatidylinositol 3-kinase; PtdIns(3,4)P₂, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P₃, phosphatidylinositol 3,4,5-trisphosphate; Pyk2, proline-rich tyrosine kinase 2; RLPK, RSK-like protein kinase; RSK, ribosomal S6 kinase; SAPK, stress-activated protein kinase; SRF, serum response factor.

Fig. 2. Quality control standards (std) for subtractive suppression hybridization (SSH) microarrays. Plant gene mRNAs (SpotReport 1–10) were spiked into total RNA samples before they were labeled for microarray analysis. SpotReport 1–3 were added in equal amounts to the Cy3 and Cy5 reactions to produce spots with a 1:1 intensity ratio (solid line). SpotReport 6 and 7 were spiked in at 2-fold greater concentrations in the Cy5 and Cy3 reactions, respectively (dashed line). SpotReport 5 and 8 were spiked into the Cy5 and Cy3 reactions with a 5-fold difference (dotted line). SpotReport 4 and 9 were spiked to show a 10-fold difference (dotted-dashed line). SpotReport 10 was added only to the Cy3 reaction. Background-subtracted median pixel intensities are plotted. Each point on the scatter plot represents one spot on the array.
Interestingly, adenosine attenuates the hypoxia-induced increase in intracellular Ca\(^{2+}\) through a mechanism that requires PKA, and membrane excitability is thought to be maintained by a compensatory decrease in PKA activity (39, 40). The Akt/phosphatidylinositol 3-kinase pathway, which is thought to be critical for cell survival, is also activated (6). The transcription factor CREB is activated through a novel, as-yet unidentified, signaling mechanism (4, 5). It is clear that these pathways are not acting independently; rather, there is a great deal of cross-talk between them (see Fig. 1).

FOCUSED CLONE SETS SIMPLIFY MICROARRAY ANALYSIS

Although traditional molecular techniques have yielded a great deal of information about the biology of oxygen sensing, the study of hypoxia-regulated genes individually by traditional methods does not provide a comprehensive picture of hypoxia-regulated gene expression. To gain an understanding of the many genes involved in the hypoxic response, a more global approach was needed. The development of high-throughput genomic technologies such as microarrays has dramatically changed the way we can evaluate regulation of genes.

**Fig. 3. Comparison between SSH microarrays and database-derived microarrays.**

A: PC12 cells were exposed to normoxia (21% O\(_2\)) or hypoxia (1% O\(_2\)) for 6 h. Total RNA was isolated, and 10 \(\mu\)g were converted to \(^{32}\)P-labeled cDNA in a standard reverse transcriptase reaction. The radiolabeled cDNA was hybridized to rat cDNA filter arrays (GeneFilter 2 array, Research Genetics, Carlsbad, CA) that contained 5,299 rat sequences. The hybridized arrays were washed and exposed to a phosphorimager screen and scanned with a Storm XXX system (Molecular Dynamics). B: for each spot on the array in A, the ratio of the signal intensities of the hypoxia sample to the normoxia sample was calculated. The graph depicts the number of genes that produced a given ratio. C: PC12 cells were exposed to normoxia (21% O\(_2\)) or hypoxia (1% O\(_2\)) for 6 h. Total RNA was isolated and converted to Cy3- or Cy5-labeled cDNA as indicated. Labeled samples were mixed and hybridized to glass arrays onto which PCR products from the SSH library had been spotted. The arrays were washed and scanned at the appropriate wavelength for each dye (532 nm for Cy3 and 635 nm for Cy5) with an Axon GenePix 4000A scanner (Axon Instruments, Union City, CA). The data are plotted as the background-subtracted median pixel intensities for each spot on the array. Note that the hypoxia-regulated genes junB, tyrosine hydroxylase (TH), and VEGF center around the line of unity in the normoxia vs. normoxia hybridization, whereas they are strongly upregulated in the hypoxia vs. normoxia hybridization. D: median Cy3-to-Cy5 ratio was calculated for each gene on the array. The number of genes that produced a given ratio are depicted.
gene expression. Use of microarrays greatly accelerates the rate of discovery of genes involved in the response to a stimulus, while also providing information about global patterns of gene regulation.

Database-derived arrays (e.g., Affymetrix and many commercially available cDNA arrays) offer the advantage of containing typically thousands to tens of thousands of genes. Despite their large size, however, there is no guarantee that the genes involved in the process under study will be included on these arrays, especially if one is working with an organism other than yeast, human, mouse, or rat (this disadvantage will disappear as the genomes of more organisms are completed). In addition, only a handful of genes are differentially expressed in response to a stimulus; therefore, in a database-derived clone set, which is essentially randomly selected, the false-positive rate is high. Other methodologies for genome-wide analysis include differential display and serial analysis of gene expression (SAGE). Differential display allows direct side-by-side comparisons of most of the mRNAs between or among two or more samples by amplifying expressed sequences in a systematic and sequence-specific manner using multiple primer-pair combinations (44). In SAGE, short sequence tags from all expressed mRNAs are generated and ligated together into long molecules that are cloned into vectors and sequenced (79). The expression of a given gene is proportional to the number of times its sequence is represented. These methods offer many advantages, chief among them being relatively low cost and no requirement for specialized equipment.

