Surgery for Congenital Heart Disease

CHD

Early gene expression profiles during intraoperative myocardial ischemia-reperfusion in cardiac surgery

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Supplemental material is available online.

Objective: The effects of cold cardioplegic arrest and reperfusion on human ventricular gene expression are unknown. We tested the hypothesis that intraoperative ischemia-reperfusion under conditions of blood cardioplegic arrest would induce a unique myocardial genomic profile indicative of a cardioprotective response.

Methods: Right ventricular samples were serially acquired during surgical repair of ventricular septal defect.

Results: Expression profiling revealed 3 patterns of gene expression: (1) increased expression above control levels within 1 hour of cardioplegic arrest, with further amplification during early reperfusion; (2) increased expression limited to the reperfusion phase; and (3) reduced expression during reperfusion. Functional annotation and network mapping of differentially expressed genes indicated activation of multiple signaling pathways regulated by phosphatidylinositide 3′-OH kinase convergent on cellular growth and reparative programs. Also observed was increased expression of genes regulating hemoglobin synthesis, suggesting a novel cardioprotective pathway evoked during ischemia-reperfusion.

Conclusion: Reversible myocardial ischemia-reperfusion during cardiac surgery is associated with an immediate genomic response that predicts a net cardioprotective phenotype.

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There are no human data describing the immediate early genomic responses to intraoperative myocardial ischemia and reperfusion (I/R). In animal models, myocardial ischemia leads to prompt and widespread changes in gene expression, particularly in cell-signaling pathways governing myocyte hypertrophy, cell survival, and repair.[1,2] The use of blood cardioplegia preceding induced myocardial ischemia, in conjunction with variable degrees of cardiac hypothermia, would be expected to elicit a molecular response that is distinct from that associated with unprotected cardiac I/R. We speculated that the measurement of sequential changes in the human ventricular transcript profile during a relatively brief, reversible interval of cardioplegic arrest would reveal activation of innately cardioprotective signaling pathways.

Patients and Methods

Materials and Subjects

Informed consent was obtained from each patient, and the study was approved by the Research Ethics Board of the Hospital for Sick Children. The study was performed on 5

Abbreviations and Acronyms

ANOVA = analysis of variance
BSA = body surface area
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
I/R = ischemia and reperfusion
IGF = insulin-like growth factor
IPC = ischemic preconditioning
MIAME = Minimum Information About a Microarray Experiment
qPCR = quantitative polymerase chain reaction
RVOT = resection of the right ventricular outflow
TRNA = total RNA
VEGF = vascular endothelial growth factor
VSD = ventricular septal defect

patients (3 boys, 2 girls) operated on for ventricular septal defect (VSD) and resection of the right ventricular outflow (RVOT) muscular obstruction. Their ages ranged from 5 months to 5 years (mean 25 months), weights from 5.6 to 21.8 kg (mean 12.1 kg), and body surface area (BSA) from 0.37 to 0.74 m^2 (mean 0.52 m^2). The cardioplegic solution was comprised of 1 part plasmalyte solution (sodium 134 mmol; potassium 25 mmol; chloride 116 mmol; magnesium 21.5 mmol; pH 5.71–5.96; osmolality 334–340 mmol/kg H_2O) and 2 parts perfusate blood; 10 mEq of $NaHCO_3$, 30 U Humulin (insulin) (Lilly, Toronto, Ontario, Canada), and 25 mOsm of 50% dextrose were added per liter of cardioplegic solution just prior to infusion.

Experimental Design and Sample Collection

Experimental design, sampling, hybridization, data analysis, and presentation were done in compliance with revised Minimum Information About a Microarray Experiment (MIAME) guidelines (www.mged.org).[3] All myocardial samples were taken from the RVOT through the tricuspid valve. The first sample was taken immediately after cardiac arrest following administration of cold blood cardioplegic solution (30 mL/kg), which was repeated (20 mL/kg) every 20 minutes. The second sample was taken at 50 minutes of cardiac arrest. The third sample was taken at 5 minutes after reperfusion prior to right atrial closure. After aortic crossclamp removal, there was spontaneous restoration of normal heart function in all patients, and none of the patients required defibrillation.

RNA Isolation

Total RNA (TRNA) was isolated from 15 myocardial samples utilizing Trizol Reagent (GIBCO/BRL, Invitrogen, Carlsbad, Calif) following the manufacturer's protocol. The quality of TRNA was assessed using the Agilent 2100 Bioanalyzer (version A.02.01S1232, Agilent Technologies, Santa Clara, Calif). Only RNA with the OD ratio of 1.99:2.0 at 260/280 was used for microarray analysis.

Affymetrix GeneChip Hybridization and Scanning

A total of 16 hybridizations were performed on the Human HG-U133A GeneChip Set (Affymetrix, Santa Clara, Calif), including 15 TRNAs from myocardial samples of 5 patients at the 3 different time points and 1 reference TRNA (Stratagene, La Jolla, Calif) as a universal control. Samples were prepared for hybridization according to standard Affymetrix instructions and performed at the Genomic Core Facility at the Hospital for Sick Children.

