Increased Atrial and Brain Natriuretic Peptides in Adults With Cyanotic Congenital Heart Disease
Enhanced Understanding of the Relationship Between Hypoxia and Natriuretic Peptide Secretion

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**Background**—Brain natriuretic peptide (BNP) levels are used in the evaluation of patients with heart disease, yet there is little understanding of the effect of hypoxia on natriuretic peptide secretion. Furthermore, recent data suggest that oxytocin may mediate stretch-induced atrial natriuretic peptide (ANP) secretion.

**Methods and Results**—Ten patients with cyanotic congenital heart defects and 10 control subjects were studied. N-terminal proatrial natriuretic peptide and N-terminal probrain natriuretic peptide levels were 4-fold \( (P=0.02) \) and 12-fold \( (P=0.03) \) greater in cyanotic patients than in control subjects. Cyanotic patients had reduced body water compared with control subjects, although the difference did not reach statistical significance \( (P=0.22) \). In a separate group of patients, cardiac myocytes were isolated from the right atrial appendage during CABG. The amount of oxygen in the buffered saline was varied to simulate hypoxia. Isolated hypoxic atrial myocytes had 43% fewer dense surface secretory granules compared with normoxic myocytes \( (P<0.0001) \). Immunohistochemical staining demonstrated decreased ANP and BNP in hypoxic compared with normoxic right atrial tissue. Isolated myocytes also degranulated when incubated with oxytocin \( (P<0.0001) \), but there was no difference in oxytocin levels in cyanotic patients compared with control subjects \( (P=0.49) \).

**Conclusions**—ANP and BNP are markedly elevated in adults with cyanotic congenital heart disease despite reduced body water. Our results show that hypoxia is a direct stimulus for ANP and BNP secretion in human cardiac myocytes. These findings may have implications for the interpretation of BNP levels in the assessment of patients with heart and lung disease. (*Circulation. 2004;109:2872-2877.)*

**Key Words:** atrial natriuretic peptide ■ heart defects, congenital ■ hypoxia ■ natriuretic peptides, brain ■ oxytocin

Myocyte stretch is thought to be the primary stimulus for natriuretic peptide secretion.\(^1,2\) However, stretch may not be a direct stimulus of natriuretic peptide secretion. Oxytocin rather than stretch itself may induce secretion of most atrial natriuretic peptide (ANP) released from rat cardiac myocytes.\(^3\) Blood volume expansion in rats stimulates baroreceptors, initiating a complex feedback loop that results in secretion of oxytocin from the posterior pituitary gland. Oxytocin receptor number parallels ANP stores in rat cardiac myocytes, and pregnancy blocks the effect of balloon-induced stretch in the rat right atrium.\(^4,5\) Thus, it has been suggested that a primary function of oxytocin in both sexes is the control of sodium and volume homeostasis.\(^3\)

Paradoxically, ANP is also increased in patients with cyanotic congenital heart disease despite low atrial pressures.\(^6,7\) This has been implicated as the cause of the reduced cardiac output seen in these patients as biventricular contractile function is typically preserved.\(^8\) Given reduced filling pressures, stretch cannot account for increased ANP levels. Whether reduced oxygen tension acts directly on cardiac myocytes or through a feedback loop involving oxytocin or another mediator is unclear. Importantly, brain natriuretic peptide (BNP) determinations provide useful diagnostic information in patients with complaints of shortness of breath, yet little, if anything, is known of the relationship between hypoxia and BNP.\(^9\) A better understanding of the precise mechanisms modulating ANP and BNP secretion is critical if one is to achieve optimal interpretation of circulating levels and potentially manipulate plasma volume in patients with congenital or acquired heart disease. Accordingly, we studied...
the relationship between hypoxia, oxytocin, and natriuretic peptides in adults with cyanotic congenital heart disease and in isolated human right atrial myocytes.

Methods

Patients Studied
Twenty patients (14 women, 6 men) were studied in the University of Vermont and Fletcher Allen Health Care General Clinical Research Center. Ten had unrepaired cyanotic congenital cardiac defects: 6 with Eisenmenger physiology (2 each with tricuspid atresia, nonrestrictive perimembranous ventricular septal defect, and complete AV canal defect), 2 with tetralogy of Fallot, and 2 with tetralogy of Fallot and pulmonary atresia. The other 5 had structurally normal hearts. Control and cyanotic patients were matched for sex, age, body mass index, and the presence of Down syndrome (3 controls and 3 cyanotic patients had Down syndrome). No cyanotic or control patients had a history of pulmonary or peripheral edema or hypertension.

