Failure of Right Ventricular Adaptation in Children With Tetralogy of Fallot

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Background—The left ventricle (LV) adapts to chronic hypoxia by expressing protective angiogenic, metabolic, and antioxidant genes to improve O2 delivery and energy production, and to minimize reoxygenation injury. The ability of the right ventricle (RV) to adapt to hypoxia in children with tetralogy of Fallot (TOF) is unknown.

Methods and Results—Gene expression using real-time polymerase chain reaction was measured in RV myocardium obtained during surgical repair of TOF from 23 patients: 13 cyanotic and 10 acyanotic. Results were compared between the 2 groups and correlated with age at surgery, severity of cyanosis, and early postoperative course. The cyanotic patients were younger at surgery compared with acyanotic (5±3 versus 9±4 months; P=0.01), had higher hematocrit (43±4 versus 38±3 grams/dL; P=0.004), and lower O2 saturations (84±4% versus 98±2%; P<0.001). Cyanotic patients had a significantly lower expression of vascular endothelial growth factor (VEGF), glycolytic enzymes, and glutathione peroxidase (GPX) (P<0.05), and a higher expression of collagen (P<0.01) compared with acyanotic patients. Gene expression correlated inversely with severity of cyanosis ie, preoperative hematocrit (P<0.01) and positively with preoperative saturation (P<0.05). The relationship between gene expression and cyanosis was independent of age at surgery. Ca2+ handling genes did not correlate with the severity of hypoxia. Lower angiogenic, glycolytic, and antioxidant gene expression correlated with increasing postoperative lactate (P<0.05).

Conclusions—The RV fails to up regulate adaptive pathways in response to increasing hypoxia in children with TOF. The implications of an early maladaptive response of the RV on long-term RV function require further investigation. (Circulation. 2006;114[suppl I]:I-37–I-42.)

Key Words: gene expression ■ hypoxia ■ right ventricle ■ tetralogy of Fallot
institution, primary complete repair using a transatrial approach is performed electively between 6 to 8 months of age and earlier for “hypercyanotic spells” or worsening cyanosis. Children with associated anomalies such as pulmonary atresia and absent pulmonary valve and those with a previous systemic-pulmonary shunt were excluded. Children with laboratory evidence of anemia and those receiving iron and/or erythropoietin preoperatively were also excluded. The study was approved by the local Institutional Review Board.

Clinical Data
Medical records were reviewed to determine patient demographics, preoperative clinical course, and severity of cyanosis. Patients were divided into 2 groups: cyanotic and acyanotic. Patients were considered cyanotic if preoperative oxygen (O2) saturations were consistently <94% on room air and preoperative hematocrit (Hct) was ≥2 SD for age. Surgical data including the type of repair, duration of cardiopulmonary bypass, and cross-clamp time were obtained. Early postoperative lactate levels measured 1 hour after surgery, need for chest tube, amount of inotropic support, and duration of intensive care unit (ICU) stay were recorded.

Tissue Studies
Sample Acquisition and RNA extraction
In all patients undergoing primary repair, muscle bundles that are routinely resected from the RV outflow tract as part of surgery were recovered. Samples were placed immediately in RNA Later (Qiagen), incubated at 2°C to 8°C for 24 hours, and stored at -80°C. RNA was isolated using RNeasy Fibrous Tissue Midi Kit (Qiagen). The manufacturer’s protocol was followed. In brief, tissue was lysed, homogenized, digested with Proteinase K, applied to RNeasy Spin Columns, contaminants were removed with DNase digest, and washed to give total RNA. Only pure RNA with an A260/A280 ratio of 1.9 to 2.1 in 10 mmol/L Tris.Cl, pH 7.5 during spectrophotometric quantification was used.

Real-Time Polymerase Chain Reaction
Sigma SYBR Green Quantitative RT-PCR Kit was used for transcription and amplification using the ABI 7900HT Thermocycler. First-strand DNA was synthesized from RNA via reverse-transcriptase (RT) at 48°C for 45 minutes, and RT was inactivated at 94°C for 2 minutes, followed by 94°C for 15 seconds. The primers annealed to the specific portion of the DNA and extension occurred in a single-step RT polymerase chain reaction (PCR) at 72°C for 1 minute. SYBR Green fluoresces when bound to double-stranded DNA. The fluorescence response was monitored as PCR product and generated over a range of 50 PCR cycles. Melting curve analysis was performed at the end of the run to analyze only the RT-PCR product of interest at 60°C to 95°C. Primers were designed using Primer 3 output program and appropriate annealing temperatures were calculated based on the product and primer melting temperatures and confirmed by gradient RT-PCR. A primer concentration of 200 to 400 nM was used. The primer sequences and conditions are outlined below in Table 1. Relative messenger RNA expression of the following genes was measured using real-time PCR: Hypoxia-inducible factor 1α (HIF 1α), VEGF (angiogenesis), Aldolase A (ALDO), adenylate kinase (AdK3) (glycolysis), Glutathione peroxidase (GPX) (anti-oxidant), Procollagen type 1 alpha 1 (COL), titin (TTN) (markers of ventricular stiffness), Sarcoendoplasmic reticulum Ca2+ ATPase type 2a (SERCA 2a), calmodulin (CALM 2), and phospholamban (PHL) (Ca2+ handling, markers of diastolic dysfunction), Brain natriuretic peptide (BNP) (hypertrophy marker), β2 Microglobulin (β2 mol/L) was used as the house keeping gene to correct for differences in transcriptional regulation across samples.

