Transcriptional Regulation of Gene Expression During Hypoxic Conditions Using the HL-1 Cardiomyocyte Cell Line

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Abstract

Currently, heart disease is the leading cause of death in the United States. Often, this involves ischemic injury to heart tissue arising from coronary occlusions. As this is a very complex disease, a need has arisen for a representative model to study the adaptive mechanisms that lead to changes in this tissue. We have utilized a unique cardiac cell line (HL-1) that has retained the phenotypic characteristics of adult cardiomyocytes. We have carried out cDNA microarray analysis of these cells as a means to prove their use as a model. Of the 15, 264 clones profiled, 614 were statistically found to be differentially expressed. From this list, 163 have no gene associated with them, 141 were ESTs and 75 were identified as RIKEN clones. The remaining 235 clones belong to a variety of genes with very diverse functions ranging from metabolic to cell cycle regulation. Thus, the results of this analysis demonstrate a pattern of gene expression that is similar to heart muscle making the cells an ideal means to investigate the molecular mechanisms regulating gene expression during hypoxic conditions *in vitro*.
A well known oxygen regulator, hypoxia-inducible factor (HIF-1) is a well documented transcription factor that responds quickly to conditions of low oxygen. Although a number of genes have been associated with this transcription factor, the global impact of HIF-1 on gene expression has yet to be determined. To this end, we will use the HL-1 model coupled with chromatin immunoprecipitation on microarrays to further examine the role of HIF-1 during both transient and chronic hypoxia at the transcriptional level. The results of this study will provide initial insights into the mechanisms leading to changes at the cellular level as a result of low oxygen and may provide leads into understanding the changes that occur during heart disease.

Introduction

Cardiac muscle is incapable of producing enough energy under anaerobic conditions to maintain cellular processes. This makes oxygen a major determinant of gene expression even under normal conditions[1]. It is also the reason why the heart is so sensitive to ischemic injury. HIF-1α is the primary adaptive response to decreases in oxygen levels and is often found at elevated levels in patients with ischemic cardiomyopathy[1]. While being continuously expressed in all cells, HIF-1α is rapidly degraded under normoxic conditions in many cell types[2,3]. Some HIF-1α may still remain present in cardiac muscle as it has been shown to play a major role in metabolism even under normoxic conditions[1,3]. Under hypoxic conditions HIF-1α stabilization is immediate[4]. Within minutes, it is possible to show accumulation in the nucleus implying its immediate impact on gene expression[4]. In the heart, these changes in expression result in adaptive measures to cope with the decrease in oxygen delivery while trying to maintain cellular processes[1]. Typical examples include increases in the expression of VEGF to promote angiogenesis in the region , GLUT-1 to increase glucose uptake and promote glycolysis and EPO to promote hematopoiesis and an increase in oxygen carrying capacity[1]. Although studies have characterized specific HIF-1α associations, no definitive analysis of HIF-1α binding on a global scale has been conducted. Here we examine in greater detail the impact of HIF-1α on gene expression under hypoxic conditions in a cardiomyocyte model.

Methods

Cell Culture: The HL-1 cell line (a gift from Dr. William Claycomb) used throughout were maintained in daily changes of Supplemented Claycomb media (JRH Biosciences) supplemented with 10% FBS (JRH Biosciences), 100 U/ml:100 mg/ml penicillin/streptomycin (Invitrogen), 0.1 mM norepinephrine (Sigma), 2 mM L-glutamine (Invitrogen). Hypoxic conditions were created in an air tight container fitted into a standard incubator. A gas comprising 2% oxygen, 5% carbon dioxide, and 93% nitrogen was pushed through the chamber for the time periods listed for each experiment.
Western Analysis: Cell lysates were isolated using RIPA lysis buffer containing protein inhibitors (0.5 mM AEBSF, 1 mg/ml aprotinin, 1 mM benzamidine, 10 mg/ml leupeptin, 10 mg/ml pepstatin (Sigma)). Proteins were resolved by PAGE (20 µg protein/lane) and transferred to a PVDF membrane (Perkin Elmer). Membranes were incubated overnight with primary antibodies for HIF-1α (Novus Biological), caspase 3 and cleaved caspase 3 (Cell Signaling) diluted 1:1000. Blots were washed and then incubated with alkaline phophatase conjugated secondary antibodies. Proteins of interest were visualized by ECL (Perkin Elmer) and exposure to film.
Gene Expression: RNA was isolated using RNEasy (Qiagen) following manufacturers protocols for isolation from cultured cells. Total RNA was quantified using the NanoDrop and qualities were determined using the Agilent 2100 Bioanalyzer. Whole Mouse Genome Arrays (Agilent) were hybridized with RNA labelled using the Low RNA Input Linear Amplification Kit (Agilent) following manufacturers protocols.
ChIP on Chip: The detailed procedure is available at http://www.microarrays.ca/support/proto.html. Alterations to this protocol were to use 2 100 mm plates for every 150 mm plate described, such that every time point was made up of 8 100 mm plates. Technical replicates were used such that each "Round A" sample was used as the template for 4 "Round B" samples creating the 4 replicates for each time point. Samples were hybridized to the Mouse CpG 7.3K array (UHN MAC). Figure 1 shows the work flow for this protocol.

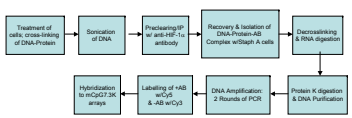

Figure 1: Work Flow of the ChIP-on-Chip Staph A cell Method. Complete protocol available at www.microarrays.ca.