N-terminal proatrial natriuretic peptide (proANP), N-terminal probrain natriuretic peptide (proBNP), blood urea nitrogen, serum creatinine, hemoglobin, arterial oxygen saturation, body mass index, resting metabolic rate, and body water were measured in all subjects the morning after an overnight fast. Total body water (TBW) was estimated with isotope dilution of 18 O-labeled water as previously described. Resting metabolic rate was measured with an open-circuit ventilated hood indirect calorimetry system. ProANP and proBNP levels were measured by radioimmunoassay. Blood urea nitrogen, serum creatinine, and hemoglobin concentration were measured in the clinical laboratory of Fletcher Allen Health Care (Burlington, VT). Arterial oxygen saturation was measured at rest with a pulse oximeter.

Because oxytocin levels may be affected by hormonal cycling and most patients in this study were women, levels were measured in 13 men whose ANP levels were reported in a previous study (6 with cyanotic congenital heart disease, 7 with noncyanotic congenital heart defects). These 13 patients were similar to the patients in the present study in age, cardiac anomaly, oxygen saturation, hemoglobin concentration, and proANP levels. Plasma oxytocin levels were measured by radioimmunoassay at the Laboratory for Pregnancy and Newborn Research, Cornell University (Ithaca, NY).

Isolation of Myocytes From Human Right Atrial Tissue and Assessment of Degranulation
Tissue from the right atrial appendage of 49 patients undergoing CABG was used. The tip of the right atrial appendage was amputated at the time of right atrial cannulation and immediately immersed in oxygenated 2,3-butanedione monoxime (30 mmol/L) Krebs-Ringer solution at room temperature (Figure 1A). None of the patients had clinical heart failure or were known to have right atrial hypertension.

Isolated, striated, rod-shaped myocytes were obtained after collagenase digestion of the right atrial tissue (Figure 1B). Viability of the isolated myocytes was assessed with the 2-color LIVE/DEAD Reduced Biohazard Viability/Cytotoxicity Kit (Molecular Probes Inc) (Figure 1C). The number of dense surface secretory granules per myocyte was determined with Hoffman modulated contrast optics and transmitted light and used as a surrogate for total granule number. Only cells with clear cross striations were selected for analysis. Freshly isolated myocytes were used to assess average number of dense surface secretory granules per myocyte, to evaluate granule stability over time, to quantify and characterize degranulation, and to evaluate the effect of oxytocin and hypoxia. To simulate hypoxia, we varied the amount of supplemental oxygen bubbled into the buffered saline. Oxygen content and partial pressure of oxygen (Po2) in the buffered saline was then determined with an AVOX system gas analyzer (Instech model 203B). Bubbling of 100% oxygen into the buffered saline resulted in a Po2 of 150 mm Hg.

Bubbling of a 50% oxygen/50% nitrogen mixture into the buffered saline resulted in a Po2 of 75 mm Hg, and bubbling of 100% nitrogen resulted in a Po2 of 34 mm Hg.

Immunohistochemistry
Tissue from the right atrial appendage of 5 patients was obtained as described above and divided in 2. Tissue blocks were incubated for 60 minutes at 37°C in buffered saline containing 1.2 mmol/L calcium with a Po2 of either 34 or 150 mm Hg. Fixed, sectioned tissue was incubated with a 1:300 dilution of either rabbit anti-human α-ANP polyclonal antibody (n=3) or rabbit anti-human BNP-32 polyclonal antibody (n=2) (Peninsula Laboratories Inc) for 18 to 24 hours at 4°C. Slides were observed with confocal scanning laser microscopy using a 680 DF 32 filter. Optical settings were not changed when hypoxic tissue and normoxic tissue were compared.

The Institutional Review Board of the University of Vermont College of Medicine approved the patient study and the use of right atrial tissue.

