Association of neonatal hypoxia with lasting changes in left ventricular gene expression: An animal model

Danny Del Duca, MD,^a Guoruey Wong, BSc,^h Phan Trieu, BSc,^g Demetra Rodaros, BSc,^b Athanasios Kouremenos, BSc,^g Artavazd Tadevosyan, MSc,^{c,d,f} George Vaniotis, BSc,^{d,e,f} Louis R. Villeneuve, MSc,^d Christo I. Tchervenkov, MD,^a Stanley Nattel, MD,^{c,d,f,g} Bruce G. Allen, PhD,^{d,e,f} Terence E. Hébert, PhD,^g and Charles V. Rohlicek, MD, PhD^{b,h,i}

Objective: Innovations in pediatric cardiovascular surgery have resulted in significant improvements in survival for children with congenital heart disease. In adults with such disease, however, surgical morbidity and mortality remain significant. We hypothesized that hypoxemia in early life causes lasting changes in gene expression in the developing heart and that such changes may persist into later life, affecting the physiology of the adult myocardium.

Methods: Microarray expression analyses were performed with left ventricular tissue from 10- and 90-day-old rats exposed to hypoxia (inspired oxygen fraction 0.12) for the first 10 days after birth then subsequently reared in ambient air and with tissue from age-matched rats reared entirely in ambient air. Changes in expression of selected genes were confirmed with real-time reverse transcriptase polymerase chain reaction. Left ventricular cardiomyocytes were isolated from adult animals in both groups, and cellular morphology and viability were compared.

Results: Microarray analyses revealed significant changes in 1945 and 422 genes in neonates and adults, respectively. Changes in genes associated with adaptive vascular remodeling and energy homeostasis, as well as regulation of apoptosis, were confirmed by real-time reverse transcriptase polymerase chain reaction. The viability of cardiomyocytes isolated from hypoxic animals was significantly lower than in those from control animals ($36.7\% \pm 13.3\%$ vs $85.0\% \pm 2.9\%$, $P = .024$).

Conclusions: Neonatal hypoxia is associated with significant changes in left ventricular gene expression in both neonatal and adult rats. This may have physiologic implications for the adult myocardium.

Supplemental material is available online.

From the Divisions of Cardiovascular Surgery^a and Cardiology,^b Montréal Children's Hospital—McGill University Health Centre; the Department of Medicine,^c Institut de Cardiologie de Montréal^d; Department of Biochemistry,^e Université de Montréal^f; and the Departments of Pharmacology and Therapeutics,^g Physiology,^h and Pediatrics,ⁱ McGill University, Montréal, Québec, Canada.

Supported by grants from the Canadian Institutes of Health Research and the Heart and Stroke Foundation of Québec (to C.V.R., T.E.H., and S.N.). T.E.H. is a Chercheur National of the Fonds de la Recherche en Santé du Québec, and D.D. holds studentships from the Fonds de la Recherche en Santé du Québec and the Montréal Children's Hospital Research Institute.

Read at the Thirty-fourth Annual Meeting of The Western Thoracic Surgical Association, Santa Kailua-Kona, Hawaii, June 25–28, 2008.

Received for publication July 2, 2008; revisions received April 1, 2009; accepted for publication April 27, 2009.

Address for reprints: Terence E. Hébert, PhD, Department of Pharmacology and Therapeutics, McGill University, McIntyre Medical Sciences Building, 3655 Promenade Sir William Osler, Room 1303, Montréal, Québec, Canada H3G 1Y6 (E-mail: terence.hebert@mcgill.ca) or Charles V. Rohlicek, MD, PhD, Department of Cardiology, Montréal Children's Hospital, McGill University Health Centre, 2300 Tupper St, Room D-365, Montréal, Québec, Canada H3H 1P3 (E-mail: charles.rohlicek@mcgill.ca).

J Thorac Cardiovasc Surg 2009;138:538–46
0022-5223/\$36.00

Copyright © 2009 by The American Association for Thoracic Surgery
doi:10.1016/j.jtcvs.2009.04.042

Innovations in the practice of pediatric cardiovascular surgery have resulted in a growing population of adults with repaired or palliated cyanotic congenital heart disease.¹ These patients may, however, have significant impairments in exercise tolerance, an increased incidence of arrhythmias, and a risk of premature death. In addition, the risks of morbidity and mortality after reoperative surgery in adults with congenital heart disease remain significant.² Although the reasons for these observations remain unclear, they may in part be related to alterations in cardiac structure and function brought on by developmental alterations during the period of hypoxemia before reparative surgery.

Our group uses a rat model of neonatal hypoxia to study the effect of cyanotic stress in early life on the physiology and biochemistry of the adult cardiovascular system as it pertains to the clinical problems related to adult congenital heart disease. With this model, we have demonstrated that a 10-day period of neonatal hypoxia is associated with significant reductions in the left ventricular peak pressure and the maximum rate of pressure increase developed in response to inotropic stimulation with dobutamine in 90-day-old adult animals.³ We also observed a marked decrease in the left ventricular protein expression of adenylyl cyclase. The latter change may provide at least a partial explanation for the observed decrease in inotropic responsiveness in the adult animals. On the basis of these

Abbreviations and Acronyms

GO = gene ontology
 RT-PCR = reverse transcriptase polymerase chain reaction

previous observations, we suggest that neonatal hypoxia may be associated with additional lasting changes in gene expression in the developing heart, and that changes in gene expression may persist into later life, thereby affecting the physiology of the adult myocardium.

The objectives of this study were as follows: (1) to apply microarray gene expression profiling analyses to an animal model of neonatal hypoxia and demonstrate that hypoxia in early life is associated with significant changes in left ventricular expression of a large number of genes, both immediately after hypoxia and persisting into adulthood; (2) to use real-time reverse transcriptase polymerase chain reaction (RT-PCR) to confirm changes in genes specifically associated with adaptive vascular remodeling and energy homeostasis, as well as the regulation of programmed cell death, in both 10- and 90-day-old animals; and (3) to use cardiomyocyte isolation experiments to provide evidence that left ventricular cardiomyocytes of adult animals exposed to neonatal hypoxia are significantly less resistant to the effects of physiologic stressors, such as ischemia–reperfusion.

