Time-course gene expression profiling of high glucose-induced endothelial cell apoptosis

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Abstract—**Diabetes is a debilitating metabolic disorder resulting from hyperglycemia and glucose intolerance. High glucose-induced endothelial dysfunction is an important contributor to vascular disease in diabetes. In this study, we profiled the global gene expression changes in human umbilical vein endothelial cells treated with high glucose at the 0-, 24-, and 48-hour intervals. Differentially expressed genes were examined through bioinformatics analysis for potential mechanisms of regulation. Our analysis uncovered novel regulatory interactions that may provide insights into the molecular transition from normal cellular activities to apoptosis under high glucose, enhancing our understanding of the mechanisms underlying vascular complications in diabetic patients.**

Keywords—high glucose, gene expression, diabetes, time-course analysis

I. INTRODUCTION

Diabetes mellitus is the major contributor of high glucose-induced endothelial dysfunction—an important pathogenic factor of vascular complications in diabetic patients [1]. The endothelium plays a prominent role in vascular homeostasis. Substantial evidence suggests that elevated glucose concentrations increase the production of reactive oxygen species, inducing endothelial apoptosis, and upsetting the balance between cell repair and injury [2, 3].

To date, several cellular pathways have been found to mediate the effect of high glucose on endothelial dysfunction. Hyperglycemia causes dysregulation of the mitochondria, increasing oxidative stress through the activation of the polyol pathway [4], diacylglycerol-protein kinase C pathway [5], and hexoamine pathway [6]. Not only does hyperglycemia result in the overproduction of reactive oxygen species in endothelial cells, but it also contributes to the formation of advanced glycation end-products (AGE) [7, 8]. AGE crosslinks with tissue proteins, lipids, and DNA, affecting their functions and turnover, contributing to a decline in endothelial function, and ultimately, diabetic vascular complications [7].

Until recently, the effect of high glucose on gene expression has only been profiled at the single gene/pathway level [9-11]. Pirola et al. [12] has examined the effect of endothelial apoptosis under hyperglycemia at both the transcriptomic and epigenomic levels. Their findings present the first global picture of hyperglycemia-induced gene expression changes occurring through histone modifications and differences in DNA methylation. This particular study has shown that the process of apoptosis during high glucose exposure is complex, involving various levels of molecular control. Their results bring to question the mechanisms underlying the transition of normal cellular activities to hyperglycemia-induced cell death. In other words, what are the key players controlling the progress towards apoptosis during high glucose treatment. If we could reach the answers, would we be able to find effective ways to enhance the protection against cell death before it occurs? Apparently, there are still questions that remain to be answered.

Over the years of studying diabetes mellitus, we have learned that cell death start to occur around 36 hours after treatment with high glucose [10]. In this study, we performed a time-course gene expression array study examining hyperglycemia-induced transcriptome changes during epithelial cell death. We attempted to identify the underlying genetic key players before and during apoptosis under high glucose. The finding is particularly invaluable to the future development of treatment for chronic diabetic complications.

II. MATERIALS AND METHODS

A. Cell culture and treatment

Growing and maintaining of human umbilical vein endothelial cells (HUVECs) were as described in [13] and cells were seeded at a density of 1 x 10^5 per 75-cm²-flask medium 199 (Gibco, Grand Island, New York), supplemented with 20 mM HEPES, 100 ug/ml endothelial cell growth substance (Collaborative Research Inc, Bedford, MA), and 20% fetal calf serum (Gibco). The cultures were maintained at 37°C with 5%CO_2-95% air mixture. All media were supplemented with 5 U/ml heparin, 100 IU/ml penicillin, and 0.1 Ag/ml streptomycin. Treatment of glucose followed Ho et al., 2006. Briefly, endothelial cells of the third to fifth passages in actively growing condition were harvested fand treated with a medium containing 5 mM glucose for 48 hours (basal glucose; control, n=3) or 33 mM glucose (high glucose; experimental) for different time intervals (24 hours, n=3; 48 hours, n=3).