Affymetrix GeneChip Data Analysis

We have previously reported the details of the microarray data analysis methodology used in this study.[4,5] Scanned raw data were processed with Affymetrix Microarray Suite version 5.0 software. The average intensity value for each probe set, which directly correlates with mRNA abundance, was calculated as an average of fluorescence differences for each perfectly matched versus single-nucleotide mismatched probe. To test the integrity of the starting RNA, we examined the signal intensity ratio for the 3′ probe set over the 5′ probe set for the housekeeping genes, *α-actin* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). For the 15 arrays used in this study, the 3′ to 5′ ratios were 1.3 ± 0.07 and 0.97 ± 0.06 for *α-actin* and *GAPDH*, respectively. Once sample quality was demonstrated, those genes with consistently present calls were considered. To monitor the expression of genes over the different experimental time points, data obtained from MAS 5.0 absolute analyses of all the individual arrays were analyzed and clustered using GeneSpring software 7.0 (http://www.agilent.com).

Statistical filtering was used to find the set of genes that show statistically significant differences in the mean normalized expression levels across all the groups. This comparison is performed for each gene, and the genes with the most significant differential expression (smallest *P* value) are returned. The parametric comparison for multiple groups used was a one-way analysis of variance (ANOVA). Calculations without the assumption of equality of variances were done using Welch's approximate *t* test and ANOVA. Briefly, a stepwise process was followed, first using a per-gene normalization to facilitate direct comparison of biologic differences. The 50th percentile of all measurements was used as a positive control for each sample; each measurement for each gene was divided by this synthetic positive control. The bottom 10th percentile was used as a test for correct background subtraction. Each gene was normalized to itself by making a synthetic positive control for that gene and dividing all measurements for that gene by this positive control (Figure 1). This synthetic control was the median of the gene's expression values over all the samples. Next, a second filter using Affymetrix data and *P* value with cutoff value of less than .005 in all conditions yielded 81 genes with differential expression.

Hierarchical Clustering

Differentially expressed genes were clustered by a hierarchical clustering algorithm by using an average linkage method in GeneSpring. Briefly, the expression values for a gene across all samples were standardized to have mean of 0 and standard deviation of 1 by linear transformation, and the distance between 2 genes was defined as $1 - r$, where r is the standard correlation coefficient between the standardized values of 2 genes. Two genes with the closest distance were first merged into a supergene and connected by branches with length representing their distance and were then deleted for future merging. The expression level of the newly formed supergene is the average of standardized expression levels of the 2 genes (average linkage) for each sample. Then the next

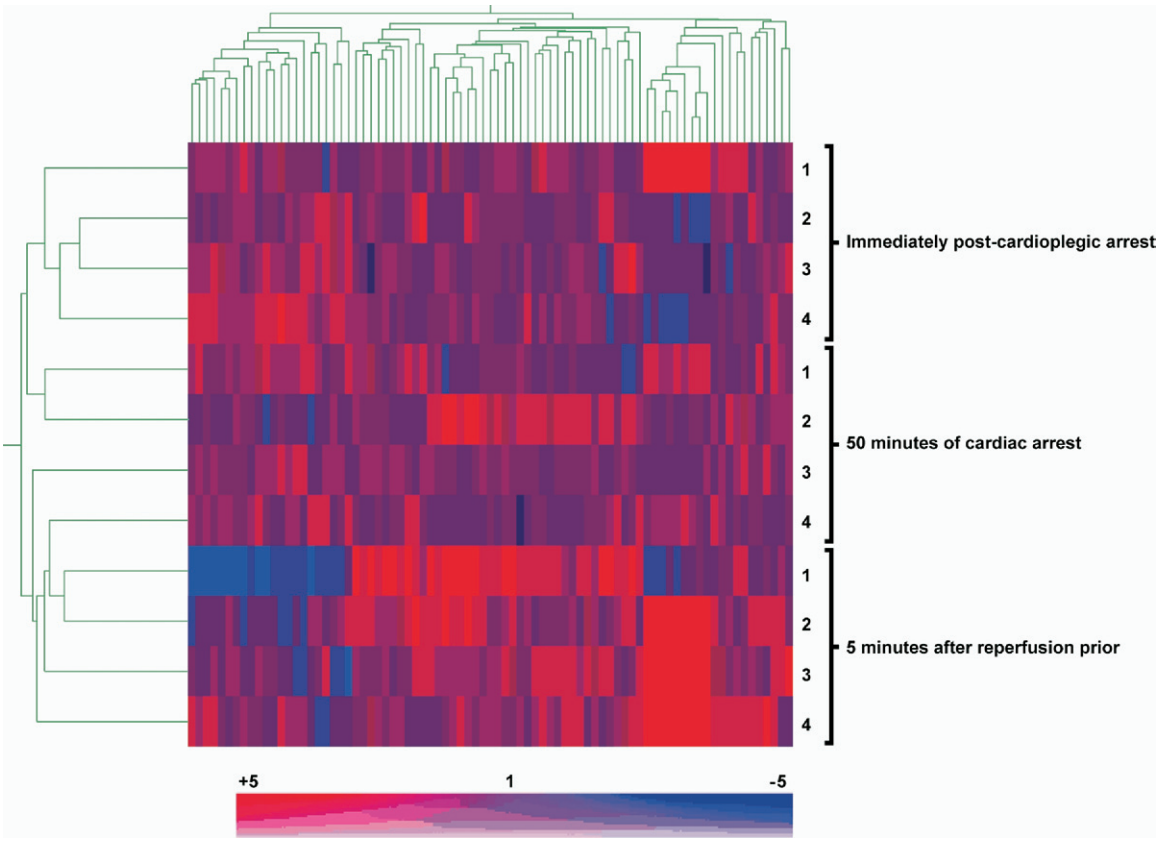

Figure 1. Hierarchical clustering depicting expression profiles of differentially regulated genes (n = 81) during repair of VSD. Samples shown on the right-hand side were acquired: (1) immediately after cardiac arrest; (2) 50 minutes following cardiac arrest; and (3) 5 minutes after reperfusion prior to right atrial closure. Unsupervised samplewise expression profiles shown on left side are highly coherent within each experimental time point. Unsupervised clustering profile of the 81 significant genes clusters is depicted above the dendrogram. *Blue* and *red* depict down- and up-regulation, respectively. *VSD*, ventricular septal defect.

