Expression of B-type Natriuretic Peptide-45 (BNP-45) Gene in The Ventricular Myocardial Induced by Systemic Chronic Hypoxia

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ABSTRACT

Aim: to investigate the expression of B-type natriuretic peptide-45 (BNP-45) gene which was induced by systemic chronic hypoxia, and whether these changes would be different from BNP-45 protein in the plasma and its mRNA in the ventricular myocardial. This study also aimed to test the hypothesis that systemic chronic hypoxia may cause heart failure.

Methods: although clinical use of BNP as a biomarker in heart failure is increasing, the specificity of BNP for heart failure is not robust, suggesting that other mechanisms beyond simple ventricular stretch stimulate BNP release. Plasma BNP levels were markedly increased in patients with coronary artery disease but without concomitant left ventricular dysfunction. Thus, elevated BNP levels do not necessarily reflect heart failure but may also result from cardiac ischemia. Sprague-Dawley male rats, weighing 220-250 g at the time of recruitment were randomly divided into 7 groups (n = 4 per group), the control normoxia group was exposed to room air, while the hypoxia group were caged in a plexiglass hypoxic chamber (8%O₂ and 92% N₂) for 1, 3, 7, 14, 21, and 28 days, respectively.

Results: our data clearly showed that plasma BNP-45 and ventricular BNP-45 mRNA concentration were markedly increased which reached its peak on day 21 after treatment.

Conclusion: regulation of BNP-45 gene expression occurred at transcription as well as post-transcription level. Systemic chronic hypoxia could result in heart failure, especially when the hypoxia is severe and prolonged.

Key words: B-type natriuretic peptipe, heart failure, chronic hypoxia, gene expression.

INTRODUCTION

Heart failure (HF) is a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood. HF is a major health care burden, and despite significant therapeutic advances, the incidence, prevalence, mortality and economic costs of HF are steadily increasing. Therefore, novel insights into pathophysiology and molecular mechanisms of HF are required to develop novel diagnostic and therapeutic approaches.

Every physician recognizes the signs and symptoms of HF, yet HF is frequently under-or overdiagnosed. The clinical diagnosis of early HF in relatively asymptomatic stages remains a major challenge. There is no single diagnostic test for HF because it is largely a clinical diagnosis that is based on a careful history and physical examination. Clinical history, symptoms and signs, when used in isolation, have only a limited value in diagnosing HF. Thus, the clinical diagnosis of HF requires careful integration of all the available information.

The natriuretic peptides (NP) are a group of hormones which are structurally similar but genetically distinct, which include atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) of myocardial cell origin and C-type natriuretic peptide (CNP) of endothelial cell origin. A fourth member of the NP, Dendroaspis natriuretic peptide (DNP), has been isolated from the venom of the green mamba snake. It has also been reported that DNP-like immunoreactivity (DNP-LI) is present in human plasma and that the plasma level of DNP-LI is elevated in patients with HF. All known natriuretic peptides share a 17-amino-acid ring.
structure formed by a disulfide bridge between two cysteine residues, essential for biological activity. NPs play important roles in the regulation of circulatory and fluid homeostasis in vertebrates.4

BNP was initially purified from porcine brain extracts and given the name brain natriuretic peptide.6 However, it was subsequently found in much higher concentrations in cardiac ventricles from patients or animals undergoing cardiac stress such as congestive HF or myocardial infarction. For this reason, it is currently referred to as BNP or “B-type natriuretic peptide,” but not “brain natriuretic peptide.”7 Human BNP is synthesized as a preprohormone of 134 residues containing a signal sequence that is cleaved to yield a 108-amino-acid prohormone. Prohormone BNP is cleaved by a circulating endoprotease, termed corin, into two polypeptides: the inactive NT-pro-BNP, 76 amino acids in length, and BNP, a bioactive peptide 32 amino acids in length. Fully processed BNP length varies between species. Human, pig, and dog BNP is of 32 amino acids, whereas rat and mouse BNP is of 45 amino acids.8