Statistical Analysis
Results are expressed as mean±SD. Two-tailed, unpaired Student’s t tests were used for comparison of variables between 2 groups and ANOVA for comparison of variables between more than 2 groups. In all cases, P<0.05 was considered significant.
Results

Natriuretic Peptides and Body Water
Two patients were excluded from analysis, 1 control patient because she had exercised vigorously before the evaluation and 1 cyanotic patient because of renal dysfunction and diabetes mellitus. The remaining 18 cyanotic and control patients were well matched for gender (6 women and 3 men in each group), age (33±12 versus 30±9 years, respectively; \(P=0.64\)), and body mass index (25.8±6.9 versus 28.3±8.2 kg/m\(^2\); \(P=0.49\)). Resting heart rate was significantly greater (85±10 versus 73±11 bpm; \(P=0.03\)), resting arterial oxygen saturation was significantly lower (82±5% versus 97±2%; \(P<0.0001\)), and hemoglobin concentration was significantly greater (20.0±2.5 versus 14.2±1.2 g/dL; \(P<0.0001\)) in cyanotic patients. There was no difference in systolic (112±10 versus 117±15 mm Hg; \(P=0.39\)) or diastolic (68±10 versus 67±6 mm Hg; \(P=0.80\)) blood pressure in the 2 groups.

Mean plasma proANP and proBNP levels were 4-fold and 12-fold greater, respectively, in cyanotic patients compared with control subjects (1817±1553 versus 477±325 pmol/L; \(P=0.02\); and 122±140 versus 10±11 pmol/L, \(P=0.03\), respectively; Figure 2). There were trends toward lower TBW (26.7±6.2 versus 34.0±9.7 kg; \(P=0.07\)) and a lower ratio of TBW to body weight (42.5±5.1% versus 46.0±6.4%; \(P=0.22\)) in cyanotic patients compared with control subjects (Figure 3). There were no differences in renal function as determined by blood urea nitrogen (17±7 versus 15±4 mg/dL; \(P=0.61\)) and serum creatinine levels (0.9±0.2 versus 0.9±0.2 mg/dL; \(P=0.99\)) or resting metabolic rate (1522±215 versus 1707±383 kcal/min; \(P=0.23\)) in cyanotic patients compared with control subjects.

Figure 2. Natriuretic peptides. Plasma levels of N-terminal proANP (A) and N-terminal proBNP (B) in patients with cyanotic congenital heart defects and noncyanotic control subjects. Bars and error bars represent mean and SD, respectively. In both cases, the difference was statistically significant (\(P<0.05\)).

Figure 3. Body water. A, TBW (kg); B, ratio of body water to body weight (percent TBW) in patients with cyanotic congenital heart defects and noncyanotic control subjects. Bars and error bars represent mean and SD, respectively. Differences did not reach statistical significance (\(P=0.07\) and \(P=0.22\), respectively).
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 Plasma proANP in the 13 male patients in whom oxytocin was measured was significantly greater in cyanotic patients than control subjects and was comparable to the levels in our current patient group (1738±411 versus 538±122 pmol/L; \( P < 0.0001 \)). Despite the 3-fold-greater proANP levels, oxytocin was not increased in the cyanotic patients (2.6±1.6 versus 3.5±2.7 pg/mL; \( P = 0.49 \)).

 Degranulation of Human Atrial Myocytes

 Figure 1B shows a representative, isolated right atrial myocyte containing visible surface granules. Viability of the isolated myocytes was confirmed (Figure 1C). The distribution of the number of dense surface granules per myocyte immediately after isolation is shown in Figure 4. The number of granules per myocyte remained stable over a 60-minute incubation period (6±3, 7±2, and 7±3 granules per myocyte at 0, 30, and 60 minutes, respectively). After establishing granule stability, we next sought to induce and quantify degranulation. We used neuromimetics and potassium chloride because these agents have been shown to induce secretion of ANP from cultured neonatal rat atrial myocytes in a calcium-dependent manner. \(^{12} \) Degranulation was inducible in the isolated myocytes. Myocytes incubated for 30 minutes with potassium chloride (50 mmol/L), phenylephrine (50 \( \mu \)mol/L), or acetylcholine (0.1 \( \mu \)mol/L) had fewer granules compared with control myocytes (5±2, 5±2, and 4±2, respectively, versus 7±2 granules; \( P < 0.001 \) in each case). Myocytes suspended in buffered saline with reduced calcium concentration (3 \( \mu \)mol/L versus 1.2 mmol/L) did not degranulate when incubated with potassium chloride, phenylephrine, or acetylcholine, confirming calcium dependence of degranulation.