MATERIALS AND METHODS**Animal Model**

Experiments were conducted with male Sprague-Dawley rats. The experimental animals were placed in a normobaric, poly(methyl methacrylate) chamber (width 63 cm, height 43 cm, depth 60 cm) into which a continuous flow of air and pure nitrogen gas mixture was delivered, resulting in a continuous inspired oxygen fraction of 0.12. The flow rates of the gases were controlled with individual flowmeters, and the inspired oxygen fraction was verified routinely with an oxygen analyzer (Applied Electrochemistry model S-3A/I; AEI Technologies, Inc, Naperville, Ill). The animals were exposed, together with their mothers, to continuous hypoxia for days 1 to 10 of life, with the day of birth considered as day 0. The degree of hypoxic exposure was selected to expose the animals to a significant hypoxic insult in early life while limiting acute hypoxia-related mortality, which our group has observed to increase significantly with lower inspired oxygen fractions and longer exposure periods. After the hypoxic exposure, animals were reared in normal ambient air. Control animals were reared in ambient air only and had no exposure to hypoxia. Analyses were carried out with the left ventricular free walls of hearts that were surgically removed from 10- and 90-day-old rats. At the time of tissue harvest, all animals were anesthetized with 60 mg/kg intraperitoneally injected sodium pentobarbital. The hearts were removed and rinsed in ice-cold physiologic saline solution, and the left ventricular free walls were isolated and immediately frozen in specimen tubes immersed in liquid nitrogen. Cardioplegia was not used at the time of tissue harvest. The experimental protocol was reviewed and approved by the Montréal Children's Hospital Research Institute committee on animal care.

Microarray Gene Expression Profiling

RNA was extracted from the left ventricular free wall tissue obtained from freshly frozen hearts of 6 neonatal and 6 adult animals, with 3 experimental

and 3 control animals in each group. Total RNA was isolated and purified with the Tri-Reagent protocol (Sigma-Aldrich, Oakville, Ontario, Canada) according to manufacturer instructions, and analyses of RNA quantity and quality were carried out with the NanoDrop 1000 spectrophotometer (Fisher Scientific, Nepean, Ontario, Canada) and agarose gel electrophoresis. Samples were used for 12 individual Affymetrix Rat Microarray RAE230 2.0 GeneChip assays (Affymetrix, Inc, Santa Clara, Calif), carried out according to established protocols.⁴ Microarray hybridization reactions and data collection were performed at the McGill University and Génome Québec Innovation Centre, Montréal, Québec, Canada. Experiments were paired for comparative gene expression assays, permitting the identification of differential gene expression patterns in response to neonatal hypoxia for both 10- and 90-day-old animals. Each microarray interrogated expression patterns with probe sets representing more than 25,000 genes in the rat genome.

Microarray expression data were subsequently analyzed using the FlexArray software package, version 1.2 (<http://genomequebec.mcgill.ca/FlexArray/index.php>). Probe intensity data were normalized across replicate arrays by robust multi-array average⁵ and differential gene expression was calculated with the significance analysis of microarrays.⁶ All values were expressed as positive or negative fold changes. *P* values were validated by calculating the false discovery rate⁷ to generate *q* values less than 0.15. The resulting lists of genes determined to be significantly upregulated or downregulated were imported into the online Database for Annotation, Visualization, and Integrated Discovery (<http://david.abcc.ncifcrf.gov>).⁸ The functional annotation tools available on this software were used for gene ontology (GO) classification according to biologic process, molecular function, and cellular component.

RT-PCR

The expression levels of genes specifically associated with adaptive vascular remodeling and energy homeostasis, as well as the regulation of apoptosis, were evaluated with Rat RT2 Profiler PCR Arrays (SuperArray Bioscience Corporation, Frederick, Md) containing defined sets of genes in these pathways. Total RNA was prepared from freshly frozen left ventricular tissue as described previously, and the SuperArray Bioscience RT2 First Strand Kit (SuperArray Bioscience) was used to synthesize complementary DNA. PCR was performed with RT2 Real-time ROX PCR Master Mix (SuperArray Bioscience) and a Stratagene Mx3000p instrument (Stratagene, La Jolla, Calif). Amplification plots, dissociation curves, and threshold cycle values were generated by the Mx3000p software after data collection, and expression fold changes were calculated for each gene by the threshold cycle method.⁹

Isolation of Cardiomyocytes

Experiments were performed with 6 adult 90-day-old rats: 3 control animals and 3 animals exposed to neonatal hypoxia. Each animal was anesthetized with ketamine and xylazine hydrochloride (both 10 mg/kg intraperitoneally) and injected with heparin (1.0 U/kg body weight intraperitoneally). The heart was exposed through sternotomy, rapidly excised, and immersed in ice-cold Tyrode solution containing 140-mmol/L sodium chloride, 5.5-mmol/L potassium chloride, 1.0-mmol/L magnesium chloride, 0.32-mmol/L potassium phosphate, 10-mmol/L dextrose, 5 mmol/L N-2-hydroxyethylpiperazine-*n*'-2-ethanesulfonic acid, 2.0-mmol/L calcium chloride, adjusted with sodium hydroxide to a pH of 7.50 and equilibrated with 100% oxygen. The ascending aorta was cannulated in a retrograde fashion, and the heart was mounted on a Langendorff system and perfused at 6.5 mL/min at 37°C in solutions equilibrated with 100% oxygen. The heart was initially perfused with Tyrode solution containing 0.2-mmol/L calcium chloride for 3 minutes, followed by perfusion with calcium-free Tyrode solution for an additional 5 minutes. Each heart was then perfused by recirculation for 40 minutes with Tyrode solution containing 0.5-mg/mL collagenase type 2 (37,700 collagenase activity units per heart; Worthington Biochemical Corporation, Lakewood, NJ), 0.1% weight/volume bovine serum albumin, and 0.07-mmol/L calcium chloride. After collagenase