The direct effect of BNP is mediated through the natriuretic peptide receptor-A (NPR-A), which, via cyclic guanosine monophosphate (cGMP), leads to vasodilation, diuresis, and natriuresis, and reduces the activities of the renin–angiotensin–aldosterone system and the sympathetic nervous system. Increasing evidence has now established that the natriuretic peptides possess cytoprotective properties on the myocardium such as anti-ischaemic, anti-hypertrophic and anti-fibrotic.9 Thus, when considered together, the actions of BNP oppose the physiological abnormalities in HF. The clearance of all three peptides is mediated by the natriuretic peptide receptor-C (NPR-C) and the degradation by the ectoenzyme neutral endopeptidase.10

Cardiac secretion of BNP increases with the progression of HF, and plasma measurement of BNP has emerged recently as a useful, for screening, diagnostic, prognostic, treatment and monitoring treatment of HF.11 Although clinical use of BNP as a biomarker in HF is increasing, the specificity of BNP for HF is not robust, suggesting that other mechanisms beyond simple ventricular stretch stimulate BNP release.12 Plasma BNP levels may increase in patients with coronary artery disease but without concomitant left ventricular dysfunction. These results are compatible with the notion that myocardial ischemia, even in the absence of left ventricular dysfunction, augments cardiac BNP gene expression and increase plasma BNP levels. Thus, elevated BNP levels do not necessarily reflect heart failure but may also result from cardiac ischemia. However, controversy persists regarding to BNP gene expression in ventricular myocardium under normal conditions and in chronic cardiac overload. Indeed, conflicting studies both in humans and in animals describe either increased or unchanged ventricular BNP mRNA levels in HF. The reason for the normal BNP mRNA levels despite constant cardiac overload is not known but could result from transcriptional and/or translational mechanisms.13 To date, there are no reports on the molecular mechanisms responsible for regulating the BNP gene expression during chronic hypoxia in vivo.

The progress made in our understanding of the pathophysiology and treatment of HF would not have been possible without a number of animal models of HF, each one having unique advantages as well as disadvantages. Nevertheless, many different models have been developed and most are useful in investigating specific aspects of HF, such as pathogenetic mechanisms or the usefulness of new pharmacological interventions. Some animal models were replaced by protocols using cultures of cardiac myocytes allowing an accurate characterization of single cell types in a wide range of experimental conditions. However, these experiments do not permit the investigation of the integrative function of the heart in the whole organism. Therefore, investigation of HF by animal studies, especially rat models will be necessary. They extend our understanding in the pathogenesis of HF and provide information, which is not available from other experimental protocols or clinical studies.14,15

The aim of this study was to examine the effects of systemic chronic hypoxia on BNP gene expression and transcription in vivo. This recent study also aimed to test the hypothesis that systemic chronic hypoxia may cause HF.

METHODS

Experimental Animals and Protocol

Sprague-Dawley male rats, weighing 220-250 g at the time of recruitment in the protocol were randomly divided into 7 groups, with four rats in each group. The control group was housed and kept in normoxic, while the hypoxia groups were exposed to a normobaric-hypoxic stimulus (8% O2 and 92% N2) for 1 (H1), 3 (H3), 7(H7), 14 (H14), 21 (H21), or 28 (H28) days, in a plexiglass hypoxic chamber, after which they were sacrificed with ether. Control rats were housed in room air for 28 days before sacrificed. The oxygen tension inside the chambers was continuously monitored by an oxygen meter (OX-12B, MEIE Shanghai, PRC). All
animals had free access to water and standard rat chow. Water and food consumption were assessed every 2 days. We used two types of chambers, the bigger was flushed with gas-mixture. When a chamber opening was required for regular cleaning, replenish food and water, or sacrifice an animal, the small compensation chamber was first flushed with hypoxic gas before the animal is transferred into it. All animals were maintained on a 12h:12-h light dark cycle. The investigation conformed with the Guide for the Care and Use of Laboratory Animals.

Cardiac Morphologic and Histopathologic Examination

Immediately after induction of general anesthesia by using ether, the chest was opened through median sternomy. The hearts were rapidly excised and then weighed. The ventricle was sliced perpendicular to the long axis and the thickness of the ventricular wall were measured by caliper digital. Sections 4 µm thick of heart tissues were cut on a microtome and stained with periodic acid-Schiff. Immunohistochemistry procedure for detecting apoptotic cardiomyocytes were performed by TUNEL assays according to the manufacturer’s instruction (In Situ Cell Death Detection kit-Roche, USA).