B. Detection of apoptosis

Apoptosis of treated HUVECs were detected by the ELISA method of cell death (Boheringer Mannheim) and assessed for morphological changes under a fluorescence microscope. Intracellular hydrogen peroxide production was measured by flow cytometry analysis.

C. RNA isolation

The control and experimental treatments were conducted in duplicates. RNA was isolated by Trizol (Invitrogen, Carlsbad, California, USA). RNA quality was determined by a 260/280 ratio of 1.9-2.1 on a spectrophotometer and by the intensity of the 18S and 28S rRNA bands on a 1% formaldehyde-agarose gel. RNA quantity was detected by a spectrophotometer. RNA integrity was examined on an Agilent Bioanalyzer.

D. Array-based gene expression profiling

Global gene expression changes associated with high glucose exposure at different time intervals were profiled on Nimblegen human gene expression 12x135k array. Each sample was run in duplicates.

E. Statistical and Bioinformatics Analysis

To identify genes showing significant differential expression associated with the differing glucose treatments and time intervals, gene expression profiles at the 24-hour and 48-hour intervals were first normalized to the control (basal glucose treatment for 48 hours). Data were processed with R/Bioconductor. Significantly differentially expressed genes (absolute value of log2 ratio > 1.0, FDR <0.05) were categorized into 24-hour- and 48-hour- specific down- and up-regulated genes. These genes were subsequently used as input in the PANTHER classification system for gene ontology (GO) enrichment analysis [14].

We employed the features in TRANSFAC [15] and miRWalk [13] to find transcription factors and miRNAs that may regulate the candidate genes, respectively. The cross-validation function in miRWalk was used to compare the prediction results with four other miRNA resources: miRanda [16], miRDB [17], RNA22 [18], and TargetScan [19]. A predicted miRNA was accepted as a potential regulatory candidate when four out of the five miRNA resources agreed that it may interact with the differentially expressed genes. Finally, we utilized the tools in BIOGRAPH to determine if the differentially expressed genes are associated with diabetes or correlated complications [20].

III. RESULTS AND DISCUSSIONS

In total, there are 93 up-regulated and 207 down-regulated genes, and 57 up-regulated and 17 down-regulated genes specific to the 24-hour and 48-hour interval, respectively. At both time points, the top three most enriched pathways appeared to be angiogenesis, apoptosis, and inflammation. Table I shows a partial list of genes belonging to these three categories. These genes are selected based on their association with diabetes and cardiovascular dysfunction according to BIOGRAPH analysis.

Many of the genes involved in the regulation of cell death and inflammation have already been identified by others. This shows that our results are consistent with others. In contrast, abnormal angiogenesis, or the impaired formation of new

TABLE I.   A LIST OF SELECTED GENES IN THE ENRICHED PATHWAYS THAT ARE ALSO ASSOCIATED WITH DIABETES AND CARDIOVASCULAR COMPLICATIONS

Pathway	24-hour	48-hour
Angiogenesis	AKT2	AKT2
	PTHN6	STAT3
Apoptosis	AKT2	AKT2
	HSPA6	RAC3
	ATF7	STAT3
	MYCL1	RPS6KB2
Inflammation	JAK3	RAC3
	CD14	STAT3

blood cells from pre-existing blood vessels, has only recently been found to cause various complications involving the retina, kidney, and cardiovascular system [21].

Table II shows a partial list of transcription factors that may regulate the differentially expressed genes as predicted by TRANSFAC. Some In particular, the AKT2 gene (v-akt murine thymoma viral oncogene homolog 2) appears to be differentially expressed at both investigated time points. The level of AKT2 expression is reduced at the 24-hour time point and subsequently increased at the 48-hour interval. AKT2 is involved in angiogenesis, anti-apoptotic activity and inflammatory response [22]. Moreover, a rare mutation in the AKT2 gene sequence has been associated with severe insulin resistance and diabetes [23].

Our results support that the AKT2 gene may also play a role in diabetes-associated vascular complications. Indeed, exposure to high concentrations of glucose can induce apoptosis in endothelial cells [7, 8], and AKT2 is known to enhance the expression of anti-apoptotic proteins during cell death [24]. That we observed a time-specific change in AKT2 expression level indicates that AKT2 gene expression may be under temporal regulation during a high glucose challenge. We furthered this finding with bioinformatics analysis of the possible regulatory mechanisms of the AKT2 expression.