digestion, the heart was again perfused with Tyrode solution containing 0.2-mmol/L calcium chloride for 5 minutes, removed from the Langendorff system, and immersed in Kraftbrühe medium containing 20-mmol/L potassium chloride, 10-mmol/L ethyleneglycotetraacetic acid, 10-mmol/L potassium phosphate, 20-mmol/L taurine, 70-mmol/L L-glutamate, 10-mmol/L glucose, 10-mmol/L β -hydroxybutyric acid, 40-mmol/L mannitol, and 0.1% weight/volume bovine serum albumin, adjusted with sodium hydroxide to a pH of 7.35. The left ventricular free wall was isolated from each heart and divided into smaller pieces with scissors. To promote cell dissociation, the solution and tissue fragments were resuspended with a transfer pipette, and the suspension was filtered through a 200- μ m nylon mesh. Cardiomyocytes were gravity sedimented for 15 minutes, the supernatant was discarded, and the cardiomyocyte pellet was resuspended several times in Kraftbrühe medium containing 0.1- to 0.2-mmol/L increases in extracellular calcium chloride until a concentration of 1 mmol/L was reached. After each increase in calcium, the cell suspension was allowed to stabilize for 5 minutes. The final cellular pellet was resuspended in 5 mL of Joklik modified Eagle medium containing 25-mmol/L sodium hydrocarbonate, 1.2-mmol/L magnesium sulfate, 1-mmol/L DL-carnitine, and 1-mmol/L calcium chloride, adjusted with sodium hydroxide to a pH of 7.45 and maintained at 37°C under 100% oxygen.

Analysis of Cardiomyocyte Viability and Morphology

Freshly isolated cardiomyocytes were immediately examined under a light microscope. Viable cells were defined as having a rodlike shape and a clear pattern of cross-striations and were counted with a hemocytometer. Nonviable cells were also counted, and the percentage viability was determined. Confocal microscopy with an unmodified Zeiss LSM-510 system (Carl Zeiss MicroImaging, Inc, Thornwood, NY), with photomultiplier tube detection and 63/1.4 oil Plan-Apochromat 10 $\times$ objective (Carl Zeiss) was used to generate high-resolution images.

Data were expressed as mean $\pm$ SEM. Significant differences in cardiomyocyte viability between experimental and control cell isolates were calculated with the Student *t* test.

RESULTS

Microarray Gene Expression Analysis

To determine the effect of neonatal hypoxia on global patterns of left ventricular gene expression both immediately after hypoxia and 3 months after the termination of the hypoxic exposure, microarray expression profiling was performed with cardiac tissue collected from 10- and 90-day-old animals. After data normalization and statistical analysis, we identified those genes for which there were greater than 1.5-fold upregulation or downregulation changes in expression level. The results of these initial analyses are summarized in Figure 1. For the 10-day-old animals, statistically significant ($P \leq .05$) changes in 1945 individual genes were recorded. Among this group of genes, 557 (28.6%) were upregulated and 1388 (71.4%) were downregulated. For the 90-day-old animals, there were 422 individual genes showing significant changes in expression. Of these, 163 (38.6%) genes were upregulated and 259 (61.4%) were downregulated (Figure 1, C).

Functional Classification of Profiled Genes

To investigate the potential biologic relevance of the changes in gene expression engendered by neonatal hypoxia

FIGURE 1. Volcano plots generated with significance analysis of microarrays illustrating global changes in left ventricular gene expression in 10-day-old (A) and 90-day-old (B) animals after neonatal hypoxia. Individual genes are represented as points, and log of fold change (x-axis) is plotted against negative log of *P* value (y-axis). Thresholds have been adjusted such that genes situated between two vertical lines have less than 1.5-fold upregulation or downregulation, and genes with *P* values greater than .05 are situated below horizontal line. Genes situated in either right or left upper quadrant (darker diamonds) are considered to be significantly upregulated or downregulated, respectively. C, Numeric representation of gene expression changes summarized for 10- and 90-day-old animals.

TABLE 1. Expression analyses for genes associated with adaptive responses to hypoxia in 10- and 90-day-old rats

Gene		10 d		90 d	
Symbol	Title	Microarray	RT-PCR	Microarray	RT-PCR
<i>Angptl4</i>	Angiopoietin-like 4	19.7 (.001)	50.9 (.001)	−1.41 (.180)	1.41 (.522)
<i>Eno1</i>	Enolase 1, α	1.73 (.0002)	1.78 (.023)	−1.26 (.030)	1.34 (.369)
<i>Hmox1</i>	Heme oxygenase (decycling) 1	2.30 (.008)	1.99 (.034)	−1.19 (.107)	1.16 (.558)
<i>Notch1</i>	Notch gene homolog 1	−1.29 (.017)	−2.55 (.001)	1.03 (.644)	1.61 (.269)
<i>Slc2a4</i>	Solute carrier family 2 (facilitated glucose transporter), member 4	−2.00 (.001)	−1.62 (.023)	1.17 (.028)	1.38 (.165)
<i>Ldha</i>	Lactate dehydrogenase A	1.66 (.021)	1.73 (.003)	1.07 (.164)	1.21 (.312)
<i>Pdia2</i>	Protein disulfide isomerase associated 2	—*	−3.03 (.017)	—*	−1.26 (.514)
<i>Pea15</i>	Phosphoprotein enriched in astrocytes 15	—*	−1.37 (.002)	—*	1.33 (.092)
<i>Hk2</i>	Hexokinase 2	−1.15 (.681)	−1.3 (.275)	1.46 (.010)	1.58 (.029)
<i>Id1</i>	Inhibitor of DNA binding 1	1.54 (.003)	1.54 (.092)	1.06 (.419)	1.46 (.031)
<i>Cdc42</i>	Cell division cycle 42 homolog	−1.35 (.110)	−2.56 (.129)	−1.24 (.330)	−1.14 (.009)