Determination of Plasma BNP-45 Levels

A blood sample was withdrawn from aorta into the Lavender Vacutaner tube which contains EDTA and aprotinin, as anti-coagulation and anti-proteinase, respectively. The extraction of peptides from plasma was conducted according to the manufacturer’s instruction using C18 SPE column (Phoenix Pharm Inc, Belmont, Ca. USA). Plasma BNP-45 levels were determined by Rat BNP-45 Immuno Assay Kit, according to manual user guide (Phoenix Pharm Inc, Belmont, Ca. USA) using VMax kinetic microplate reader, which has a built-in SOFTmax Pro 4.0 Drug Discovery Edition Program for the calculation of the results.

BNP-45 Gene Expression Analysis

Total RNA was isolated from 10 mg fresh ventricular tissue using Aqua Pure RNA Isolation kit according to the manufacturer’s instruction (Bio-Rad) which the final volume of the isolated RNA was 50 µl. Total RNA concentrations were determined by UV spectrophotometer. Real-Time quantitative RT-PCR were conducted using 100 ng of total RNA for each sample with iScript One-Step RT-PCR SYBR Green kit, on the MJ Mini Opticon Real-Time PCR Detection System (Bio-Rad). Specific primer sequences for Real time qRT-PCR: BNP-45 (Access # M25297) and β-Actin (Access # NM031144) as the gene of interest and endogenous control (housekeeping) gene, respectively (Table 1), were designed by using Jelly Fish v3.2 (LabVelocity Inc, USA). RT-PCR reaction was carried out following manufacturer’s standard protocol. Briefly, PCR reaction consisted of: 25 µl 2x SYBR Green RT-PCR reaction mix, 1.5 µl of each primer, and 19 µl nuclease-free H₂O, 2 µl RNA template, and 1 µl iScript Reverse Transcriptase. PCR conditions were: synthesis cDNA at 50°C for 10 minutes; inactivation of iScript reverse transcriptase at 95°C for 5 minutes, 39 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Real-time PCR data analysis was conducted by using Comparative C_T(2-ΔΔC_T) method.

Statistical Analysis

Statistical analysis was performed by using GraphPad Prism 5.0, San Diego, CA. All data are expressed as mean ± SE. Mann-Whitney test was used for comparison of data between groups. The correlation between continuous variables was calculated by the method of Spearman. Linearity of a relationship between two variables was assessed by linear regression analysis. P-values <0.05 were considered statistically significant.

<p>| Table 1. Primer sequences for quantitative real-time RT-PCR |</p>
<table>
<thead>
<tr>
<th>Functionality</th>
<th>Genes</th>
<th>Sequences</th>
<th>Location and size</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNP-45 Forward</td>
<td>5’-TGG-GCA-GAA-GAT-AGA-CCG-GA-3’</td>
<td>357-376 =20nt</td>
<td></td>
</tr>
<tr>
<td>BNP-45 Reverse</td>
<td>5’-ACA-ACC-TCA-GCC-CGT-CAC-AG-3’</td>
<td>399-418 =20nt</td>
<td></td>
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<tr>
<td>β-Actin Forward</td>
<td>5’-ACC-ACA-GCT-GAG-AGG-GAA-ATC-G-3’</td>
<td>685-706 =22nt</td>
<td></td>
</tr>
<tr>
<td>β-Actin Reverse</td>
<td>5’-AGA-GGT-CTT-TAC-GGA-TGT-CAA-CG-3’</td>
<td>939-961 =23nt</td>
<td></td>
</tr>
<tr>
<td>Product length:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>685-961 =277bp</td>
<td></td>
</tr>
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</table>
RESULTS

Cardiac Morphometric Evaluation

Table 2 shows that left ventricle (LV) and right ventricle (RV) wall thickness become hypertrophic in proportion with the duration of treatment. Significant increase was found from the seventh day of treatment in both LV and RV, but the ventricular thickness increase ratio (hypertrophy index) of the RV wall is higher compared to LV. Rats at the 21-day hypoxia treatment (P6), hypertrophy index: 48.26% vs 32.26%, while in the 28-day hypoxia-treated group (P7), hypertrophy index: 48.8% vs 42.97%. Heart weight (HW) gradually increased early in the course of treatment, so that at the 28-day hypoxia group (P7), HW had increased by 19% compared to the control group (normoxia). Due to BW decrease and HW increase, the HW/BW ratio increased, and at the end of treatment the ratio had increased by 80% compared to control group (5.74 vs 3.19).

