Influence of Hypoxia on Nitric Oxide Synthase Activity and Gene Expression in Children With Congenital Heart Disease: A Novel Pathophysiological Adaptive Mechanism

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Background—Chronic hypoxia has been shown to modulate nitric oxide (NO) responses in different cell models, but the relationship between hypoxia and NO synthase (NOS) regulation in humans was not studied. We studied the relationship between endothelial and inducible NOS (eNOS and iNOS) activities and expression and chronic hypoxia in children with cyanotic and acyanotic congenital heart defects.

Methods and Results—Right atrial tissue was excised from 18 patients during cardiac surgery. eNOS and iNOS activities were measured by conversion of L-[H3]arginine to L-[H3]citrulline. Gene expression of eNOS and iNOS was quantified by competitive reverse transcription–polymerase chain reaction. The eNOS activity and expression were significantly reduced in cyanotic hearts compared with acyanotic hearts: 0.38 ± 0.14 versus 1.06 ± 0.11 pmol · mg⁻¹ · min⁻¹ (P < 0.0001) and 0.54 ± 0.08 versus 0.80 ± 0.10 relative optical density (ROD) of cDNA (P < 0.0001), respectively. In contrast, iNOS activity and expression were significantly higher in cyanotic than in acyanotic children: 7.04 ± 1.20 versus 4.17 ± 1.10 pmol · mg⁻¹ · min⁻¹ (P < 0.0001) and 2.55 ± 0.11 versus 1.91 ± 0.18 ROD of cDNA (P < 0.0001), respectively.

Conclusions—Hypoxia downregulates eNOS activity and gene expression in cardiac tissue from patients with cyanotic congenital heart defects. By contrast, iNOS activity and expression are increased in cyanotic children and may represent an alternative mechanism to counteract the effects of hypoxia in the cardiovascular system. Therefore, a novel adaptive mechanism during hypoxia is suggested.

Key Words: nitric oxide synthase • hypoxia • heart defects, congenital

Nitric oxide (NO) is one of the most important regulatory factors of the cardiovascular system. NO is synthesized from L-arginine by NO synthases (NOS), a family of isoenzymes with distinct functional, biological, and regulatory properties. The neuronal type I (nNOS) and the endothelial type III (eNOS) are both constitutively expressed and Ca²⁺-calmodulin–dependent enzymes and are regulated predominantly at the posttranslational level, being expressed in specific tissues. The nNOS and eNOS genes are localized in chromosomes 12 and 7, respectively. The inducible type II (iNOS) is expressed in macrophages and leukocytes in response to appropriate stimuli and is localized on chromosome 17.

Small amounts of NO are involved in signaling of neurotransmission and vascular tone regulation. Much larger NO concentrations are provided by iNOS; in fact, iNOS activation is associated with 10²- to 10³-fold larger NO output than eNOS. The iNOS is a cytokine or bacterial endotoxin-inducible and Ca²⁺-independent enzyme. The iNOS activity is sustained, in part, because of its Ca²⁺ independence. NO produced by iNOS has been implicated in many pathophysiological states leading to myocardial dysfunction.

Chronic hypoxia modulates NO responses in different cell models, but the relationship between hypoxia and NOS regulation in cardiac tissue is not well understood, and it has not yet been investigated in humans. McQuillan et al found decreased NO production and eNOS expression in endothelial cells exposed to chronic hypoxia. In contrast, Archer et al showed that induction of iNOS is resistant to hypoxia in mesangial cells. Recently, Toporsian et al demonstrated, in rat aorta, that eNOS expression is downregulated by prolonged hypoxia. In humans, a possible role of NO in hypoxia has not been studied. It is conceivable that pathophysiological changes in reactive nitrogen species might modulate the consequences of hypoxia. In this context, children with cyanotic heart defects are exposed to chronic hypoxia.
congenital heart disease offer a unique opportunity to investigate these issues. Hence, the aim of the present study was to analyze the relationship between the expression of NOS isoforms and hypoxia in cardiac tissue of infants with cyanotic and acyanotic congenital heart defects, to test the hypothesis that NOS expression is influenced by oxygen tension.

