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Research Article

Tetrahydrobiopterin Concentrations in Normal and Coronary Artery Diseased Heart Tissue

Abstract

Background: Tetrahydrobiopterin (BH4) is a cofactor that plays a major role in cardiovascular health and disease. BH4 levels in the human heart have not been previously reported.

Objective and Methods: Using a novel LC-MS/MS method we measured BH4 and BH2 levels in human heart tissue from subjects with (n=19) and without (n=19) coronary artery disease (CAD).

Results: The concentration of BH4 was significantly lower in CAD subjects compared to controls (p=0.0006). There was a trend for a decrease in BH4/BH2 ratio (p=0.09). mRNA expression of *GCH1, NOS3* and *QDPR* was not significantly different between CAD and controls.

Conclusions: We conclude that the decrease in BH4 concentration in the left ventricular wall of subjects with CAD may be a causative factor or consequence of the failing heart.

Abbreviations

BH4: Tetrahydrobiopterin; BH2: Dihydrobiopterin; NOS: Nitric Oxide Synthase; eNOS: endothelial Noxide Synthase; nNOS: Neuronal Nitric Oxide Synthase; iNOS: Inducible Nitric Oxide Synthase; CAD: Coronary Artery Disease; LC-MS/MS: Liquid Chromatography and Tandem Mass Spectrometry; GTCH1: Guanine Triphosphate Cyclohydrolase; NOS3: Nitrous Oxide Synthase 3 – Endothelial; QDPR: Quinoid Dihydropteridine Reductase; PCA: Perchloric Acid;

Introduction

Tetrahydrobiopterin (BH4) is an essential cofactor that controls the enzymatic activity of aromatic amino acid hydroxylases as well as all forms of nitric oxide synthase (NOS), which include endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) isoforms [1]. In coronary arteries, reduced availability of BH4 in the endothelium causes decreased production of nitric oxide and an increase in eNOS-derived production of reactive oxygen species including superoxide anion and hydrogen peroxide [2,3]. Studies have demonstrated that oxidative stress causes uncoupling of eNOS and endothelial dysfunction, which is associated with chemical inactivation of BH4 and its oxidation to dihydrobiopterin (BH2) [4]. There is mounting evidence that this mechanism plays a particularly important role in coronary artery disease (CAD). For instance, low BH4 and BH4/BH2 ratio have been found to be decreased in plasma of patients with CAD and in blood vessel wall [5]. BH4 levels are reported to be reduced in heart tissue from rodent models of cardiac ischemia that results in NOS decoupling [6-8]. Both eNOS and nNOS are expressed in heart tissue, unlike in vascular and inflammatory cells [9]. However, neither BH4 nor BH2 concentrations in normal nor diseased human heart tissue have been previously reported. Here we describe the measurement of BH4 and BH2 in heart tissue from subjects with and without CAD using a novel liquid chromatography and tandem mass spectrometry (LC-MS/MS) method. Based on existing data in hearts from animal models and human coronary arteries, we hypothesized that BH4 and BH4/BH2 ratio in heart tissue will be decreased in patients with CAD compared to non-CAD controls. We also investigated mRNA expression of three key enzymes involved in BH4 synthesis, guanine triphosphate cyclohydrolase (GTCH1), nitric oxide synthase 3 – endothelial (NOS3) and quinoid dihydropteridine reductase (QDPR) in human heart tissue.

Materials and Methods

Patient samples

Detailed description of each subject is described in Supplementary Table 1. Human heart tissue (left ventricular tip, full wall thickness) was obtained at the time of transplantation from patients with end-stage heart failure due to CAD. Control samples from subjects without CAD were obtained from hearts harvested for transplantation, but unutilized for non-cardiac reasons. Both CAD and non-CAD subjects were randomly selected. The control group were comprised of subjects with cerebrovascular accident (n=10), cerebrovascular stroke (n=4), intracranial hemorrhage (n=1), cardiac arrest (n=1), suicide (n=1), head trauma (n=2) and self-inflicted gunshot (n=1). Tissue was flash frozen in liquid nitrogen according to methods we have previously described [10]. The Institutional Review Boards at the University of Colorado approved the research.

Analysis of BH4 and BH2

Standards for (6R)-5,6,7,8-tetrahydrobiopterin (BH4) and L-7,8-

dihydrobiopterin (BH2) and labeled stable isotopes ^{15}N-BH4 and ^{15}N-BH2 were obtained from Schircks Laboratories (Switzerland). Formic acid (Sigma Aldrich), methanol (Fisher Sci), dithiothreitol, heptafluorobutyric acid, ammonium acetate and water were all LC-MS/MS grade.