pair of genes (supergene) with the smallest distance was merged, and the process was repeated to cover all genes (Figure 1). Additionally, K-means clustering, self-organization map, and principle component analysis were used for further confirmation.

The complete MIAME-compliant dataset (including all "description files") has been submitted and accepted by Gene Expression Omnibus at National Center for Biotechnology Information; accession number GSE6381.

Microarray Validation

Microarray results were confirmed by using real-time quantitative polymerase chain reaction (qPCR) on 5 randomly selected genes that demonstrated altered postinterventional expression. The principle of real-time qPCR has been described in detail.[6] Predesigned FAM-labeled TaqMan primer sets were constructed against: *hemoglobin beta* (Hs00758889_s1), *dual specificity phosphatase 1* (Hs00610256_g1), *cysteine-rich angiogenic inducer 61* (Hs00155479_m1), *early growth response 1* (*Egr-1;* Hs00152928_m1), and *insulin-like growth factor 1* (*IGF-1;* Hs00153126_m1, Applied Biosystems, Branchburg, NJ). Amplicon abundance was determined in real time normalized against a *GAPDH* control. Fold changes were determined as a ratio of sample RNA to that of the average RNA expression in the initial biopsy following aortic occlusion.

The functional properties of significant genes were inferred from several sources, including standard Gene Ontologies (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch), PubMed citations as provided in the Discussion, and network mapping algorithms using Pathway Assist software version 2.53 (Ariadne Genomics, Inc., Rockville, Md). Pathway Assist is a software application for the graphical depiction of biologic pathways, focusing on cell signaling networks and based on information extracted from PubMed citations using a Natural Language Processing engine.

Results

Aortic crossclamp time ranged from 51 to 63 minutes (mean 56 minutes). Total cardiopulmonary bypass time ranged from 67 to 95 minutes (mean 79 minutes). All patients had

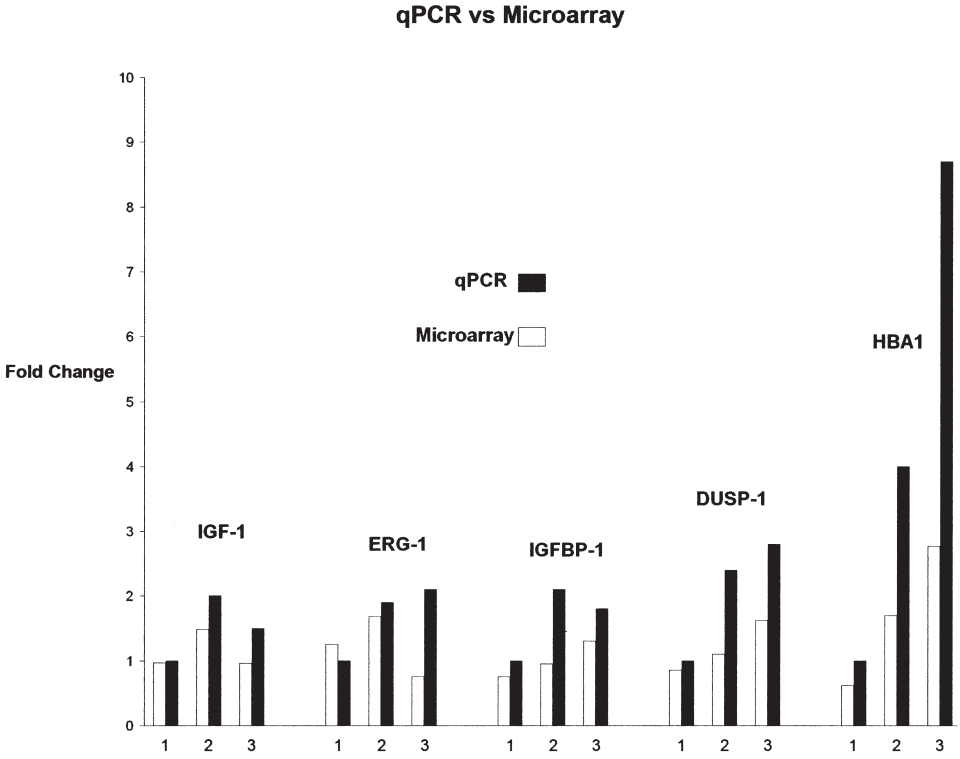

Figure 2. qPCR versus microarray. There was a high degree of correlation in expression levels of 5 genes differentially regulated during I/R as determined by qPCR and microarray analysis. The prime points for sample measurements are indicated on the *X-axis:* (1) immediately postcardioplegic arrest; (2) end ischemia; and (3) 5-minute reperfusion. Among the 5 genes sampled, the fold changes in expression were directionally the same as determined by qPCR and microarray at end- I/R compared with baseline in 9 of 10 measurements. Correlation coefficient, based on median R^2 slope of linear fit of per gene fold changes, measured by qPCR versus microarray = .86; $P < .05$. *IGF1*, Insulin-like growth factor-1; *Egr-1*, early growth response-1; *IGFBP-1*, insulin-like growth factor binding protein-1; *DUSP-1*, dual-specificity phosphatase-1; *HBA1*, α-hemoglobin.

an uneventful surgery, were weaned off the inotropic support within the first 6 hours, remained in the intensive care unit for less than 24 hours, and were discharged without complications.