Statement of Responsibility
The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

Statistical Analysis
Data were expressed as mean±SD. Clinical variables and gene expression were compared between the cyanotic and acyanotic TOF groups using Student t test. Gene expression was then correlated with the severity of pre-operative hypoxia and postoperative lactate and clinical course using Pearson correlation and Fisher exact test. Multivariable regression analysis was performed to assess the influence of age on gene expression. P≤0.05 was considered significant.

Figure 1. Left ventricular adaptive response to hypoxia. Hypoxia activates hypoxia inducible transcription factors. This leads to the transcription of target genes that respond by increasing O2 delivery and energy production. Upregulation of glutathione peroxidase protects against reoxygenation injury.
Results

Clinical Profile
Twenty-three TOF patients were studied; 13 were cyanotic and 10 acyanotic as shown in Table 2. Cyanotic patients had significantly lower O2 saturations (84±4% versus 98±2%; P<0.001) and higher mean preoperative Hct (43±4% versus 38±3%; P=0.004) when compared with the acyanotic group. The mean age at the time of repair in months was lower in the cyanotic group (4.9±3 months versus the acyanotic group 3±0.3 months); 96% of the children in the cyanotic group received a transannular patch compared with 50% in the acyanotic group. The cardiopulmonary bypass time and cross-clamp time were not different between the groups.

TABLE 1. Primer Sequence and Annealing Temperatures

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5’–3’</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxia-inducible factor 1α (HIF 1α)</td>
<td>+GACAAGCCACCTGAGGAGAAG</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>–GAAAGGACAACCCCAGAGGTA</td>
<td></td>
</tr>
<tr>
<td>Vascular endothelial growth factor (VEGF)</td>
<td>+AAGGGAGGAGGGAGAAGAATCAT</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>–CACACAGGTGCCCTGAAGA</td>
<td></td>
</tr>
<tr>
<td>Aldolase A (ALDO A)</td>
<td>+TGCTAATCCACGAACCACATC</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>–ATGGTCCACGATGCATATC</td>
<td></td>
</tr>
<tr>
<td>Adenylate kinase 3 (AdK3)</td>
<td>+TTGGTCTCAGACGATGTGA</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>–TTGCGTCTGCTACTATGCTCC</td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase (GPX)</td>
<td>+ATGCTGGCAATAGCCTCTC</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>–AGATGACCAAGACGAAATGC</td>
<td></td>
</tr>
<tr>
<td>Collagen (COL)</td>
<td>+TCAGCTTCCAGTCGACGAC</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>–CCAGCTTCCCTGCAAGACCC</td>
<td></td>
</tr>
<tr>
<td>Titin (TTN)</td>
<td>+AGACGGAGGAGGCAAAATCA</td>
<td>58.5</td>
</tr>
<tr>
<td></td>
<td>–ATCCGCTTCAACAAATCC</td>
<td></td>
</tr>
<tr>
<td>Sarcoendoplasmic reticulum Ca2+ ATPase type 2a (SERCA 2a)</td>
<td>+AGGGTTACCTCATGATTTGCA</td>
<td>58.5</td>
</tr>
<tr>
<td></td>
<td>–CTGCTGATGCTACTCCCTCC</td>
<td></td>
</tr>
<tr>
<td>Calmodulin (CALM 2)</td>
<td>+AGTGAGAGAGGAGGCTGAGA</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>–AGTCAATTCGTGAGCATGTC</td>
<td></td>
</tr>
<tr>
<td>Phospholamban (PHL)</td>
<td>+TCAGCTTCCAGACGCTACC</td>
<td>58.5</td>
</tr>
<tr>
<td></td>
<td>–TGGTCTTGCTGGTCCGTA</td>
<td></td>
</tr>
<tr>
<td>Brain natriuretic peptide (BNP)</td>
<td>+AGCTCCTCCAGAGCACTGGA</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>–AGAAGGCGAGATGGAAGAGA</td>
<td></td>
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<tr>
<td>β2 Microglobulin (β2 mol/L)</td>
<td>+GTCGCTGCCTACTTCTCTCT</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>–TCAATGCTGGAATGGATGAAA</td>
<td></td>
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</table>