Numeric values indicate calculated fold change in gene expression. *P* values are indicated in parentheses. RT-PCR, Reverse transcriptase–polymerase chain reaction. *Expression data for these genes were not available from the microarray experiments because corresponding probe sets were not included on the GeneChip arrays.

in both 10- and 90-day-old animals, a detailed evaluation of our gene lists was performed with the Database for Annotation, Visualization, and Integrated Discovery (DAVID). This permitted the classification of large groups of genes according to molecular function and associated biochemical pathways. Neonatal hypoxia was associated with significant changes in genes involved in a number of GO classifications at both 10 and 90 days. These included coordinate changes in a total of 148 and 143 individual biologic processes at 10 and 90 days, respectively. We also observed changes in different cellular components (28 at 10 days and 70 at 90 days), as well as molecular functions (62 at 10 days and 78 at 90 days). We focus here on GO terms that implicated the regulation of apoptosis altered in the neonatal animals and energy homeostasis altered in the adult animals (Table E1). These two areas were chosen for further study.

RT-PCR Validation of Gene Expression Analysis

Expression patterns of selected genes specifically associated with adaptive vascular remodeling and energy homeostasis, as well as the regulation of programmed cell death, were subsequently studied in greater detail with RT-PCR to validate the results of the microarray expression profiling experiments. Table 1 presents the expression analyses for a series of genes associated with adaptive vascular remodeling and energy homeostasis, particularly those associated with hypoxia. The genes listed were included if concordance in direction of fold change, in addition to statistical significance, was observed between data from microarray and RT-PCR experiments. A limited number of additional genes of interest for which there were statistically significant changes in expression by RT-PCR only were also included. In the latter cases, concordance in direction of fold change by both experimental approaches was generally observed. The data are consistent with the global changes in gene expression observed by genome-wide microarray analyses

(Figure 1) in which more changes in gene expression were recorded at 10 days than at 90 days. In addition to the greater statistical significance, the magnitudes of fold changes, particularly by RT-PCR, were also higher at 10 days. Interestingly, the genes with expression significantly changed at 90 days were generally not changed significantly in the 10-day-old animals, suggesting that these changes unfolded through development after the initial period of hypoxia. As shown in Table 1, neonatal hypoxia was associated with significant changes in the expression of three genes associated with adaptive changes to hypoxia, including the regulation of endothelial cell function and vascular remodeling, in the 10-day-old animals. There were significant upregulations of angiopoietin-like 4 (*Angptl4*) and heme oxygenase 1 (*Hmox1*) and downregulation of notch gene homolog 1 (*notch1*). Neonatal hypoxia was also associated with significant changes in the expressions of four genes involved in glucose metabolism and energy homeostasis in the 10-day-old animals. Enolase 1α (*Eno1*) and lactate dehydrogenase A (*Ldha*) were upregulated, whereas solute carrier 2a4 (*Slc2a4* or *Glut4*) and phosphoprotein enriched in astrocytes 15 (*Pea15*) were downregulated. Finally, there was a 3-fold downregulation according to RT-PCR of protein disulfide isomerase 2 (*Pdia2*), a gene that encodes an endoplasmic reticular protein that functions in the structural arrangement of peptides during their maturation.¹⁰ The mentioned changes in left ventricular gene expression observed at 10 days were not maintained in the 90-day-old animals. Neonatal hypoxia resulted in significant changes according to RT-PCR in three additional genes at 90 days, however, including upregulation of hexokinase 2 (*Hk2*), a key glycolytic enzyme that plays an adaptive role in regulating adenosine triphosphate balance in the context of hypoxic stress,¹¹ and the inhibitor of DNA binding 1 (*Id1*), which supports adaptive tissue angiogenesis in the adult phenotype.^{12,13}

TABLE 2. Expression analyses for genes associated with the regulation of apoptosis in 10- and 90-day-old rats

Gene		10 d		90 d	
Symbol	Title	Microarray	RT-PCR	Microarray	RT-PCR
<i>Nfkb1</i>	Nuclear factor of κ light polypeptide gene enhancer in B cells 1	−1.47 (.029)	−3.05 (.048)	−1.08 (.139)	1.26 (.548)
<i>Bax</i>	<i>Bcl2</i> -associated X protein	−1.22 (.074)	−1.73 (.035)	1.14 (.039)	1.73 (.038)
<i>Bik</i>	<i>Bcl2</i> -interacting killer	−2.72 (.004)	−2.36 (.009)	1.21 (.082)	−1.08 (.658)
<i>Casp3</i>	Caspase 3	−1.19 (.055)	−6.34 (.078)	−1.02 (.875)	1.21 (.469)
<i>Casp9</i>	Caspase 9	−1.41 (.049)	−2.07 (.040)	1.06 (.323)	1.19 (.211)
<i>Pycard</i>	PYD and CARD domain containing	−1.91 (.009)	−4.31 (.059)	−1.16 (.046)	1.07 (.571)
<i>Sphk2</i>	Sphingosine kinase 2	−1.65 (.003)	−3.37 (.037)	1.01 (.840)	1.34 (.296)
<i>Tnfrsf12</i>	Tumor necrosis factor ligand superfamily member 12	−1.53 (.015)	−5.39 (.017)	1.10 (.138)	1.84 (.091)
<i>Traf2</i>	Tumor necrosis factor receptor–associated factor 2	−2.33 (.004)	−2.15 (.026)	1.12 (.033)	1.67 (.256)
<i>Traf4</i>	Tumor necrosis factor receptor–associated factor 4	−1.49 (.034)	−2.66 (.034)	1.16 (.176)	1.29 (.339)
<i>Bnip3</i>	<i>Bcl2</i> /adenovirus E1B 19 kd–interacting protein 3	2.21 (.013)	−2.37 (.255)	1.28 (.036)	1.75 (.006)
<i>Gadd45a</i>	Growth arrest and DNA damage–inducible 45 α	−1.14 (.457)	−8.11 (.114)	1.55 (.003)	1.71 (.049)
<i>Prlr</i>	Prolactin receptor	1.31 (.025)	1.55 (.623)	−1.07 (.290)	−1.36 (.026)
<i>Tp53</i>	Tumor protein 53	—*	−3.20 (.004)	—*	1.73 (.032)