Sample preparation

Stock calibration standards and internal standards were prepared individually for BH4, BH2, ^{15}N-BH4 and ^{15}N-BH2 solutions at 1 mmol/L in Milli-Q water containing 0.2% dithiothreitol and stored at -80°C. Working calibrators were prepared in water containing 0.2% dithiothreitol over the range of 6.25 – 200 nmol/L. A deproteinization solution containing internal standard concentrations of ^{15}N-BH4 and ^{15}N-BH2 were prepared in 0.4M perchloric acid (PCA) containing 0.2% dithiothreitol at a final concentration of 200 nmol/L each. Sample preparation involved deproteinizing approximately 20mg of heart tissue in 4-fold 0.4M PCA internal standard solution and homogenizing with pestle using an overhead stirrer. Following PCA deproteinization the sample was centrifuged at 14800 rpm at 4°C for 10 minutes. 20 µl of supernatant was combined with 180 µl of water containing 0.2% dithiothreitol. Sample was then loaded in a 96-well microtiter plate for analysis.

LC-MS/MS of BH4 and BH2

Heart tissue BH4 and BH2 were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described with slight modifications [11]. Liquid chromatography was performed on a Shimadzu Nexera system by reversed-phase HPLC (EZ-faast AAA-MS column 250 x 2mm 3µm, Phenomenex) equilibrated at 40°C. The system consisted of a binary gradient: water (0.1% formic acid and 0.1% heptafluorobutyric acid) (Eluent A) and methanol (0.1% formic acid) (Eluent B). The flow rate was 0.20 ml/min and 10µl of processed sample was injected for analysis. The LC gradient was optimized to retain the pterin compounds on the column while eluting other ion-suppressing moieties. The initial composition of the gradient was 95% A:5% B and increased linearly to 25% A:75% B during the first 4 minutes. The concentration of eluent B was increased to 100% at 4.1 minutes and held until 5.0 minutes. At 5.1 minutes eluents were returned to initial conditions for equilibration. The total run time was 10 minutes per sample. Eluent flow from the column was diverted to waste at the beginning and end of each run and was only directed to the source for the period from 5 – 7.5 minutes. The mass spectrometry data was acquired and processed using Analyst 1.5.2 software (ABSciex).

Quantitative RT-PCR

Total RNA was isolated from human heart tissue (20 – 30 mg) in a subset of 10 control and 8 CAD subjects, using the RNeasy mini Kit (Qiagen). Reverse transcription was performed using DNase I-treated 0.25 µg total RNA with Super Script III (Invitrogen). Quantitative PCR was performed using pre-designed TaqMan gene expression assays (QDPR [Hs00165610_m1], GCH1 [Hs00609198_m1] and NOS3 [Hs01574659_m1]) on a StepOnePlus Real time PCR system (Applied Biosystems), while using 18S rRNA as internal control.

Results

LC-MS/MS analysis of BH4 and BH2 in heart tissue

The current LC-MS/MS method described was able to detect BH4 and BH2 levels in heart tissue. Representative MS chromatograms for BH4 and BH2 levels in heart tissue from a control subject is shown in Figure 1. The mean concentration of BH4 was significantly lower in heart tissue from the group with CAD compared to controls (Figure 2). Heart BH2 levels were not significantly reduced in the CAD group compared to controls, and consequently there was only a trend for a reduction in BH4/BH2 ratio (Figure 2).

mRNA expression

We found no significant difference in the levels of mRNA expression for *GCH1, NOS3*, and *QDPR* between 8 samples from patients with CAD and 10 samples from control subjects (data not shown).

Discussion

We report for the first time levels of BH4 and BH2 in human heart tissue using a highly sensitive and specific LC-MS/MS method. Compared to studies in other species, human heart BH4 levels are approximately 10-fold lower than in rat and pig heart [7,8], but similar to those found in human endothelial cells and mammary artery or saphenous vein [5,12]. Importantly, BH4 levels were found to be significantly reduced in patients with CAD. Although the mean BH4/BH2 ratio was lower in patients with CAD it did not reach statistical significance. In our analysis we looked for gender differences, however we were unable to draw any definitive conclusions since there were only a small number (n=3) of female subjects with CAD.