The expression profile of 81 genes exhibiting differential expression during the I/R phases is shown in Figure 1. This analysis indicates that samplewise self-organizing clusters (shown on the left of dendrogram) are highly coherent within each experimental time point (shown on the right of dendrogram). The clustering profile of gene expression intensities of the 81 differentially expressed genes is indicated at the top of Figure 1. Detectable changes in mRNA levels of several genes were evident within approximately 1 hour of induction of myocardial ischemia. The expression levels of almost all of the genes exhibiting ischemic induction increased significantly further during reperfusion. These genes clustered into several major functional groups, including those involved in the immediate early response to stress and the regulation of cellular hypertrophy, repair, and apoptosis. A small number of genes showed decreased expression during the reperfusion phase.

The changes in expression levels measured by qPCR and microarray analysis of the 5 randomly selected genes were found to be proportional and directionally identical in 9 of 10 time point comparisons (Figure 2).

We observed an increase (less than twofold) in *Egr-1*, *vascular endothelial growth factor* (*VEGF*), and tissue factor message levels during reperfusion. The induction of immediate early genes is in line with the in silico–generated network map implicating c-*jun* and *elk1*[7] as common targets of differentially expressed several genes during I/R (Figure 3).

Unlike the expression pattern of *Egr-1* and transcripts encoding classical growth factors such as *VEGF* and *tyrosine kinase with immunoglobulin and epidermal growth factor (tie),* which exhibited reperfusion-mediated elevation, *IGF-1* transcript levels were increased at the end of the ischemic interval and decreased after a brief interval of