Numeric values indicate calculated fold change in gene expression. *P* values are indicated in parentheses. RT-PCR, Reverse transcriptase–polymerase chain reaction. *Expression data for this gene were not available from the microarray experiments because corresponding probe sets were not included on the GeneChip arrays.

The influences of neonatal hypoxia on the left ventricular expression of genes associated with the regulation of apoptosis in the 10- and 90-day-old animals are summarized in Table 2. These results also demonstrate general trends consistent with data from the genome-wide microarray analyses, as well as the expression patterns of genes specifically associated with vascular remodeling and energy homeostasis (Table 1); that is, a greater number of significant changes were observed in the 10-day-old animals. In addition, changes in particular genes were recorded at 90 days that were not significant immediately after the termination of the hypoxic exposure. Notably, there were two genes—*Bcl2*-associated X protein (*Bax*) and tumor protein 53 (*Tp53*)—whose expressions remained significantly altered at both 10- and 90-days, despite a reversal in the direction of their fold change.

In the 10-day-old animals, neonatal hypoxia resulted in the significant downregulation of five proapoptotic genes, including *Bax*, *Bcl2*-interacting killer (*Bik*), caspase 9 (*Casp9*), tumor necrosis factor ligand superfamily member 12 (*Tnfrsf12*), and tumor necrosis factor receptor–associated factor 4 (*Traf4*). The proapoptotic caspase 3 (*Casp3*) and PYD and CARD domain–containing gene (*Pycard*) also showed trends toward significant downregulation (−6.34, *P* = .078, and −4.31, *P* = .059, respectively, by RT-PCR), but these trends did not reach statistical significance. Neonatal hypoxia was also associated with the significant downregulation of three antiapoptotic genes, including nuclear factor of κ light polypeptide gene enhancer in B cells 1 (*Nfkb1*), sphingosine kinase 2 (*Sphk2*), and tumor necrosis factor receptor–associated factor 2 (*Traf2*). As shown in Table 2, neonatal hypoxia was associated with significant changes at 90 days in the expressions of four genes involved with the regulation of apoptosis. There was significant

upregulation observed in three genes involved in mediating programmed cell death, including growth arrest and DNA damage–inducible 45 α (*Gadd45a*), *Tp53*, and *Bcl2*/adenovirus E1B 19 kd–interacting protein 3 (*Bnip3*). Finally, we noted downregulation of the gene encoding the prolactin receptor (*Prlr*). Prolactin modulates aspects of immune system function by stimulating cell proliferation and prolonging cell survival. The expression of prolactin and its receptor has been reported to confer cellular protection against apoptosis.¹⁴

Neonatal Hypoxia and Cardiomyocyte Fragility

To study the potential physiologic implications of the described changes at the single-cell level, the effect of isolated heart ischemia–reperfusion on cardiomyocyte morphology and viability was examined in the 90-day-old animals. To study their cellular morphology in greater detail, the freshly isolated cardiomyocyte populations were examined with confocal microscopy. Immediately after cardiomyocyte isolation from control animals, dense groups of viable, rod-shaped cells were observed (Figure 2, A), suggesting that the isolation protocol was well tolerated. In contrast, cells isolated from age-matched animals exposed to neonatal hypoxia underwent hypercontracture and death during the cardiomyocyte isolation (Figure 2, B). This suggests that neonatal hypoxia resulted in a reduction of the ability of adult left ventricular cardiomyocytes to withstand particular physiologic stresses, including ischemia–reperfusion. As shown in Figure 2 (C), the proportion of viable cardiomyocytes isolated from the left ventricle of adult animals exposed to neonatal hypoxia was markedly lower than that isolated from age-matched control animals (36.7% $\pm$ 13.3% vs 85.0% $\pm$ 2.9%, *P* = .024).

A

B

C

DISCUSSION

This study provides evidence that neonatal hypoxia is associated with significant changes in a series of genes associated with the regulation of vascular remodeling. Three of these changes (*Angptl4*, *Hmox1*, *Notch1*) were observed in the 10-day-old animals, whereas one gene (*Id1*) was significantly changed at 90 days. Remarkably, we observed a 50-fold upregulation, as determined by RT-PCR, of *Angptl4*, a gene that encodes a protein that functions in endothelial cells to control angiogenesis through the transduction of survival and migration-inducing signals.¹⁵ Although the gene was originally identified as being predominantly expressed in liver and adipose tissue,¹⁶ it is clear that *Angptl4* expression in the heart is induced by neonatal hypoxia. Significant upregulation was also observed in the expression of *Hmox1*, a gene that encodes a cytoprotective protein with anti-inflammatory functions that stimulates both proliferation of endothelial cells and angiogenesis.¹⁷ The expression of *Notch1* was significantly downregulated. *Notch1* plays an important role in the regulation of endothelial cell development and proliferation.¹⁸ Finally, we observed a significant upregulation of *Id1*, which plays an important role in the regulation of cellular differentiation and cell cycle progression, as well as supporting adaptive tissue angiogenesis in the adult phenotype.^{12,13} We did not observe changes in the levels of the hypoxia-inducible factor type 1 or 2 at the level of messenger RNA either in the microarray analyses or by RT-PCR (data not shown). In preliminary experiments, however, we have demonstrated that levels of hypoxia-inducible factor 1 α protein were increased in hearts isolated from adult animals (D.D., C.V.R., and T.E.H, unpublished observations), suggesting that this key regulator of hypoxia-mediated changes may be relevant in our model.