**Methods**

**Subjects**

The right atrial appendage (30 mg) was excised from 18 consecutive infants undergoing cardiac surgery, before cardiopulmonary bypass. Patients were divided into 2 groups: A, 9 infants with acyanotic congenital heart disease without pulmonary hypertension; and B, 9 infants with cyanotic congenital heart disease with SaO2<90%.

In all cases, the atrial tissue was obtained, immediately frozen in liquid nitrogen, and stored at −80°C for subsequent biochemical analysis.

2D echocardiography was used to confirm the diagnosis in all infants and to estimate pulmonary arterial pressure in the acyanotic group. Preoperative cardiac catheterization also was performed in 7 infants in the cyanotic group.

Hemodynamic data were obtained at the time of surgery before cardiopulmonary bypass onset. Heart rate was obtained by direct electric monitoring with a Hewlett-Packard (HP) monitor; arterial blood pressure was obtained by a direct invasive method using a pressure transducer (HP-104). Blood samples from a radial or femoral arterial catheter were obtained in both groups at the time of tissue biopsy to determine PaO2, PaCO2, SaO2, and pH by direct measurement (Meter EML-100). Infants were ventilated during surgery in a Drager EVITA 2 ventilator, in volume control modality, with FiO2=100%.

Informed consent was obtained from all parents before the procedure. The protocol was approved by the human subjects review committee.

**NOS Activity Measurement**

NOS activity was measured in supernatants from right atrial tissue as described by McKeel et al., with some modifications. The NOS assay is based on the biochemical conversion of L-arginine to L-citrulline by NOS.

The tissue was homogenized in ice-cold Tris-HCl buffer (mmol/L: Tris-HCl 20, EDTA 10, EGTA 10; pH 7.4) with a Teflon homogenization buffer (HEPES 50 mmol/L, EDTA 10 mmol/L; pH 5.5). Cation-dependent (eNOS) activity was calculated as the difference of samples of human right atrium homogenates were determined by the Bradford Coomassie brilliant blue method with BSA as the standard and homogenization buffer as blank. This method does not allow separation of neuronal and endothelial components of NOS; because the neuronal component is probably small and mRNA assessment is specific for eNOS (see below), we refer to the activity here as eNOS.

**NOS Synthase Expression: Reverse Transcription–Polymerase Chain Reaction**

Total cellular RNA was isolated from human right atrium with Trizol reagent (Gibco BRL, Life Technologies). After DNA digestion (QI DNAse RNAase-free, Promega Corp), 1 μg of total RNA from each preparation was reverse transcribed in the presence of RNAase inhibitor (RNAsIn, Promega Corp) in a reaction volume of 20 μL containing 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3.0 mmol/L MgCl2, 10 mmol/L DTT, 2.0 mmol/L dNTP, 200 U of Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Gibco BRL), and 1 μg of oligo(dT)12 to 18 primer. The reaction was carried out at room temperature for 10 minutes and at 37°C for 60 minutes and terminated by heating at 100°C for 5 minutes. The reverse-transcribed cDNA (2 μL) was amplified in a final volume of 50 μL by polymerase chain reaction (PCR) under standard conditions (1.5 mmol/L MgCl2, 450 μmol/L dNTP, 2.5 U Taq polymerase) with specific primers for human eNOS and iNOS and GAPDH designed on the basis of published cDNA sequences (Teng et al.14). GAPDH was used as an internal control for the coamplification. To identify the optimal amplification conditions, a series of pilot studies was performed with a thermal cycler with temperature gradient at the annealing step (Eppendorf Mastercycler gradient, Ependorf-Netheler-Hinz), various amounts of reverse transcription (RT) products from 2 to 200 ng RNA, and 20 to 35 cycles of PCR amplification. The primers and experimental conditions for RT-PCR are summarized in Figure 1. The amplification was carried out with an initial denaturing cycle at 94°C for 5 minutes and the subsequent cycles as follows: denaturation at 94°C, annealing (as described in Table 1); and extension, 45 seconds at 72°C. PCR products (10 μL per lane) were electrophoresed with 1% agarose gel containing ethidium bromide 0.5 μg/mL. The gel was subjected to ultraviolet light and photographed. The band intensities were measured by use of a software package (Kodak Digital Science, Eastman Kodak Co), and the signals were expressed relative to the intensity of the GAPDH amplicon in each coamplified sample.