 Hypoxia, Oxytocin, and Myocyte Degranulation

 Isolated right atrial myocytes degranulated in response to reduced oxygen in the buffered medium. On average, there were 43% fewer surface granules in myocytes incubated in buffered saline with a \( P_0_2 \) of 34 mm Hg compared with myocytes incubated in buffered saline with a \( P_0_2 \) of 150 mm Hg (4±2 versus 7±2; \( P < 0.0001 \)). Myocytes incubated in buffered saline with a \( P_0_2 \) of 75 mm Hg had an intermediate number of granules (5±2; \( P < 0.0001 \) compared with \( P_0_2 \) of 150 and 34 mm Hg; Figure 5A). Hypoxic myocytes (\( P_0_2 \), 34 mm Hg) incubated in buffered saline with reduced calcium concentration (3 \( \mu \)mol/L) did not degranulate, consistent with a physiological rather than a nonspecific response to hypoxia (Figure 5B). The number of secretory granules was also significantly decreased in myocytes incubated with 0.01, 0.1, and 1.0 \( \mu \)mol/L oxytocin compared with control myocytes (5±2, 5±2, and 4±2, respectively, versus 8±2 granules; \( P < 0.0001 \) in all cases; Figure 6).

 Immunohistochemical staining of right atrial tissue demonstrated decreased ANP and BNP in hypoxic compared with normoxic tissue, confirming both degranulation and secretion of natriuretic peptides in hypoxic myocytes (Figure 7).

 Discussion

 Both ANP and BNP are stored in secretory granules of atrial and ventricular myocytes and may be elevated in patients with congenital and acquired heart disease. \(^{1,2,6} \) Natriuretic peptides counteract fluid retention, a characteristic of heart failure, but coincide with volume depletion in patients with cyanotic congenital heart disease. We reported that ANP during the cyanotic period was significantly less than that in the noncyanotic control patients (4 versus 7 mm Hg) despite systemic-level right ventricular pressure or univentricular hearts in the cyanotic group. \(^{6} \) ANP levels were

 Figure 5. Hypoxia and myocyte degranulation. A, Isolated right atrial myocytes from 10 patients were incubated in buffered saline solution containing 1.2 mmol/L calcium and 3 different oxygen tensions (\( P_0_2 \), 150, 75, or 34 mm Hg) at 37°C for 60 minutes. \( * P < 0.0001 \) vs \( P_0_2 \) 150 and 34 mm Hg. \( ** P < 0.0001 \) vs \( P_0_2 \) 150 mm Hg. B, To confirm that degranulation was not nonspecific response to hypoxia, isolated right atrial myocytes from 3 additional patients were incubated in buffered saline solution containing 3 \( \mu \)mol/L calcium and \( P_0_2 \) of either 150 or 34 mm Hg. Bars and error bars represent mean and SD, respectively. On average, 118 myocytes per patient were evaluated to determine granule number in normoxic and hypoxic cells.
increased whether pulmonary hypertension was present (Eisenmenger physiology) or not (tetralogy of Fallot physiology). Importantly, there were highly significant inverse relationships between ANP and resting arterial oxygen saturation, systemic cardiac index, and systemic oxygen transport.

In the present study, both proANP and proBNP were markedly elevated in the cyanotic patients compared with noncyanotic controls. The 4-fold increase in proANP was similar to the finding in our previous report. Even more striking was the 12-fold increase in proBNP levels in cyanotic patients. We found an 8% relative reduction in percent TBW in the cyanotic patients, although this trend did not reach statistical significance. To put the magnitude of this difference in perspective, an 80-kg person with 42% TBW will have approximately 3.2 L less water than an equivalent person with 46% TBW or approximately 300 mL less plasma volume. Reduced plasma volume may contribute to reduced ventricular filling, stroke volume, and cardiac output and increased blood viscosity. Consistent with reduced preload, transmitral Doppler studies have revealed reduced E-to-A wave ratio and prolonged deceleration time in adult patients with cyanotic congenital heart disease, findings that are very similar to those of the present study.

When cardiac output is reduced in adults with cyanotic congenital heart disease, it is because of volume depletion rather than ventricular dysfunction, unlike patients with heart failure.