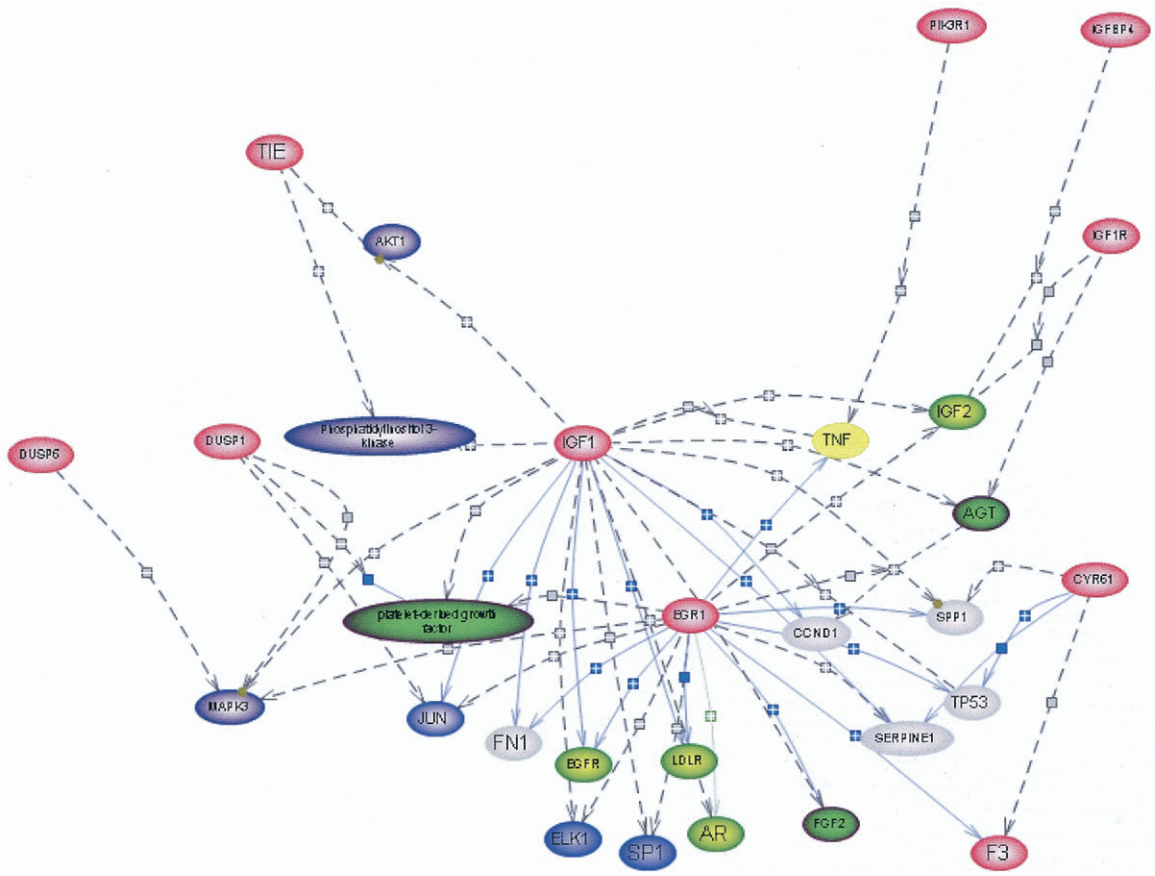

Figure 3. Network map showing common targets of those genes that showed significant up-regulation during reperfusion. Common targets were identified as those genes or proteins subject to upstream regulation, based on available PubMed citations, by at least 2 of the genes or corresponding proteins that exhibited significantly increased expression during reperfusion. Each node in the network map is linked to an html page showing the protein annotation based on available public databases. *Small square boxes* connecting 2 nodes are linked to an html page showing the type of effect (positive or negative) and the supporting Medline references. Nodes corresponding to genes/proteins that exhibited significantly increased expression during reperfusion are shown in *red.* Target genes or proteins subject to regulation by genes that exhibited induction during reperfusion are color-coded to reflect functional annotation as growth factors *(pink),* transcription factors *(blue),* PI3K signaling *(purple),* and miscellaneous function *(gray).*

reperfusion. Repression of insulin-like growth factor (IGF)-mediated signaling during reperfusion was further suggested by the coincident increased expression of *IGF-binding protein 4* (*IGFBP4*), which is a potent inhibitor of IGF-1. We observed an increase in mRNA corresponding to 4 different phosphatases during reperfusion, including the dual-specificity phosphatase *(MKP1).* An increase in both *tie-1* and *PI3K1* gene expression was evident during reperfusion. PI3K, MAPK3, and PKB/Akt were implicated by protein network mapping, further evidence in support of activation of the PI3K pathway during reperfusion (Figure 3). The reperfusion transcript profile implicates the induction of myocardial cytoskeletal repair genes, particularly *talin 1* (*TN1*), a critical gene for building early mechanical linkages during sarcomere repair,[8] as does the up-regulation of 2 other key structural proteins, *actin-binding LIM protein 1 (ABLIM)* and *actinin alpha 4 (ACTN4)*. We observed a twofold up-regulation of *MCT-2* mRNA during early reperfusion. Monocarboxylic acid transporters play a critical role in maintaining intracellular pH and normal lactic acid transport in cardiomyocytes during I/R,[9] and blockade of lactate transport exacerbates postischemic cellular damage.[10] There was a marked increase in several hemoglobin genes during the ischemic interval with further significant elevation during reperfusion. The rapid increase in mRNA of postnatal (α, β) hemoglobin genes during I/R is notable as this process is

normally tightly controlled by upstream gene locus regulatory elements, and non-erythroid globin gene synthesis is unprecedented.[11].

Protein Network Mapping

To uncover potential regulatory network invoked during I/R, network maps were constructed using Pathway Assist software to reveal potential genes or proteins subject to regulation by the 10 genes showing the highest differential expression during reperfusion (Figure 3). We searched for interacting genes/proteins based on the filter that they represent literature-validated targets of at least 2 genes identified as exhibiting differential expression during reperfusion by microarray analysis. This in silico approach identified several transcription factors and interacting growth factor receptors, as well as PI3K signaling molecules, which have been broadly implicated in cytoprotection.

Discussion

Principal Findings

Cardioplegic arrest elicits a cardioprotective transcriptional network. The present study provides evidence that myocardial ischemic stress associated with repair of VSD induces a net protective transcriptional response. This study is the first to describe the genomic response in the human ventricle elicited during the I/R phases. One unresolved issue in the interpretation of genomic expression data is determining whether the endogenous transcriptional response to potentially injurious stimuli is protective or, antithetically, whether it reflects activation of a genetic program that mediates a maladaptive injury response. Using a stringent filtering approach, we show that the majority of genes up-regulated in response to cardioplegic arrest have literature-precedented cytoprotective properties, including several that have been previously validated as endogenous mediators of ischemic preconditioning (IPC). We speculate that the brief period of ischemia imposed under conditions of blood cardioplegic arrest as employed in the present study induces a stress response that is similar in magnitude to that resulting from repetitive, brief (eg, 3-5 minutes) periods of normothermic ischemia and intervening reperfusion associated with classical IPC. Thus, the molecular response to myocardial ischemia imposed in the context of controlled cardioplegic protection reveals specific targets and functional pathways, which may have a role in the prevention of irreversible cellular injury associated with unprotected myocardial ischemia. Our results further reveal the novel finding, based on the construction of network maps generated from the reperfusion transcript profile, that activation of growth factor signaling represents a dominant higher-order functional theme during reperfusion following reversible myocardial ischemia.

Egr-1 and other immediate early genes exhibited an increase in expression levels during reperfusion. Network maps based on an unbiased PubMed search algorithm reveals a number of interactions of *Egr-1* with transcription factors, as well as a growth factors and growth factor receptors (Figure 3). Gene expression profiling experiments in animal models of myocardial ischemia have previously implicated increased expression of the *Egr-1* gene as an element of the protective response associated with nonlethal ischemia (reviewed by Simkhovich et al[12]).

Cardioplegic arrest activates growth factor signaling. Increased expression of growth factor signaling genes emerged as a dominant functional theme, which was especially marked during the reperfusion phase. This was evident by the increased expression levels of prototypical growth factors, *IGF-1* and *VEGF,* and the *IGF-1 receptor,* as well as components of PI3K membrane lipid kinase signaling cascade. Activation of growth factor signaling is cardioprotective against I/R injury (reviewed by Hausenloy et al[13]). IGF-1 peptide confers protection against I/R via mitochondria-dependent mechanisms.[14] Myocardial IGF-1 gene expression is also increased by short- and long-term exercise.[15] We have previously reported the transcriptional up-regulation of ventricular *Erg-1, PTPA,* and *IGF* genes during repair of congenitally obstructive right heart lesions.