**Figure 1.** Primers and experimental conditions (RT-PCR). S indicates sense; AS, antisense.

**Table 1.** Arterial Blood Gas Values (Room Air) Before Surgery: Cyanotic Group

<table>
<thead>
<tr>
<th>Patient</th>
<th>SaO2 %</th>
<th>PaO2 mm Hg</th>
<th>PaCO2 mm Hg</th>
<th>pH</th>
<th>Arterial</th>
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Statistical Analysis
Multivariate ANOVA was used to compare eNOS and iNOS activities and expression between acyanotic and cyanotic groups. The comparisons of age, weight, and sex distributions between cyanotic and acyanotic groups were done with the Kruskal-Wallis test for continuous variables and Pearson $\chi^2$ test for categorical variables. Pearson correlation coefficients were calculated to study the relation of eNOS and iNOS activity and expression and levels of $\text{SaO}_2$, $\text{PaO}_2$, $\text{PaCO}_2$, and pH. A value of $P<0.05$ was considered significant.

Results
Patient Characteristics
Among the 9 infants with acyanotic congenital heart disease, there were 5 girls and 4 boys. The underlying diagnoses were atrial septal defect (ASD) secundum, 3 patients; ventricular septal defect (VSD) perimembranous, 4 patients; and VSD associated with ASD, 2 patients.

Among the 9 infants with cyanotic heart defects, 5 were boys and 4 girls. The basic diagnoses were tetralogy of Fallot, 4 patients; complete transposition of the great arteries, 2 patients; tricuspid atresia, 1 patient; pulmonic atresia, 1 patient; and hypoplastic left heart syndrome, 2 patients. Patient characteristics are shown in Tables 1, 2, 3, and 4.

eNOS and iNOS Activities
The eNOS activity was significantly lower in cyanotic than in acyanotic hearts: $0.38 \pm 0.14$ versus $1.06 \pm 0.11$ pmol · mg$^{-1}$ · min$^{-1}$; $P<0.0001$ (Figure 2a). In contrast, iNOS activity was significantly augmented in the cyanotic compared with the acyanotic group: $7.04 \pm 1.20$ versus $4.17 \pm 1.10$ pmol · mg$^{-1}$ · min$^{-1}$; $P<0.0001$ (Figure 2b).

NOS Activities and Expressions and Blood Gases
There were no statistically significant correlations between $\text{SaO}_2$, $\text{PaO}_2$, $\text{PaCO}_2$, or pH and NOS activities and expressions in both groups, and there also were no statistically significant correlations between arterial blood gas values before surgery in the cyanotic group and NOS gene expression.

Discussion
The present study shows that activity and gene expression of eNOS assessed in the right atrial appendage of infants with congenital heart disease are significantly lower in cyanotic than in acyanotic infants; in contrast, activity and expression of iNOS are significantly higher in cyanotic than in acyanotic children. To the best of our knowledge, this is the first study documenting these facts in human beings. Hypoxia elicits a variety of adaptive responses at the tissue, cellular, and molecular levels; these responses require an $O_2$ sensor coupled to a signal transduction system, which in turn activates the functional response. Although much has been learned about the signaling systems activated by hypoxia, no consensus exists regarding the nature of the underlying $O_2$ sensors. This study showed that changes in the pattern of NOS isoforms underlie the pathophysiological consequences of...
hypoxia in cyanotic congenital heart disease. This raises the possibility that changes in reactive nitrogen species may be involved in adaptive mechanisms to chronic hypoxia. It should be noted that the acyanotic children were older than the cyanotic ones, although the difference was not statistically significant; however, the nature of our study does not permit us to exclude or confirm the influence of age on the results.