TABLE 2. Clinical Profile of Cyanotic and Acyanotic TOF Patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cyanotic TOF (n=13)</th>
<th>Acyanotic TOF (n=10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoperative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O2 saturations %</td>
<td>84±4</td>
<td>98±2</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Pre op Hematocrit %</td>
<td>43±4</td>
<td>38±3</td>
<td>0.004*</td>
</tr>
<tr>
<td>Operative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at surgery (m)</td>
<td>4.9±3</td>
<td>9.1±4</td>
<td>0.01*</td>
</tr>
<tr>
<td>Transannular patch</td>
<td>96%</td>
<td>50%</td>
<td>0.07</td>
</tr>
<tr>
<td>Duration of CPB (min)</td>
<td>90±17</td>
<td>78±16</td>
<td>0.1</td>
</tr>
<tr>
<td>Duration of cross-clamp (min)</td>
<td>53±8</td>
<td>48±13</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Gene Expression
Relative gene expression was expressed as the fold difference in gene expression in cyanotic versus acyanotic right ventricles. HIF 1α expression was not significantly different between the cyanotic and acyanotic groups.

Angiogenic and Metabolic Genes
VEGF, ALDO A, and AdK showed a 2- to 5-fold lower expression in the cyanotic group (P<0.05) (Figure 2a).

Antioxidant Genes
Expression of glutathione peroxidase was 25-fold lower in the cyanotic group (P<0.001) (Figure 2b).

Structural Genes
There was a 4-fold higher collagen expression in the cyanotic group (P=0.005) but no difference in titin expression (P=0.9) (Figure 2b). There was no significant difference in expression of the Ca2+ handling genes, SERCA 2a (P=0.06), calmodulin (P=0.5), phospholamban (P=0.1), and BNP (P=0.17), between the cyanotic and acyanotic groups.
These differences were independent of age at the time of repair.

Correlation With Severity of Cyanosis

The expression of metabolic and anti-oxidant genes correlated inversely with severity of cyanosis, i.e., positively with preoperative saturations ($P<0.05$) (Figure 3) and negatively with preoperative Hct ($P<0.01$) (Figure 4). Collagen expression correlated negatively with preoperative saturations ($r=-0.6, P=0.01$) (Figure 3d). The expression of titin and Ca$^{2+}$ handling genes did not correlate with the severity of cyanosis.

Correlation With Early Postoperative Course

Gene expression correlated significantly with post-operative lactate levels. Lower expression of VEGF ($P=0.03$), ALDO A ($P=0.007$), and GPX ($P=0.02$) was associated with higher lactate levels (Figure 5). This was independent of the cardiopulmonary bypass time and cross-clamp time. In this cohort of patients, there was no correlation of gene expression with the duration of postoperative inotropic support in hours (28 versus 27; $P=0.95$), need for chest tube drainage (8% versus 40%; $P=0.2$), and duration of ICU stay in days (3±2 versus 5±2.5; $P=0.1$).