We have used the subtractive suppression hybridization (SSH; PCR-Select kit, Clontech, Palo Alto, CA) method to create a cDNA library enriched in hypoxia-regulated genes and have used this library to create microarrays that allow us to examine hundreds of genes at a time (7, 68). SSH libraries provide a powerful biological approach for obtaining clones that are differentially expressed between two populations. The libraries consist of a subset of genes that are involved in mediating the phenotype of interest. The protocol combines high-subtraction efficiency with an equalized representation of abundant and rare mRNAs (20, 21, 30). The method can be used with any two populations whose gene expression profiles differ (e.g., treated vs. untreated, different developmental states, different tissue types). In addition, our library, which is enriched for genes upregulated by 6 h of hypoxia (1% O_2) in PC12 cells, contains about 250 known genes and ~60 unidentified genes and expressed sequence tags. Among the identified genes are many genes that have previously been associated with hypoxic regulation, including TH (17), VEGF (74), and junB (53, 55) (Table 1). The frequency of these genes in the library corresponds well with their induction by hypoxia. In addition to the prototypical hypoxia-regulated genes TH, junB, and VEGF, the SSH library contains genes involved in glucose metabolism, apoptosis, and neurotransmission. One adaptation to hypoxia involves generating ATP via the glycolytic pathway instead of oxidative phosphorylation. Several enzymes in the glycolytic pathway, such as pyruvate kinase (52), phosphoglycerate kinase (52), and hexokinase II (66), have HIF-1 binding sites and are regulated by hypoxia. One of the apoptosis-related proteins identified by microarray analyses was Bnip-3, a recently described proapoptotic member of the BH3-only Bcl-2 protein family (see Ref. 85 for review). These few

![Graph A](image1.png)

![Graph B](image2.png)

Fig. 4. Validation of gene regulation by real-time PCR. PC12 cells were exposed to normoxia (dashed lines) or hypoxia (1% O_2, solid lines) for 6 h. Total RNA was isolated, and quantitative real-time PCR (SYBR Green I dye intercalation method) was performed as described in the text. Growth curves are plotted for TH (A) and GAPDH (B). The threshold cycles (C_t) were calculated by the second-derivative method and represent the point at which the growth curves enter log-linear phase. In this experiment, GAPDH is unregulated, whereas TH is strongly induced. Insets: melt curves, which indicate the formation of single products.

Table 2. Validation of genes isolated in the SSH library

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Microarray*</th>
<th>PCR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>JunB</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TH</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VEGF</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hexokinase II</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bnip3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A_s adenosine receptor</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>1.67-fold</td>
<td>+</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>1.99-fold</td>
<td>NS</td>
</tr>
</tbody>
</table>

For microarray, + indicates induction of >2-fold (average of minimum of 2 experiments). For PCR, + indicates statistically greater levels in hypoxia samples than in normoxia samples (P < 0.001 by t-test; n = at least 4). NS = not significant.
examples touch only briefly on the many phenotypic alterations that occur in response to hypoxia.

Microarrays have been produced by amplifying the clone inserts from the SSH library and spotting the PCR products onto glass slides (32, 61, 71). All of the widely used microarray normalization methods (56) assume that the average expression ratio for all the genes on the array is equal to one. This is not the case for microarrays made from SSH libraries because the gene content is biased. To avoid normalization and scanner artifacts, we have included 10 exogenous plant genes (SpotReport 1–10, Stratagene, La Jolla, CA) scattered throughout the body of the array. SpotReport 1, 2, and 3 are also spotted at the top of the array, and their mRNAs are added in equal amounts (5 ng, 0.5 ng, and 0.05 ng per 100 μg of total RNA, respectively) to each sample labeling reaction to provide a known set of genes that should have a ratio of 1:1. After the slide has been scanned, the SpotReport 1–3 spots at the top of the array are quickly analyzed. The scanner settings are adjusted, and the slide is rescanned until the ratios for these spots are as close as possible to 1:1 (we generally accept ratios of 0.95–1.05). The SpotReport 1–3 spots in the body of the array are used to demonstrate that there is no positional bias on the arrays. The other seven mRNAs are spiked into the two labeling reactions at different known ratios. When the background-subtracted fluorescence values for each wavelength are plotted, these spots fall along their predicted lines (Fig. 2). This system provides a good control for all the technical aspects of microarray analysis (labeling, hybridization, and scanning).

By beginning with a phenotype instead of a database for gene selection, this hybrid approach allows one to specifically study genes of interest while reducing much of the biological noise associated with microarray analysis. Comparison of array results showed that the percentage of genes upregulated at least twofold by hypoxia is an order of magnitude higher with our focused array (49%) than with a commercial filter-based array (4.6%) (Fig. 3). When the absolute number of genes under consideration is reduced and one can bias the clone set in favor of genes related to a phenotype, biological noise is minimized and data analysis is greatly simplified. Reproducibility is also high, with >40% of genes showing twofold or greater upregulation in at least two of three replicate experiments (not shown).