Hypoxia occurs commonly in cardiopulmonary diseases. Previous studies in failing hearts indicated that eNOS activity and expression in cardiac tissue can be downregulated in low contractile states. Our study suggests another mechanism that seems to downregulate eNOS activity by oxygen tension. In both mechanisms, shear stress and oxygen seem to work as input signals.

Studies of the effects of hypoxia on eNOS activity and expression, however, have yielded conflicting results. Hypoxic incubation (zero O2 for 24 hours) decreased eNOS activity and expression in endothelial cells from human umbilical cords and saphenous veins. In contrast, Arnet et al. reported that eNOS activity and expression were increased in bovine aortic endothelial cells after 24 hours of incubation at 1% O2. This variability probably reflects differences in species and vascular beds, methods used to maintain the cells, and duration and severity of the hypoxic exposures. Our findings of downregulation of eNOS activity and expression in cardiac tissue of hypoxic patients is in agreement with the findings of Toporsian et al. These authors showed, in rat aorta in vivo, that hypoxic downregulation of eNOS expression is a relevant mechanism used by endothelium to allow a predominance of substances that enhance vascular contraction through the removal of an inhibitor of vasoconstriction, seeking to redistribute the flow to vital organs in hypoxic conditions. We found, in cardiac tissue, the same relationship as occurred in the rat aorta. One possible mechanism to explain the downregulation of eNOS in hypoxic cardiac tissue would be the production of NO by increasing iNOS expression and activity.

Studies of iNOS expression under hypoxic conditions offered contradictory results in animal species as well. Thus, Kacimi et al. found that hypoxia is a negative regulator of iNOS in neonatal rat myocytes. Jung et al. and Kitakaze et al. however, also using rat myocytes, found the opposite. In addition, Melillo et al. found increased iNOS expression by hypoxia in a macrophage cell line. Human cardiac cells had not been investigated, however, under hypoxic conditions with regard to iNOS activity and gene expression.

The meaning of higher iNOS expression and activity found in the cyanotic group compared with the acyanotic group remains speculative. We hypothesize that it may represent an alternative pathway to equilibrate the balance between relaxing and constrictor factors in cardiovascular system subjected to chronic hypoxia.

In the present study, although samples from atrial appendages of patients with acyanotic heart diseases cannot be considered matched controls, they also show a clear presence of iNOS activity, although their levels were lower than in cyanotic
children. One unanswered question is what the levels of iNOS expression in normal children would be. It is possible that our patients have a systemic activation of cytokines due to their underlying cardiac diseases, or there might be a mechanism to increase NO production by the iNOS pathway to counterbalance vasoconstrictor substances in some diseases.

Study Limitations

Some limitations must be considered. For instance, demonstration of NOS activities in tissue homogenates cannot identify the cell type expressing NOS within the myocardium. We applied the citruline assay to NOS activities as established by Moncada’s group.18 This approach to differentiating constitutive and inducible activity, based on the Ca2+ independence to iNOS, has its limitations. The data obtained by measurement of NOS expression are considered more specific for eNOS. Also, it cannot be assumed a priori that the release of free NO is enhanced in cyanotic hearts, because high-output iNOS activity may be associated with cofactor deficiency and release of superoxide and other reactive NO species.27 Finally, the potential effect of mechanical stress on the right atrium cannot be excluded. Right atrial pressure and/or size was not measured in either group of children; therefore, such a potential influence on our results remains speculative.

Conclusions

Our data indicate that hypoxia downregulates eNOS expression in cardiac tissue from patients with cyanotic congenital heart disease, supporting the idea that O2 tension probably regulates eNOS activity and expression. The higher values of iNOS expression in cyanosis may indicate an alternative mechanism to compensate for the diminished production of NO by endothelial cells in response to hypoxia. Overall, the data unravel a novel adaptive mechanism of the cardiovascular system in hypoxic conditions.

References

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