Histopathologic Examination

The following figures are the microphotographic images of histology sections, taken using digital camera and light microscope.

Immunohistochemistry examination showed the morphological alterations typical for apoptotic cell death, as determined by TUNEL assays.

Plasma BNP-45 Levels

Figure 3 shows significant increase in plasma BNP level from the first day of treatment, although still within normal range. After three days of treatment (H3) the concentration reached 0.98 ± 0.04 ng/ml, near the cut off point (100 ng/ml). The increase persisted, reaching the peak on day 21 (H21), 0.715 ± 0.07 ng/ml, then declined sharply at the end of treatment (H28).

Table 2. Cadiac morphologic evaluations

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normoxia</th>
<th>Hipoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day</td>
<td>3 days</td>
</tr>
<tr>
<td>LV wall Thickness, mm</td>
<td>2.79 ± 0.08</td>
<td>2.86 ± 0.09</td>
</tr>
<tr>
<td>LVH (%)</td>
<td>2.51</td>
<td>10.04</td>
</tr>
<tr>
<td>RV wall Thickness, mm</td>
<td>1.72 ± 0.06</td>
<td>1.80 ± 0.06</td>
</tr>
<tr>
<td>RVH (%)</td>
<td>4.05</td>
<td>15.12</td>
</tr>
<tr>
<td>Heart weights, mg</td>
<td>811.5 ±42.6</td>
<td>844.3 ±65.4</td>
</tr>
<tr>
<td>Body weights, g</td>
<td>254.0 ± 3.87</td>
<td>206.3 ± 9.66</td>
</tr>
<tr>
<td>HW/BW mg/g</td>
<td>3.19 ± 0.011</td>
<td>4.09 ± 0.007</td>
</tr>
</tbody>
</table>

Mean values ± SE, * (Significant difference versus normoxia P<0.05, Mann Whitney test)

Figure 1. Normoxia: 400x magnification - PAS staining. Normal cell size, clear nucleus, no signs of nuclear degeneration or necrosis

Figure 2. Hypoxia 21 days - 400x magnification - PAS staining. Massive hypertrophic-1, some nucleus disappeared and degenerated-2, perinuclear space-3, necrosis-4 and fibrosis in a number of locations-5

Impression: Histopathologic features are in line with cardiomyotic hypertrophy with signs of angiogenesis, congestion, necrosis, and fibrosis (ventricular remodeling), due to chronic systemic hypoxia.
Quantitative Real-Time RT PCR
The RT-PCR analysis shows the changes in BNP gene expression in the form of upregulation, as the increased of BNP-45 mRNA level. The increase in mRNA concentration occurred gradually and reaching the peak at 7.26 fold (H21) compared with the control group (N), then decreased again at the end of treatment, as seen in figure 4. Statistical analysis showed correlation between the concentration of BNP-45 mRNA and the plasma BNP-45 concentration, where the Spearman correlation test found $r = -0.8929$ and $P$ (two-tailed) = 0.0123, while the simple linear regression test found $r^2 = 0.920$, and $P = 0.001$ as seen in figure 5.

DISCUSSION
The heart showed a severe cardiac hypertrophy, because at the end of treatment the HW/BW ratio increased to almost 80%. Although both ventricular walls were hypertrophied, the RV hypertrophy was more dominant compared to the LV. This is thought to be due to pulmonary hypertension. Hypoxia triggered adaptation mechanism through stimulation of sympathetic nerve system (SNS), which objective is to cause vasoconstriction in the peripheral tissue including lungs, and on the other hand causing vasodilatation to vital organs such as the heart and the brain. Stimulation of SNS causes an increase of RAS, especially angiotensin II which causes vasoconstriction, thickening and remodeling of pulmonary vasculature, which at the end causes pulmonary hypertension.18 Pulmonary hypertension will subsequently increase the RV burden in pumping blood to the lungs, which in turn will cause hypertrophy. If this condition lasted for a long time, it will cause an increase in LV burden in the effort to pump the blood to systemic circulation, which at the end causes LV hypertrophy as well.19