Discussion

This study showed that the RV has an impaired ability to upregulate adaptive pathways to chronic hypoxia in children with TOF. This was associated with higher collagen expression in the RV as well as higher postoperative lactate levels. This early maladaptive response of the RV may influence long-term RV function in TOF patients. Mammalian cells including left ventricular myocytes adapt to hypoxia by upregulating HIF 1α, which leads to the transcription of angiogenic and metabolic genes. This has been shown in animal models of hypoxia and in ischemic human myocytes.4,8–10 Further proof of the central role of HIF 1α and its downstream targets in maintaining oxygen homeostasis stems from work on myocyte-specific deletion of HIF 1α in mice.7 HIF 1α deletion resulted in downregulation of angiogenic and metabolic genes resulting in myocardial fibrosis, altered Ca$^{2+}$ handling, and subsequently LV systolic and diastolic dysfunction. Upregulation of HIF 1α through gene therapy was shown to upregulate VEGF expression and promote new blood vessel formation in rat myocardial infarction models.11,12 The mechanisms of adaptation to hypoxia have not been described in the RV. Our findings show that the RV in TOF failed to mount an adaptive response to hypoxia. Increasing hypoxia was associated with downregulation instead of upregulation of angiogenic and metabolic genes. To elucidate the impact of the RV maladaptive process in TOF on diastolic function, we studied the expression of genes implicated in diastolic dysfunction such as the structural genes, collagen and titin, and the Ca handling genes. Molecular changes in sarcomeric and interstitial proteins such as titin and collagen have been shown to result in increased chamber stiffness.13 Titin N2B-based stiffness acts in concert with collagen-based stiffness to worsen diastolic stiffness.14,15 Our findings show an upregulation of procollagen type 1 alpha 1 gene expression, which is one of the major forms of collagen in endomyocardial tissue with no change in titin gene expression. Endomyocardial fibrosis has been described in TOF after late repair and it has been postulated that this may cause a restrictive RV physiology, which adversely
impacts the early postoperative course. An increase in myocardial fibrosis was also seen in HIF 1α null mice. We found no difference in expression of genes that regulate Ca²⁺ cycling such as SERCA, calmodulin, and phospholamban. SERCA 2a regulates Ca²⁺ reuptake by the sarcoplasmic reticulum and is dependent on ATP for its activity. Phospholamban inhibits SERCA 2a and calmodulin facilitates SERCA 2a. Together, these genes maintain intracellular Ca²⁺ homeostasis. Structural remodeling, as in fibrosis, downregulates these genes, thereby causing ventricular dysfunction. It is possible that alterations in these genes only occur late in the maladaptive process and were therefore not seen in patients undergoing early TOF repair. However, because intracellular Ca²⁺ transients were not measured, it is not known if the activity of these Ca²⁺ cycling pathways was affected in the cyanotic RV. Hypertrophy secondary to pressure overload can also induce angiogenic and metabolic genes to meet the increased demand for nutrients. We sought to differentiate this by studying the expression of BNP, a marker of ventricular hypertrophy. Pressure overload is accompanied by overexpression of both ANP and BNP in the left ventricle. The production of the second messenger cGMP via the biologically active ANP-A receptor is the same for both ANP and BNP in human and rat tissue. Because BNP gene expression is 10-times more in human ventricles than ANP gene expression, we sought to consider BNP as a marker of hypertrophy in this study. BNP expression was not different between the cyanotic and acyanotic groups suggesting that these differences in gene expression were more likely secondary to differences in severity of hypoxia rather than severity of pressure overload. It is, however, concerning that this failure of the RV to upregulate protective genes may represent a maladaptation to both hypoxia and pressure overload in TOF patients.

We compared our results with those of Konstantinov et al, who highlighted age-related differences in gene expression profile in the right ventricle of children undergoing right ventricular outflow tract resection. They found an effect of age but not of hypoxia on gene expression. There are several differences between their article and ours. The subjects in their study were more heterogeneous and included all etiologies of right ventricular outflow tract obstruction rather than only TOF patients, thereby reflecting a less pure anatomic subset. Their study included a wider age range including patients up to 12.5 years of age. Although the expression of HIF and its target genes is developmentally regulated with decreasing message levels from neonate to the adult, our
study included only infants which may explain the absence of a primary effect of age on candidate gene expression in our study.

Antioxidant Effects
It has been shown that transient hypoxia upregulates genes encoding antioxidant enzymes in the LV. This forms the basis of the beneficial effects of hypoxic preconditioning. In our study, the expression of glutathione peroxidase, an antioxidant enzyme, was lower in the RV in patients with more severe hypoxia. This was consistent with earlier work by Li et al on cultured RV myocytes from patients with TOF, which also showed decreased antioxidant tissue levels in response to increasing hypoxia. This may make the RV more susceptible to reoxygenation injury related to oxidative stress at the time of TOF repair. The higher postoperative lactate levels in patients with lower expression of metabolic and antioxidant genes further indicate an impaired ability of the RV to handle oxidative stress related to surgical repair. We were unable to assess the influence of these alterations in gene expression on RV diastolic function because these parameters were not routinely obtained immediately postoperatively in patients after surgical repair. Within this relatively small cohort of patients, early repair was not associated with a prolonged postoperative course, ie, chest tube drainage, inotropic support, or length of ICU stay, suggesting possible benefits of early repair in severely cyanotic patients.

Clinical Implications
Because the RV in TOF has an impaired ability to adapt to chronic hypoxia, early surgical correction to relieve cyanosis may be cardioprotective. Our findings also have implications for interventions that can protect against reoxygenation injury including use of antioxidants and/or free radical scavengers during cardiopulmonary bypass. We speculate that early intervention may reduce the development of early postoperative restrictive physiology, thereby contributing to improved early postoperative outcome seen in the current era of TOF repair. The long-term consequences of early primary repair both at the molecular and functional level require further investigation.

Limitations
Because myocardium was obtained primarily from the RV outflow tract, it may not be representative of the remainder of the RV. We did not study normal or nondereased RV myocardium to determine if gene expression was different compared with normal RV. Because of limited amount of tissue available for study per patient, we were unable to study the effect of differences in gene expression on protein expression and activity.

Disclosures
None.

References
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