Genetic up-regulation of the PI3K pathway resulting from PTEN deficiency,[16] or expression of a constitutively active p110α[17] or IGF-1 receptor[18] transgene in the heart, results in compensated or physiologic forms of cardiac hypertrophy. Increased expression of *VEGF* message plays an important role in acute postischemic myocardial recovery,[19] and *VEGF*-deleted mice exhibit impaired cardiac functional recovery following I/R evident as increased diastolic dysfunction.[19] The finding of increased *VEGF-B* levels in the current study is also consistent with activation of PI3K/Akt signaling, which has been shown to result from the interaction of *VEGF-B* and its corresponding receptor in postischemic vascular endothelium.[20] Thus, concerted elevation of *PI3K, tie-1, RADD, VEGF-B,* and the pleckstrin homology domain, *TAPP1,* suggests that activation of the PI3K/Akt pathway is a feature of the molecular response to I/R in human myocardium.

Cardioplegic arrest activates ischemic preconditioning pathways. PKB/Akt is a downstream target of PI3K.[21] The PI3K/PKB pathway can be activated via toll-like receptors[21] or growth factor receptors. The lipid product of PI3K, phosphatidylinositol-3,4,5-triphosphate, recruits PKB/Akt via its (PH) domains to the cellular membrane, where it is activated. PKB/Akt-mediated phosphorylation and nuclear translocation of the p65 subunit of nuclear factor kappa B[22] has been implicated as a critical event in both the early and late phases of IPC[23,24] and in cardioprotection during acute isch-

emia.[25] An increase in phosphorylation of PKB/Akt during reperfusion is necessary for IPC-induced cardioprotection.[26]

The increased expression of several structural proteins that serve as molecular scaffolds suggests they also participate in the transduction of growth factor signaling,[27] which suggests a novel control point regulating reperfusion survival pathways. The finding of rapid up-regulation of *frizzled-related proteins, FRP* and *SFRP1*, is of interest because they have been implicated in postischemia myocardial repair.[28,29] These proteins are also agonists for the canonical Wnt pathway, which is subject to regulation by PI3K,[30] and have been shown to play a critical role in self-renewal in human and mouse embryonic stem cells.[31]

Novel role for globin gene synthesis during cardioplegic arrest. Increased globin gene synthesis evident during ischemia before reperfusion rules out the trivial explanation that this finding results from an influx of erythroid cells during reperfusion. We reported a similar finding in isolated cardiomyocytes during simulated I/R,[32] which is also indicative of ectopic (nonhematologic) origin of hemoglobin gene transcription. In addition to O_2 and CO_2 transport, hemoglobins play a role in minimizing nitrosative stress by sequestration of excess nitric oxide generated during ischemia through the formation of hemoglobin-NO adducts.[33,34] Further studies will be required to understand the potential therapeutic significance of up-regulation of myocardial hemoglobin gene expression in response to I/R injury.

Limitations of the Study

This study is focused on the transcriptional profile in the human myocardium during I/R in the unique context of cardioplegic arrest and cardiopulmonary bypass. Although validation using polymerase chain reaction was performed in a limited number of genes, DNA microarray platforms have been shown to exhibit high correlation with quantitative gene expression values, as determined by reverse-transcriptase polymerase chain reaction assays.[35] The inferences drawn from these data are subject to the caveat that functionally important changes in protein expression and/or posttranslational modification during I/R are unknown. However, it is reasonable to assume that the transcriptional profile is ultimately modulated by the corresponding proteomic response and, as such, faithfully represents an important element of the integrated molecular response to I/R. This limitation is also offset to some degree by the use of an unbiased network mapping approach, which utilizes PubMed citations relating to interactions among differentially expressed genes at both transcriptional and posttranscriptional levels of regulation.

This study is focused on the changes in myocardial gene expression induced by I/R. These changes are superimposed on those resulting from cardiopulmonary bypass, which cannot be ascertained from this study. Nevertheless, the changes in gene expression reflect the effects of incremental I/R with each patient serving as their own control. The human transcriptome associated with I/R in this study is based on measurements derived from the endocardial layer of the right ventricular outflow tract and may not be representative of that in the left ventricle given that genetic segmentation is known to occur during cardiac development. Our previous study showed that activation of signaling pathways measured from the right ventricular endocardium was highly similar to that observed from left ventricular endocardial tissue.[36] The contributions from each of the various constituent myocardial cell types, which account the observed global myocardial expression profile, are unknown. It should be pointed out that expression of cardiogenic transcription factors governing ventricular trabeculation and compaction, such as NKx2.5, may be abnormal in patients with VSD,[37] so that that the expression profile in response to I/R may be specific to this entity and differ from that of a structurally normal heart.

Conclusions

This study describes the sequential changes in gene expression in the human ventricle during surgically imposed I/R. The annotation of several genes exhibiting differential expression suggests that the elicited transcriptional response in this context is compensatory and adaptive. In silico functional clustering of several genes comprising this response revealed dominant up-regulation of transcripts encoding elements of prohypertrophic cellular growth factor pathways, involving multiple levels of regulation, including receptors, cognate signaling kinases, and programmatically linked transcription factors. Further studies are warranted to test the prediction that the transcriptional network resulting from controlled I/R is broadly cardioprotective. Of particular interest are the mechanisms that account for ectopic activation of hemoglobin gene synthesis within the heart during I/R.