That the quantity of significant changes in gene expression decreased after termination of the neonatal hypoxic stress and maturation to adulthood might have been predicted. Our observation of significant changes in the expression of certain genes at 90 days that were not found to be altered at 10 days was less expected. This suggests a temporal evolution in the expression patterns of different groups of genes initiated by neonatal hypoxia that may influence the development of the cardiovascular system in the adult exposed to a hypoxic insult in early life. The developmental implications of changes occurring during and immediately after hypoxic exposure compared with those observed long after the termination of the insult remain to be determined.

FIGURE 2. Representative confocal microscopy images of freshly isolated left ventricular cardiomyocytes from 90-day-old control animals (A) and age-matched animals exposed to neonatal hypoxia (B). C, Percentage viability of cardiomyocytes after isolation procedure. Data presented are mean $\pm$ SEM of 6 independent experiments. Asterisk indicates $P = .024$.

Tissue hypoxia may prompt vascular remodeling and development in response to secreted growth factors and chemokines.¹⁸ Human beings living at high altitude who are exposed to chronic hypoxia have been shown to have numerous secondary arterial branches originating from the main coronary arteries, in addition to an increase in the number of peripheral arterial ramifications.¹⁹ Cyanotic congenital heart disease has been associated with adaptive remodeling of the coronary microcirculation, leading to greater arteriolar diameter.²⁰ Furthermore, carotid artery intima-media thickness, a surrogate measure of general atherosclerosis risk and progression, has been found to be directly correlated with the severity of hypoxic stress in patients with obstructive sleep apnea.²¹ We have previously demonstrated significant changes in the luminal diameter and intima-media thickness of left ventricular arterioles isolated from adult animals exposed to neonatal hypoxia.²² Taken together, these observations suggest that hypoxic stress promotes vascular remodeling that may be associated with lasting changes in vascular morphology. The molecular basis underlying the control of arterial collateral growth and vascular remodeling is still incompletely understood. We provide evidence, however, that the changes in vascular architecture observed after hypoxic stress at least in part might be correlated with alterations in gene expression that would favor adaptive vascular remodeling.

A major cellular adaptation to hypoxia is the transition from oxidative phosphorylation to glycolysis as the principle mechanism of generating adenosine triphosphate.²³ Our study demonstrates that neonatal hypoxia is associated with changes in the expression of four genes involved in glucose metabolism and energy homeostasis in 10-day-old animals. We observed upregulations of *Eno1* and *Ldha*, two genes encoding important glycolytic enzymes. We also observed a downregulation of *Slc2a4*, which encodes the insulin-sensitive glucose transporter 4 (*Glut4*) involved in insulin-activated glucose uptake.²⁴ We also observed the downregulation of *Pea15*, which encodes a ubiquitously expressed cytosolic phosphoprotein implicated in glucose metabolism and the pathophysiology of insulin resistance.²⁴ Although these changes were not observed at 90 days, there was significant upregulation of *Hk2*, an additional key glycolytic enzyme involved in the cellular uptake and utilization of glucose as well as the regulation of adenosine triphosphate balance in the context of hypoxic stress.¹¹

Our results suggest not only that neonatal hypoxia is associated with significant changes in genes associated with energy homeostasis immediately after the hypoxic insult but that certain changes in additional genes may occur in the adult phenotype, long after the termination of the hypoxic period. The upregulation of left ventricular *Hk2* expression at 90 days may be associated with adaptive changes in glucose use and myocardial energetics in the adult phenotype. There is accumulating evidence suggesting that energy

metabolism and cell survival are closely related processes and that mitochondrial hexokinases may be the central link between them.²⁵ Specifically, the interaction between hexokinases and the mitochondrial outer membrane has been associated with stabilization of mitochondrial integrity and protection against cytochrome c release triggered by proapoptotic signals, including *Bax*.²⁶ The upregulation of *Hk2* induced after neonatal hypoxia may therefore be a form of cardioprotection. Indeed, the concept of hypoxic preconditioning, and its influence on different biologic systems, has been previously described.²⁷ The detailed mechanisms underlying these effects, however, remain to be elucidated. Furthermore, whereas preconditioning is usually associated with short or intermittent hypoxic exposures, the animal model used here differs significantly in that it represents a relatively longer, sustained hypoxic exposure.

Cardiomyocyte apoptosis has been observed in the context of myocardial infarction, as well as in the setting of ventricular hypertrophy, dilatation, and heart failure.²⁸ For these reasons, we were interested in understanding the influence of neonatal hypoxia on the left ventricular expression patterns of specific biochemical regulators of apoptosis. In the 10-day-old animals, neonatal hypoxia resulted in significant downregulations of five proapoptotic genes, as well as three antiapoptotic genes. Neonatal hypoxia was also associated with significant changes in the expressions of four additional genes controlling the regulation of apoptosis in the adult animals. These changes included the upregulations of three genes known to have proapoptotic function and the downregulation of one gene that confers protection against apoptosis. Notably, whereas the changes recorded at 10 days were mixed with regard to favoring or protecting against apoptosis, those observed at 90 days showed a clear trend toward predisposing the cell toward apoptosis.