BH4 has been implicated in cardiac hypertrophy, ischemia, diastolic dysfunction and cardiac autonomic function [13]. BH4 acts as a critical regulator of eNOS activity and nitric oxide levels, which modulate endothelial and vascular function. Experimental evidence indicates that limited BH4 availability may contribute to eNOS dysfunction. It has been shown that in vivo coronary artery occlusion is associated with decreased eNOS dependent vasoreactivity.

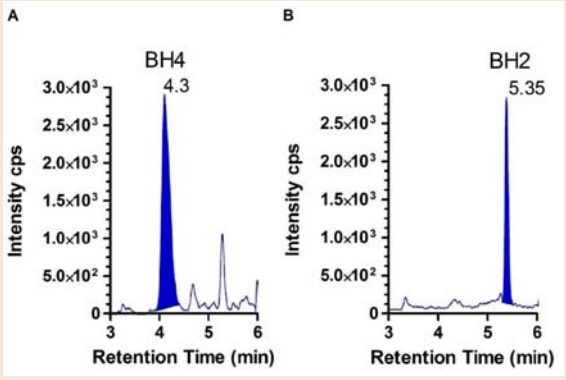

Figure 1: Representative LC-MS/MS chromatogram of (A) BH4, 76 pmol/g; and (B) BH2, 46 pmol/g, measured in human heart tissue from a control subject.

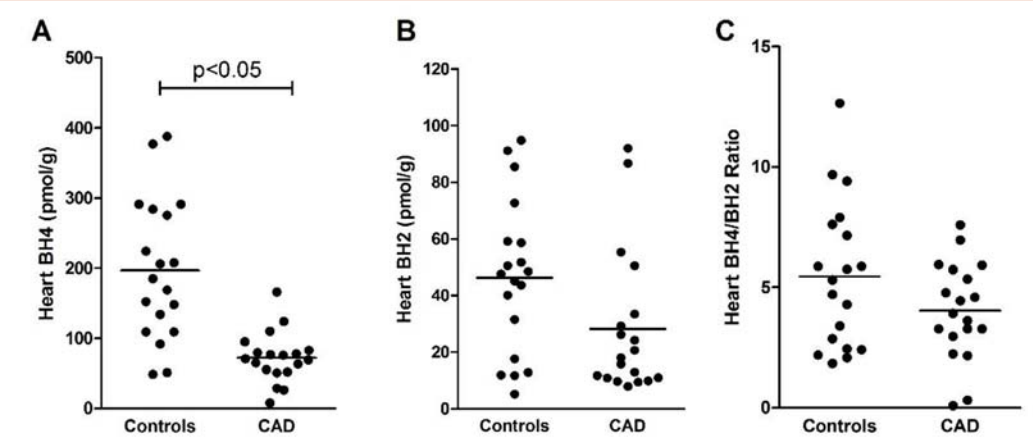

Figure 2: Heart tissue concentrations of (A) BH4; (B) BH2 and (C) BH4/BH2 ratio in control and CAD cases.

Furthermore, experimental studies in rats have demonstrated that myocardial ischemia results in depletion of BH4 levels and a subsequent loss of NOS activity that induces superoxide radical production. Our finding of low BH4 levels in the heart of subjects with CAD is consistent with these experimental observations. We did not measure BH4 levels in the coronary artery themselves and could not definitively explain the mechanism of BH4 reduction in CAD. The expression of *GCH1*, the rate limiting enzyme in the synthesis of BH4 was not different than controls as was the expression of *NOS3* and *QDPR* that recycles BH2 [13]. However, it is possible that transcriptional or post-translational changes are present that explain in part the low levels of BH4. Because of insufficient heart tissue material we were unable to measure reactive oxygen species in our study. Future studies in which oxidative stress markers are included may confirm the relationship between BH4 levels and formation of superoxide radicals in failing heart tissue.

In summary, our finding of low BH4 in the myocardium of CAD parallels the previously described abnormality in the vascular wall observed in these patients. Our findings suggests that BH4 deficiency, and possibly secondary eNOS and nNOS decoupling is present in the patients cardiovascular system in general and is not limited to the blood vessel wall and may contribute to heart failure in patients with advanced CAD. The LC-MS/MS analytical technique described will be useful for future investigations of this important vasoreactive cofactor.

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Competing Interests

None of the authors have any competing interests.

Authors' Contributions

EA developed the BH4 and BH2 analytical method, analyzed the samples and drafted the manuscript. BDL and MRRG provided the samples and collected subject data and edited the manuscript. XM performed and interpreted the quantitative PCR assays, RS initiated and organized the study and edited the manuscript. TB supervised the project, interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

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