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Appendix E1: Supplemental Methods

Brief General Information on Microarrays

A DNA microarray (also commonly known as genechip, DNA chip, or gene array) is a collection of microscopic DNA spots attached to a solid surface, such as glass, plastic, or silicon chip, forming an array to monitor expression levels for thousands of genes simultaneously, referred to as gene expression profiling. The affixed DNA segments are known as probes; thousands of probes can be placed in known locations on a single DNA chip.

In spotted microarrays (or 2-channel or 2-color microarrays), the probes are oligonucleotides, cDNA, or small fragments of polymerase chain reaction products corresponding to different mRNAs. This type of array is hybridized with cDNA from two samples to be compared (ie, diseased and normal), which are labeled with 2 different fluorophores (usually Cy3 and Cy5). The samples can be mixed and hybridized to 1 single microarray/chip that is then scanned, allowing the visualization of up-regulated and down-regulated genes on the chip. However, in this system, the absolute levels of gene expression cannot be measured.

In oligonucleotide microarrays (or single-channel or 1-color microarrays), the probes are designed to match parts of the sequence of known or predicted mRNAs. In this case, the individual sample is labeled (usually with biotin) and hybridized to the chip. This system provides mesurements of the absolute levels of gene but requires 2 chips for the comparison different time points or experimental conditions.

In this study we used Affymetrix GeneChip Human Genome HG-U133A, a high-density in situ oligonucleotide array with a total of 22,282 probe sets that includes unique oligonucleotide features covering 13,900 of the best-characterized human genes and 18,720 of full-length transcripts. Sequences were selected from GenBank, dbEST, and RefSeq. Sequence clusters were created from Build 133 of UniGene.

Methods in Detail (Based on MIAME Standard)

Oligonucleotide arrays (hybridization and staining). A total of 15 hybridizations were performed on the Human HG-U133A GeneChip Set (Affymetrix, Santa Clara, Calif) with the 15 TRNA derived from 5 heart samples at 3 time points. Samples were prepared for hybridization according to Affymetrix instructions. Briefly, a primer encoding the T7 RNA polymerase promoter linked to oligo-dT_{17} was used to prime double-stranded cDNA synthesis from each mRNA sample using Superscript II RNase H^- reverse transcriptase (Life Technologies, Rockville, Md). Each double-stranded cDNA sample was purified through adsorption to silica (Qiaquick kit, Qiagen) according to manufacturer's instructions and then in vitro transcribed using T7 RNA polymerase (T7 kit; Enzo), incorporating biotin-UTP and biotin-CTP (Enzo Biochemicals, New York, NY) into the resulting copy RNA. These copy RNA transcripts were purified using RNEasy (Qiagen) and quantitated by measuring absorption at 260/280 nm. Samples were fragmented at 95°C for 35 minutes in 10 mmol/L $MgCl_2$ to a mean size of ~50 to 100 nucleotides, added to hybridization buffer, and hybridized to the Chip for 16 hours at 45°C. GeneChips were washed and stained with streptavidin-R-phycoerythrin. The chips were scanned using the GeneArray scanner (Affymetrix) and output files were visually inspected for hybridization artifacts. Arrays lacking artifacts were analyzed using GCOS (GeneChip Operating Software, Affymetrix) and then scaled to an average intensity of 150 per gene and analyzed independently. The expression value for each gene was determined by calculating the average of differences (perfect match intensity minus mismatch intensity) of the probe pairs in use for the gene. To determine whether the measured transcript is detected (Present, P) or not detected (Absent, A), a detection algorithm uses probe pair intensities to generate a Detection *P* value and assign Present or Absent call. Affymetrix default value of Tau = 0.015 was used for the calculation of a detection *P* value. The expression analysis files created by GCOS software were then transferred to GeneSpring (Silicon Genetics) for further analysis. Hybridization, staining, scanning, and scaling were performed at the core facility of the Hospital for Sick Children at University of Toronto.

Data analysis. Data analysis was performed using GeneSpring 7.0 software (Agilent). To identify differentially expressed transcripts, statistically significant genes ($P < .05$) were selected for further analysis. Briefly, a stepwise process was followed to achieve per-gene normalization to facilitate direct comparison of biologic differences. The 50th percentile of all measurements was used as a positive control for each sample; each measurement for each gene was divided by this synthetic positive control, assuming that this was at least 10. The bottom 10th percentile was used as a test for correct background subtraction. Each gene was then normalized to itself by making a synthetic positive control for that gene and dividing all measurements for that gene by this positive control, assuming it was at least 0.01. This synthetic control was the median of the gene-specific expression values over all the samples.

Brief description of hierarchical clustering. Hierarchical clustering generates a heat map that is a graphical representation of data values in a 2-dimensional color gradient map, often used to represent the level of expression of many genes across a number of comparable samples (eg, cells in different states, samples from different patients or time points; Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A.* 1998;95:14863-8).