TABLE II.   A PARTIAL LIST OF POTENTIAL TRANSCRIPTION FACTORS REGULATING DIFFERENTIALLY EXPRESSED GENES UNDER HIGH GLUCOSE EXPOSURE

	gene	expression	transcription factor
24 hour	AKT2	up	MYOD
	ATP2A3	up	ETS-1
	CYP1B1	up	ER-α
	CD14	down	SP1
	IGFBP6	down	GLI

	gene	expression	transcription factor
48 hour	ADM	up	AP-2 α
	CDK5	down	AP-1
	STAT3	down	CREB1

According to TRANSFAC [15], MYOD (myogenic differentiation 1) acts as an enhancer for AKT2. Interestingly, our data indicate that MYOD and AKT2 share the same expression pattern in response to the 24-hour high glucose treatment. However, MYOD expression level after 48 hours of hyperglycemia did not appear to differ significantly from the 24-hour time point. This suggests that other regulatory mechanisms may be regulating AKT2 expression at the 48-hour interval.

To search for additional regulatory molecules, we utilized the features in mirWalk [13] to find putative microRNAs that may modulate the expression of the differentially expressed transcripts observed in our data analysis. The microRNA-gene interactions are selected based on experimentally validated relationships between the two biomolecules, and the association of these microRNAs with diabetes and related complications. An example of our analytical results is illustrated in Fig.1. The putative regulatory microRNAs connecting the genes AKT2 and CYP1B1 (Cytochrome P450, Family 1, Subfamily B, Polypeptide 1) may represent another potential mechanism underlying hyperglycermia-induced apoptosis. In particular, microRNAs may reveal different molecular signatures that are not apparent in gene expression profiling.
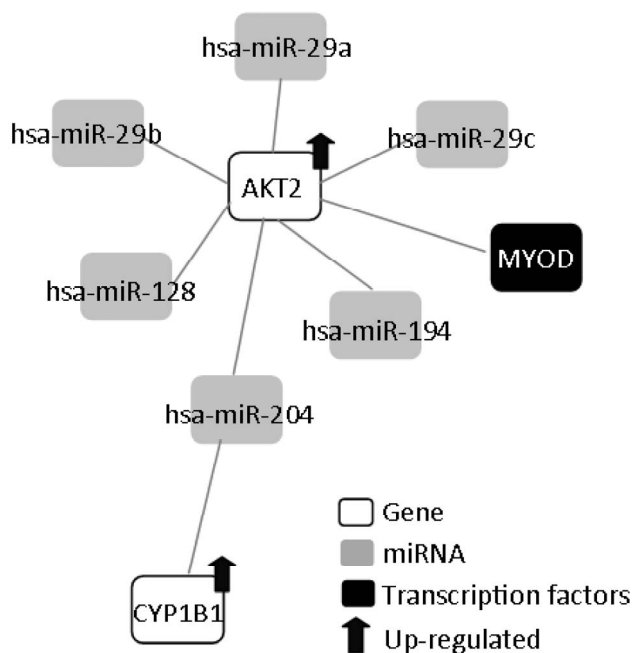

Fig. 1.   A microRNA-gene interaction network representing the potential regulatory mechanisms underlying high-glucose induced gene expression changes of apoptosis

In conclusion, we have uncovered a number of differentially expressed genes associated with the transition from normal cellular activities to apoptosis during high glucose exposure. Our findings are consistent with other studies in that many of the candidate genes are involved in apoptosis and inflammatory pathways. However, we have identified genes that show temporal gene expression patterns under high glucose condition. Moreover, by integrating available bioinformatics tools, we have attempted to construct a map of the potential regulatory mechanisms involving transcription factors and microRNAs modulating these candidate genes. Future validation of these preliminary results shall enhance our understanding of the molecular underpinnings of endothelial cell apoptosis induced by high glucose, and therefore, diabetes associated vascular dysfunction.

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