Plasma ANP is also increased in patients with sleep apnea, and levels decrease with continuous positive airway pressure treatment. As in patients with cyanotic congenital heart disease, plasma volume is decreased, and there is an inverse relationship between plasma ANP and oxygen saturation.

Data from animals suggest that stretch-induced natriuretic peptide secretion may be more complex than previously thought and that the increase in ANP is mediated by oxytocin. However, there are no data on the effect of oxytocin in human cardiac myocytes. We developed a simple, reproducible model to evaluate and quantify degranulation of human atrial myocytes. Right atrial tissue was selected because it can be readily and safely obtained from patients undergoing cardiac surgery and because atrial myocytes are known to contain both ANP and BNP. Indeed, we found that isolated atrial myocytes degranulate in response to oxytocin. This suggests that oxytocin receptors are present on human cardiac myocytes and that oxytocin may be a mediator of natriuretic peptide secretion in patients with heart failure. However, it is clear from the present study that increased ANP and BNP levels in our cyanotic patients are not mediated by circulating oxytocin. In fact, we found a trend toward lower oxytocin levels in cyanotic patients.

Immunostaining of atrial myocytes revealed reduced ANP and BNP in hypoxic human myocytes, supporting the premises that the number of visible, dense, surface secretory granules is representative of total granule number and that decreases in number represent secretion. Our findings are

**Figure 6.** Oxytocin and myocyte degranulation. Atrial myocytes from 12 patients were incubated in buffered saline for 30 minutes at 37°C (control myocytes) or in same solution containing 3 different concentrations of oxytocin (0.01, 0.1, and 1.0 μmol/L). Bars and error bars represent mean and SD, respectively. Number of granules at all 3 oxytocin concentrations was significantly less than that in control myocytes (P<0.0001). On average, 44 myocytes per patient were evaluated to determine granule number in control and stimulated cells.

**Figure 7.** Immunohistochemistry. Right atrial tissue from 5 patients was divided in 2 and incubated in buffered saline solution with PO₂ of 150 or 34 mm Hg. Immunofluorescence-stained sections from 2 representative patients are depicted. A and B, Right atrial tissue incubated in buffered saline with PO₂ of 150 and 34 mm Hg, respectively, and stained for ANP. C and D, Right atrial tissue incubated in buffered saline with PO₂ of 150 and 34 mm Hg, respectively, and stained for BNP. All 4 sections were imaged under low-power objective (×20).
consistent with those reported in animal and cell culture preparations. Hypoxia induces secretion of ANP from isolated perfused animal hearts and cultured animal cells. Chen et al identified a 120-bp hypoxia responsive element in the promoter site of the ANP gene in cultured mouse atrial myocytes. From our results, it is reasonable to propose that hypoxia is a direct stimulus of ANP and BNP secretion in chronically hypoxic adults with congenital heart disease. These findings raise questions about the specificity of BNP assays in differentiating patients with complaints of shortness of breath secondary to heart failure from those with shortness of breath secondary to lung disease because BNP might also be elevated in patients with chronic hypoxia secondary to lung disease.

Conclusions

Optimal manipulation of natriuretic peptides and interpretation of levels in patients with and without heart disease require detailed understanding of the mechanisms controlling their secretion. We have demonstrated that oxytocin and hypoxia directly stimulate natriuretic peptide secretion. These findings suggest that oxytocin receptors are present on human right atrial myocytes and that the human ANP and BNP genes also contain a hypoxia responsive element. In the future, it will be important to determine whether oxytocin and hypoxia induce secretion of natriuretic peptides from human ventricular myocytes, a potentially large reservoir of natriuretic peptides. Manipulation of oxytocin may allow enhanced secretion of natriuretic peptides in patients with congestive heart failure. Increased red cell mass and decreased plasma volume are responses to chronic hypoxemia in adults with cyanotic congenital heart disease. Although the increase in red blood cell mass is desirable, the reduction in plasma volume may not be. We believe that blood volume expansion may significantly improve systemic oxygen transport and therefore quality of life in adults with cyanotic congenital heart disease. Consequently, inhibition of the natriuretic and diuretic effects of natriuretic peptides is a potential therapeutic target in adults with cyanotic congenital heart disease.

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