Cluster analysis is used to visualize a set of samples or genes by organizing them into a phylogenetic tree, often referred to as a dendrogram. One way of analyzing microarray data is to look for the cluster (group) of genes with a similar pattern of expression across many experiments (time points). The coregulated genes within such groups are often found to have related function. The distance between 2 branches of a tree is a measure of the correlation between any 2 genes in the 2 branches. This is a powerful application that allows a researcher to find experimental conditions (eg, various drug treatments, classification of disease states) that have similar effects. All measurements of the gene expression data set were used for clustering analysis. After filtering genes based on a *P* value $< .05$, differentially expressed genes were clustered and ordered by a hierarchical clustering algorithm by using an average linkage method in GeneSpring. Briefly, the expression values for a gene across the all samples were standardized to have mean 0 and standard deviation 1 by linear transformation, and the distance between 2 genes was defined as $1 - r$ where r is the standard correlation coefficient between the standardized values of 2 genes. Two genes with the closest distance were first merged into a

supergene and connected by branches with length representing their distance and were then deleted for future merging. The expression level of the newly formed supergene is the average of standardized expression levels of the 2 genes (average linkage) for each sample. Then the next pair of genes (supergene) with the smallest distance was merged, and the process was repeated to cover all genes. Additionally, K-means clustering, self-organization map, and principle component analysis were used for confirmation of the overall pattern of gene expression value relatedness identified by clustering analysis.

K-means clustering. K-means clustering divides genes into distinct groups based on their expression patterns. Genes are initially divided into a number (k) of user-defined and equally sized groups. Centroids are calculated for each group corresponding to the average of the expression profiles. Individual genes are then reassigned to the group in which the centroid is the most similar to the gene. Group centroids are then recalculated, and the process is iterated until the group compositions converge. A wide selection of similarity measures (parametric and nonparametric correlations, Euclidean distance, etc) is available in different software.

Self-organization maps. Self-organization map is a clustering technique similar to K-means clustering. However, self-organization maps illustrate the relationship between groups by arranging them in a 2-dimensional map in addition to dividing genes into groups based on expression patterns. Self-organization maps are useful for visualizing the number of distinct expression patterns in the data and determining which of these patterns are variants of one another.

The self-organization map algorithm in GeneSpring begins by creating a 2-dimensional grid of nodes in the space of gene expression. In each iteration, 1 gene is selected and all of the nodes within a user-defined "neighborhood" are moved closer to it. This process is repeated with each gene in the selected gene list until the maximum number of iterations has been reached. With each iteration, the "neighborhood radius" is incrementally reduced and nodes are moved by smaller and smaller amounts to produce convergence. In this way, the grid of nodes is stretched and wrapped to best represent the variability of the data while still maintaining similarity between adjacent nodes. After the iteration is complete, genes are assigned to the nearest node, and a display grid of gene expression graphs is generated, corresponding to the initial grid of nodes. As the iteration proceeds, the neighborhood radius decreases smoothly, so that points move more independently later in the process. The neighborhood radius is expressed in terms of Euclidean distance in grid units relative to the abstract grid of the expression patterns.

Statistical group comparison. GeneSpring uses a filter tool that statistically compares mean expression levels between 2 or more groups of samples. The object is to find the set of genes for which the specified comparison shows statistically significant differences in the mean normalized expression levels. This comparison is performed for each gene, and the genes with the most significant differential expression (smallest P value) are returned. The parametric comparison for multiple groups is performed using one-way ANOVA. Calculations without the assumption of equality of variances were done using Welch's approximate t test and ANOVA.

For each gene separately, GeneSpring calculates the following: Let i be the index over the G groups formed by distinct levels of the comparison parameter. Let X_{ik} be the expression values, with k running over the replicates for each situation, interpreted according to the current interpretation (ratio, log of ratio, fold change). Let

N_i = the number of nonmissing data values for each group,

$$\overline{X}_i = 1/N_i \sum_{k=1}^{N_i} X_{ik} \quad \text{be the groups means, and}$$

$$SS_i = \sum_{k=1}^{N_i} (X_{ik} - \overline{X}_i)^2 \quad \text{be the within-group sum of squares.}$$

In all calculations here, missing (NaN) values are left out of the sums, not propagated. If any of the N_i are 0, that parameter level is dropped from the analysis, and G is readjust accordingly. If G is not at least 2, exit (P value = 1).

For the parametric test without assuming variances equal: First check that each group has N_i greater than or equal to 2 and SS_i greater than 0; if not, remove it from consideration and recompute G again. If G is not at least 2, exit (P value = 1).

Then compute:

$$w_i = N_i \left(\frac{N_i - 1}{SS_i} \right) \quad \text{the group weights}$$

$$W = \sum_{i=1}^{G} w_i \quad \text{the sum of weights}$$

$$\widetilde{X} = \frac{\sum_{i=1}^{G} w_i \overline{X}_i}{W} \quad \text{the weighted mean}$$

$$BSS = \sum w_i (\overline{X}_i - \widetilde{X})^2 \quad \text{the between-groups sum of squares}$$

$$d_1 = G - 1 \quad \text{the numerator degrees of freedom}$$

$$BMS = BSS/d_1 \quad \text{the between-groups mean square}$$

$$Z = \frac{1}{G^2 - 1} \sum_{i=1}^{G} \left(1 - \frac{w_i}{W} \right)^2 / (N_i - 1)$$

$$d_2 = \frac{1}{3Z} \quad \text{the denominator degrees of freedom}$$

if d_2 is not greater than 0, then exit (P value = 1).

$$WMS = 1 + 2(G - 2)Z \quad \text{the within-group mean square}$$

$$W = BMS/WMS \quad \text{the test statistic.}$$