Results

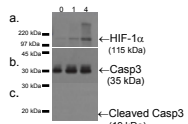

Figure 2: Western Analysis of HL-1 cells. Increasing amounts of HIF-1α are present with increased exposure to a hypoxic environment (a). Presence of caspase-3 (b) but absence of cleaved caspase-3 (c) demonstrates that the cells, while stressed by hypoxia are not apoptotic.

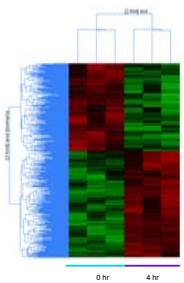

Figure 3: Gene Expression Analysis of HL-1 cells under Hypoxic Conditions. Shown is an unsupervised cluster representing the three biological replicates for each 0 hr and 4 hr exposure to a hypoxic environment. The cluster shows separation of the 2 time points demonstrating that there is a significant separation between the expression of the genes under each condition. Furthermore, there is a distinct separation of genes which become up-regulated or down-regulated with the exposure to hypoxia.

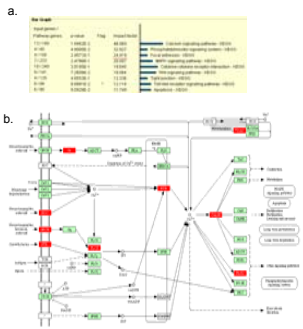

Figure 4: Pathway Analysis of Gene Expression Data. Pathway Express (Wayne State University) was used to group the significant genes determined by gene expression analysis (a). This software ranks pathways by taking into account the ratio of significant genes to the total number of genes within that pathway represented on the array. It also takes into account the location of the genes within the pathway such that higher impact is given if genes are located in the initial stages of a cascade. The most significant pathway, the Ca^{2+} Signalling Pathway is shown in full (b) with the significantly up regulated genes highlighted in red. This pathway is shown to affect other pathways such as contraction and apoptosis both of which will have an impact on how hypoxia will affect the cardiac muscle.

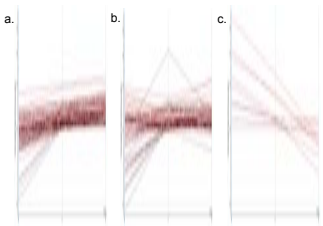

Figure 5: ChIP-on-Chip Analysis of HL-1 Cells under Hypoxic Conditions. Statistically significant CpG islands are shown over the 3 time points of 0 hr, 1 hr and 4 hr. Each time point represents 4 technical replicates where the intensity is normalized versus the "no-antibody control". The graphs show the various types of binding patterns of HIF-1α after its activation by hypoxia. The majority of the genes show a pattern of increased binding or increased binding followed by a period of levelled off binding (a). There are also genes that demonstrate a rapid increase of binding at the 1hr time point followed by a significant drop off at the 4 hour time point (b). Finally, there are a few genes that show a decrease over time. The implication is that the genes controlled by the binding sites within these CpG islands are following similar expression patterns.

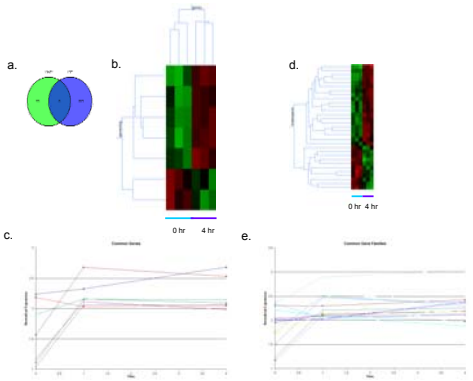

Figure 6: Correlation of Gene Expression and ChIP-on-Chip. The number of genes shown to be significant in both methods of analysis are represented with a Venn Diagram (a). While there is not a large number of overlapping genes it should be noted that the genes represented by both methods are significantly different so that this is not unusual. The genes common to both methods are shown as an subset of the cluster analysis shown previously (b) while ChIP-on-Chip data is shown as a graphical representation (c). There were also a number of gene families that were represented across both methods and these are shown in a similar capacity (d, e).

Discussion

The novel combination of gene expression and ChIP-on-Chip to study the impact of HIF-1α has created both expected and unexpected results. Due to the limited number of features on the CGI array used there was less overlap between the two technologies than we had anticipated. However, while there were known targets of HIF-1α pulled out by each of these analyses, many were lost due to the stringent filtering we performed on the data. For example, both COL5A1 and ADRA1B were pulled out of the ChIP-on-Chip data as being significant, however, the distance between the gene and the CGI was greater than we would have expected. The same is true of the gene expression data. Many of the known targets of HIF-1α were present on the array but were not shown to be up-regulated. This may be a factor of the time points used. As HIF-1α has such an immediate response it may be useful to re-examine the gene expression at a 1 hour time point to determine if any of these genes are being expressed early but not maintained at these levels over the long term.
Despite the stringency of our analyses, we were able to identify genes that were significant by both methods which were not previously linked to HIF-1α. Of these, genes related to transcriptional regulation were identified which is logical as HIF-1α is already known to target other transcription factors and cofactors[5]. Another gene of interest was a calcium channel component further confirming the pathway analysis of the gene expression data demonstrating the high impact on calcium signalling.
Unexpectedly, some of the gene expression data demonstrated opposite trends to that of the ChIP-on-Chip data. To date, HIF-1α has been primarily described as an activator of transcription so it was unexpected to see that a gene showing a steady increase over time in the ChIP-on-Chip analysis would be shown to be down-regulated in the gene expression data. We are currently investigating this relationship.
It should be noted that the data presented is preliminary. It is standard practice for all microarray analysis to be confirmed by a secondary method. For gene expression analysis this will be executed using qPCR while the ChIP-on-Chip data will be confirmed by standard ChIP and IP methods.

References

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