The processes of ischemia–reperfusion are of central importance with respect to patients undergoing cardiac surgery, and three basic forms of injury may occur during cardiopulmonary bypass: myocardial stunning, apoptosis, and myocardial infarction. There is increasing evidence that apoptotic cell death in cardiomyocytes as a result of ischemia–reperfusion contributes significantly to the development of infarction, as well as to the loss of cells surrounding the infarct area.²⁹ These observations may be particularly relevant for adults with a history of cyanotic congenital heart disease undergoing reoperation, because these patients are known to face a significant risk of complications and mortality during such interventions.² Our study suggests that neonatal hypoxia results in increased fragility of cardiomyocytes in procedures designed to isolate them. In the latter experiments, transient global myocardial ischemia is invariably engendered during the surgical excision of the heart, until perfusion is reinstituted with warm, oxygenated physiologic buffers. Hypoxic stress in neonatal cardiomyocytes has been associated with morphologic and

biochemical changes characteristic of apoptosis, as well as with the activation of proapoptotic intracellular signaling pathways.³⁰ The influence of neonatal hypoxia on the adult phenotype with regards to (1) the expressions of genes associated with the regulation of apoptosis and (2) the isolated cardiomyocyte responses to physiologic stress have not been previously described. It may be reasonable to speculate that the considerably higher mortality observed in cardiomyocytes isolated from adult animals exposed to neonatal hypoxia could be correlated, at least in part, with changes in the expression paradigms of genes controlling the regulation of programmed cell death such as *Bnip3*.³¹

Several limitations of this study must be acknowledged. First, our animal model subjects not only newborn rats but also their mothers to hypoxic conditions for 10 days. Hypoxia in the mother, the only source of nutrition for her offspring, may alter her ability to nourish the litter, which could also affect gene expression in the young and should be considered a potential confounder. We have demonstrated that prolonged systemic hypoxia in the immature rat is associated with immediate as well as long-term changes in gene expression. The genome wide microarray investigation provides a global indication of the numbers of genes affected by neonatal hypoxia. Caution must be exercised, however, in the interpretation of changes in specific genes. In this regard, GO mapping suggests that at the very least changes are occurring in groups of genes with important regulatory functions. Real-time RT-PCR provides confirmation of changes in gene expression, but from a practical standpoint its application is limited to small sets of genes. Of significance is that both genome wide microarray analysis and smaller scale RT-PCR indicated similar patterns of change. Despite this confirmation, it remains to be established that such changes in gene expression are ultimately reflected in changes in protein expression and physiologic function. The changes in gene expression described after neonatal hypoxia were confirmed to be significant at the transcriptional level by two independent techniques. Message stability, posttranslational modifications, or alterations in the rate of degradation of the corresponding proteins may, however, influence the ultimate biologic effects of changes in gene expression per se.

Further care must be taken in translating results obtained in rats to the human situation. Clearly, there are important similarities as well as differences in the cardiovascular systems of the two species. From a developmental standpoint, there are also inherent differences in fetal maturity at birth, as well as the subsequent rates and patterns of development of rats and human beings, making direct extrapolation from a 10-day-old rat to a specific human infant age difficult. Nevertheless, it should be considered that the hypoxic stress in our work was applied to immature rats for the first 10 days of life, with weaning occurring only at 21 days, and that myocardial tissue was obtained from adult rats at 3 months,

at least 1 month after they had achieved sexual maturity. Finally, we recognize that systemic hypoxia is an imperfect model of cyanotic heart disease. In this study, however, we investigated changes in gene expression in the left ventricular free wall. The left ventricular free wall is predominantly composed of myocardium and should be equally affected by oxygen lack produced by systemic hypoxia and by the hypoxemia associated with cyanotic heart malformations.

In conclusion, we have shown with this animal model that neonatal hypoxia is associated with significant global changes in left ventricular gene expression immediately after the termination of hypoxia and also later in adulthood. Functional classification indicates that these genes are involved in a number of important metabolic and regulatory signaling pathways associated with diverse aspects of cellular function. More specifically, our findings suggest that neonatal hypoxia results in significant alterations in the expressions of genes associated with the regulation of adaptive vascular remodeling, the maintenance of energy homeostasis, and the control of apoptotic cell death. A limited number of previous investigations involving gene expression analyses after whole-organism hypoxia have demonstrated changes in the expressions of significant numbers of genes in various tissues.³² To our knowledge, however, this is the first report to demonstrate that neonatal hypoxia is associated with lasting changes in gene expression in the adult left ventricular myocardium.

References

1. Marelli AJ, Mackie AS, Ionescu-Ittu R, Rahme E, Pilote L. Congenital heart disease in the general population: changing prevalence and age distribution. *Circulation*. 2007;115:163-72.
2. Berdat PA, Immer F, Pfammatter JP, Carrel T. Reoperations in adults with congenital heart disease: analysis of early outcome. *Int J Cardiol*. 2004;93:239-45.
3. Rohlicek CV, Viau S, Trieu P, Hébert TE. Effects of neonatal hypoxia in the rat on inotropic stimulation of the adult heart. *Cardiovasc Res*. 2005;65:861-8.
4. Tipps RS, Mumtaz M, Leahy P, Duncan BW. Gene array analysis of a rat model of pulmonary arteriovenous malformations after superior cavopulmonary anastomosis. *J Thorac Cardiovasc Surg*. 2008;136:283-9.
5. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*. 2003;4:249-64.
6. Tusher VG, Tibshirani R, Chu G. Significant analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A*. 2001;98:5116-21.
7. Dudoit S, Shaffer JP, Boldrick JC. Multiple hypothesis testing in microarray experiments. *Stat Sci*. 2003;18:71-103.
8. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4:44-57.
9. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25:402-8.
10. Ko HS, Uehara T, Nomura Y. Role of ubiquitin associated with protein-disulfide isomerase in the endoplasmic reticulum in stress-induced apoptotic cell death. *J Biol Chem*. 2002;277:35386-92.
11. Ye G, Donthi RV, Metreveli NS, Epstein PN. Overexpression of hexokinase protects hypoxic and diabetic cardiomyocytes by increasing ATP generation. *Cardiovasc Toxicol*. 2005;5:293-300.
12. Kim HJ, Chung H, Yoo YG, Kim H, Lee JY, Lee MO, et al. Inhibitor of DNA binding 1 activates vascular endothelial growth factor through enhancing the stability and activity of hypoxia-inducible factor-1α. *Mol Cancer Res*. 2007;5:321-9.