There are several other important mechanisms that could explain ventricular hypertrophy as a result of hypoxia, among them are oxidative stress due to increased ROS formation. Increased ROS beyond the capacity of antioxidants could cause a number of changes in cell components, such as protein, lipid, and nucleotides, causing cell damage and even cell death, both through apoptosis mechanism or as a result of necrosis or autophagia.20 In addition, chronic hypoxia is known to increase the formation and activity of growth factors, and activate the hypertrophic signal. Furthermore, hypoxia causes fibrosis of the vascular endothelium, both as a direct effect or through stimulation of Ang II secretion from SNS and the one produced by the heart.21

Histopathologic examination showed ventricular hypertrophy and cardiomyocytes accompanied by structural alteration that could be observed as fading of the horizontal line structure and intercalated disk (ID).
ID is a vital structure in the heart, which serves as a connector between heart cells, forming a kind of syncytium. Damaged or faded ID structure is an evidence of ventricular remodeling, which could be caused by angiogenesis and fibrosis due to continuous RAS (Ang II) stimulation, as a result of hypoxia treatment. This also shows that the hypertrophy was no longer an adaptive-compensatory mechanism, but more likely a maladaptation due to chronic overload burden to the heart. The molecular mechanism of the event is because the ID consists of 3 main structures: gap junction, adherence junction, and desmosome. In cardiac hypertrophy due to pressure overload such as in hypoxia, the number of gap junctions decreases, while the number of adherent junctions increases, causing communication disturbance between the heart cells and heart stiffness.

Lemler performed a hypobaric-hypoxia study in one- to two-day old neonate calves exposed to 430 mm Hg barometer pressure. The calves experienced RV hypertrophy, and subsequently HF after 15 days of treatment. In the histopathology, perinuclear space was found, along with ID and horizontal line structure damage and fibrosis, while the echocardiography examination found RV dilatation. They also studied neonate calves born in the lowlands, which were then raised in a 3000-meter highland. The calves experienced RV hypertrophy and HF, causing death after 9 months of age.

Besides severe hypertrophy, structural damage, and fibrosis, immuno-histochemistry evaluation also found apoptosis of cardiomyocytes (positive TUNEL test). As known before, cardiomyocyte differentiation ends soon after birth. The growth stimulus by RAS as an effort to minimize the overload burden of the heart stimulated a compensatory hypertrophy response, not hyperplasia, and causing apoptosis in the future. Therefore every apoptosis event will reduce the number of cardiomyocytes, causing further decrease of cardiac function.

A number of studies stated that fibrosis and apoptosis is a sign of transition from adaptive hypertrophy to maladaptive hypertrophy that progresses towards HF, and some even stated that apoptosis was found in advanced HF. Ritter & Neyses stated that 4 characteristic histopathologic changes in HF are: cardiomyocyte hypertrophy, fibrosis, apoptosis, and cardiomyocyte slippage. Because these four alterations were also found in this study, we might be able to conclude that the rats in H6 and H7 group in this study, have histopathologically experienced HF. As a comparison, Hessel et al. reported that hypobaric hypoxia with 30 mg/kgBB monocrotaline (MCT) injection for 4 weeks causes compensated hypertrophy, while the 80 mg/kgBB dose administration causes HF. Corno, et al treated rats with normobaric hypoxia (10% O2) for 2 weeks, and found it to cause ventricular and cardiomyocyte hypertrophy, but did not report HF. From these results we we can conclude that there is a dose-response relationship in the pathophysiology of hypoxia-induced HF. That means, once the pathologic hypertrophy signal tract is activated, sooner or later it will lead to HF. This fact also proves the progressive nature of the pathophysiology of HF.