Fig. 5. Schematic of modified loop design for microarray analysis of signal transduction pathways. Thick solid arrows indicate hybridizations that would be performed in a Kerr and Churchill loop design. The arrowhead represents one dye (either Cy3 or Cy5), and the ball at the other end indicates the other dye. Thin solid arrows represent corresponding dye-flip hybridizations, in which the Cy3 and Cy5 dyes are reversed. Dashed arrows indicate additional hybridizations recommended as part of the modified loop design.

Fig. 6. Microarray analysis of the dependence of hypoxic gene regulation on extracellular Ca2+. PC12 cells were incubated for 6 h under normoxic (21% O2; A) or hypoxic (1% O2; B) conditions, in the presence (C) or absence (D) of extracellular Ca2+ as indicated. Total RNA was isolated and converted to Cy3- or Cy5-labeled cDNA as indicated. Labeled samples were mixed and hybridized to glass arrays onto which PCR products from the SSH library had been spotted, as described in Fig. 3. Data are plotted as the background-subtracted median pixel intensities for each spot on the array.
VALIDATION OF RESULTS

Microarray analysis requires validation by a second methodology. Northern blots (traditional and virtual), SAGE, differential display, and quantitative end-point RT-PCR have all been widely used to validate microarray results. We have used Northern and Western blot analyses to confirm the hypoxic regulation of MKP-1 (69). In addition, the relatively new technique of quantitative real-time RT-PCR is a valuable tool for large-scale analysis of microarray results (reviewed in Ref. 10). The SYBR Green I dye intercalation method is particularly well-suited for this purpose because it does not require special primers and is therefore far less time consuming and expensive than other real-time PCR methods. Primers are designed to produce a short amplicon (~80–100 bp), and the amount of product is measured in each cycle by measuring the fluorescence emitted by dye bound to the double-stranded DNA. A threshold cycle \( (C_t) \) is generated by measuring either the cycle in which the growth curve crosses a predetermined threshold or the cycle in which the growth curve enters the log linear phase. The \( C_t \) is related to the relative abundance of a particular gene in the original RNA sample. A lower \( C_t \) in one sample relative to another indicates a greater abundance of the gene in that sample. Calibration curves can be generated to relate a change in \( C_t \) value to a change in gene expression.

Although absolute quantification is rarely necessary, it is also possible by generating standard curves consisting of samples containing known amounts of mRNA for the gene of interest. Specificity is determined by analysis of the melting point of the PCR product. It is also desirable to verify the sequence of the PCR product. Figure 4 shows typical real-time PCR growth curves for a gene that is upregulated by hypoxia in PC12 cells (TH) and a gene that is not regulated (GAPDH). Preliminary analysis of 55 genes isolated in our SSH library indicates a 75% agreement between real-time PCR and microarray results (Table 2).

ANALYSIS OF SIGNAL TRANSDUCTION PATHWAYS

The use of microarray analysis as a screening tool to identify interesting genes for further study is relatively straightforward. Analysis of gene expression patterns related to hypoxia-responsive signal transduction pathways is considerably more complicated because multiple treatments (e.g., a signal transduction pathway activator/inhibitor and hypoxia) are given to each sample. In the simplest scenario, four different conditions are possible (vehicle + normoxia, vehicle + hypoxia, drug + normoxia, and drug + hypoxia). In a single-gene analysis experiment, expression levels of the gene of interest would be measured in each sample and statistical comparison would be made by two-way ANOVA. In a two-color experiment in which PC12 cells were incubated in the presence or absence of extracellular \( Ca^{2+} \) under either normoxic or hypoxic (1% \( O_2 \)) conditions. The hybridizations in the basic loop design (solid arrows in Fig. 5) were performed. Removal of extracellular \( Ca^{2+} \) had a small effect on the resting (normoxic) gene expression levels (Fig. 6A) and a somewhat larger effect on hypoxic gene expression levels (Fig. 6B). In contrast, the presence or absence of extracellular \( Ca^{2+} \) has a dramatic effect on hypoxia-induced gene expression (Fig. 6, C and D).

Differential analyses such as these, using pharmacological and oligo-based inhibition (e.g., RNA interference) of specific signal transduction pathways, offer a powerful tool to examine hypoxia-responsive signal transduction pathways and their gene targets.

FUTURE DIRECTIONS: RELATING GENE EXPRESSION PATTERNS TO PHENOTYPES

The ultimate goal of gene expression analysis is to elucidate gene expression patterns associated with a particular phenotype. We have begun to try to relate gene expression patterns with specific phenotypic outcomes in response to hypoxia, namely, cell survival or death. The coupling of focused microarray analysis described above with high-throughput, dye-based assays for cell survival and apoptosis offers a rapid approach for discovering validated therapeutic targets for the treatment of cardiovascular disease, stroke, and tumors.

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