13. Sakurai D, Tsuchiya N, Yamaguchi A, Okaji Y, Tsuno NH, Kobata T, et al. Crucial role of inhibitor of DNA binding/differentiation in the vascular endothelial growth factor-induced activation and angiogenic processes of human endothelial cells. *J Immunol.* 2004;173:5801-9.
14. Clevenger CV, Freier DO, Kline JB. Prolactin receptor signal transduction in cells of the immune system. *J Endocrinol.* 1998;157:187-97.
15. Augustin HG, Koh GY, Thurston G, Alitalo K. Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Nat Rev Mol Cell Biol.* 2009;10:165-77.
16. Kersten S, Mandard S, Tan NS, Escher P, Metzger D, Chambon P, et al. Characterization of the fasting induced adipose factor FIAF, a novel peroxisome proliferator-activated receptor target gene. *J Biol Chem.* 2000;275:28488-93.
17. Bussolati B, Mason JC. Dual role of VEGF-induced heme-oxygenase-1 in angiogenesis. *Antioxid Redox Signal.* 2006;8:1153-63.
18. Phng LK, Gerhardt H. Angiogenesis: a team effort coordinated by notch. *Dev Cell.* 2009;16:196-208.
19. Arias-Stella J, Topilsky M. Anatomy of the coronary circulation at high altitude. In: Knight J, ed. High altitude physiology: cardiac and respiratory aspects. London: Churchill-Livingstone; 1971. p. 149-51.
20. Dedkov EI, Perloff JK, Tomanek RJ, Fishbein MC, Guterman DD. The coronary microcirculation in cyanotic congenital heart disease. *Circulation.* 2006;114:196-200.
21. Minoguchi K, Yokoe T, Tazaki T, Minoguchi H, Tanaka A, Oda N, et al. Increased carotid intima-media thickness and serum inflammatory markers in obstructive sleep apnea. *Am J Respir Crit Care Med.* 2005;172:625-30.
22. Akhavein F, Seifert EL, Rohlicek CV. Effects of neonatal hypoxia on coronary arteriolar morphology in adult rats [abstract]. *Cardiol Young.* 2005;15(Suppl S2):26-7.
23. Webster KA, Gunning P, Hardeman E, Wallace DC, Kedes L. Coordinate reciprocal trends in glycolytic and mitochondrial transcript accumulations during the in vitro differentiation of human myoblasts. *J Cell Physiol.* 1990;142:566-73.
24. Lima GA, Anhe GF, Giannocco G, Nunes MT, Correa-Giannella ML, Machado UF. Contractile activity per se induces transcriptional activation of SLC2A4 gene in soleus muscle: involvement of MEF2D, HIF-1a, and TR transcriptional factors. *Am J Physiol Endocrinol Metab.* 2009;296:E132-8.
25. Robey RB, Hay N. Mitochondrial hexokinases: guardians of the mitochondria. *Cell Cycle.* 2005;4:654-8.
26. Hammerman PS, Fox CJ, Thompson CB. Beginnings of a signal-transduction pathway for bioenergetic control of cell survival. *Trends Biochem Sci.* 2004;29:586-92.
27. Engelman DT, Chen CZ, Watanabe M, Kulshrestha P, Das DK, Rousou JA, et al. Hypoxic preconditioning enhances functional recovery after prolonged cardioplegic arrest. *Ann Thorac Surg.* 1995;59:428-32.
28. Sabbah HN. Apoptotic cell death in heart failure. *Cardiovasc Res.* 2000;45:704-12.
29. Mentzer RM Jr, Jahania MS, Lasley RD. Myocardial protection. In: Cohn LH, Edmunds LH Jr, eds. Cardiac surgery in the adult. 2nd ed. New York: McGraw-Hill; 2003. p. 413-38.
30. Long X, Boluyt MO, Hipolito ML, Lundberg MS, Zheng JS, O'Neill L, et al. p53 and the hypoxia-induced apoptosis of cultured neonatal rat cardiac myocytes. *J Clin Invest.* 1997;99:2635-43.
31. Graham RM, Frazier DP, Thompson JW, Haliko S, Li H, Wasserlauf BJ, et al. A unique pathway of cardiac myocyte death caused by hypoxia-acidosis. *J Exp Biol.* 2004;207:3189-200.
32. Rupert JL. Genomics and environmental hypoxia: what (and how) we can learn from the transcriptome. *High Alt Med Biol.* 2008;9:115-22.

TABLE E1. Functional classification of changes in gene expression after neonatal hypoxia

Biological process	Cellular component	Molecular function
Age 10 d		
Programmed cell death (181)	Mitochondrion (195)	Ubiquitin–protein ligase activity (33)
Response to hypoxia (36)		
Negative regulation of programmed cell death (67)		
Regulation of programmed cell death (140)		
Negative regulation of apoptosis (66)		
Apoptotic program (21)		
Regulation of apoptosis (139)		
Cell death (184)		
Tricarboxylic acid cycle intermediate metabolic process (10)		
Glycolysis (29)		
Glucose metabolic process (65)		
Antiapoptosis (48)		
Apoptosis (180)		
Regulation of caspase activity (18)		
Age 90 d		
ATP biosynthetic process (13)	Mitochondrial membrane (67)	Ubiquitin–protein ligase activity (31)
Regulation of metabolic process (293)	Mitochondrial inner membrane (54)	Oxygen transporter activity (6)
Oxidative phosphorylation (17)	Mitochondrial part (93)	
Protein import into mitochondrion (6)	Mitochondrial outer membrane (15)	
Protein targeting to mitochondrion (10)	Mitochondrial envelope (71)	
ATP metabolic process (15)	Mitochondrion (201)	
Response to reactive oxygen species (11)		
Fatty acid oxidation (13)		

Values in parentheses represent the total number of genes associated with the corresponding process/component for which a significant change in expression was recorded. *ATP*, Adenosine triphosphate.