The other important finding in this study is the significant increase of plasma BNP-45 level on day 21 of treatment (H21), i.e. 0.715 ng/ml, which means almost 30-fold increase compared to control group or > 7 fold compared to the cut off value. Considering the high level of plasma BNP, HF is thought to already occur on day 21. Therefore, on day 14 the heart disorder is still on ventricular dysfunction state and on day 21 progresses to HF. This idea is supported by the NYHA heart failure classification which states that the increase of plasma BNP level more than 750 ng/ml is classified as class III-IV HF. Desjardins and Toussaint used the marked increase in plasma BNP level ( > 10 times higher) as a hallmark of HF. This increase is thought to occur due to chronic hypoxia which causes ischemia and damage of myocardial structure, which causes pressure overload and stretched cardiac wall, which serves as a stimulus for BNP-45 secretion. The underlying mechanism of cardiac pressure overload might be related to hypoxia pulmonary vasoconstriction (HPV) and vascular remodeling. The combination between vasoconstriction and vascular remodeling will result in pulmonary hypertension, RV hypertrophy which eventually results in HF. This also shows that the hypoxia could be severe and long-term (chronic) to be able to cause significant pressure overload.

The RT-PCR analysis showed changes in BNP gene expression in the form of upregulation, i.e. the increased mRNA BNP-45 level, along with hypoxia treatment. The increase was relatively insignificant (< 2 fold) up to day 7 (H7) and significant upregulation occurred on day 14 (H14), i.e. 2.96 times. However on the 21st day of treatment (H21) drastic increase of mRNA concentration is observed by 7,26 fold compared to control group, then plummeted at the end of treatment, as seen in figure 3. This shows that in chronic hypoxia, the regulation of BNP-45 gene expression is also found in transcription level, especially after 14 days of treatment. As a comparison, Suo et al. used angiotensin II infusion in the study of HF model due to
pressure overload in rats. They found the increase in plasma BNP, accompanied by increased BNP mRNA B level with 5.2 times peak compared with the control group. This supports the view that ventricular dysfunction starts on day 14 (H14) and HF eventually occur on day 21 (H21), due to the gradual increase of cardiac overload. This result is similar to a report from Langenickel, in the study of HF model due to volume overload with aortacaval shunt, which stated that transition of compensated state to decompensated HF is mainly attributable to changes in gene expression in BNP mRNA level leap, not by plasma BNP-45. This is logical because the increase in plasma BNP level could be found in conditions such as ischaemia, although the increase is not as high as in HF. Therefore, the measurement of plasma BNP level mainly serves as screening tool and predictor of mortality.

Statistical analysis shows (figure 5) strong correlation between plasma BNP level and ventricular mRNA, which provides the prediction that regulation of BNP expression is done by HIF-1α, which serves as a master regulator in oxygen homeostasis. This view is supported by a study by Weidemann, et al. in the rat cardiomyocyte culture, exposed to CO 0.1%. Through EMSA (electrophoretic mobility-shift assay) proved that BNP promoter contains HRE (hypoxia response element) of HIF-1α.

From this result the BNP regulation in chronic hypoxia could be illustrated as follows: When the burden of the heart increases due to various causes, the pressure in the ventricular cavity raises. This raised pressure will cause ventricular wall to stretch, which also act as a trigger for BNP release into the bloodstream, to help maintain the cardiovascular homeostasis and fluid volume. When the heart burden increases and starts to show functional failure, the second line protection will be activated, and for this aim the BNP transcription will be accelerated, so that the mRNA BNP level in the ventricular tissue increases. Then, through translation and post-translation modification process, the mRNA increase will provide additional plasma BNP supply, in the effort to repair the cardiac function. Therefore the BNP is considered as a friend within our heart.

The significant decrease in plasma BNP-45 level and ventricular mRNA level at the end of treatment (H28) might be because the cardiac function have reached terminal stage at that time, so that generally the transcription, translation, or post-translation system have become minimized, or perhaps due to other unexplained factors.

**CONCLUSION**

Systemic chronic hypoxia could result in increased plasma BNP-45 level along with the increase in BNP-45 mRNA level in the ventricle. Markedly increased of plasma BNP-45 and BNP-45 mRNA showed that systemic chronic hypoxia could result in HF, especially when the hypoxia is severe and prolonged. There were strong correlation between plasma BNP-45 and BNP mRNA. These findings suggest that the BNP-45 gene is a target for HIF-1, as a master regulator in oxygen homeostasis.

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