Suppressed expression of GTP cyclohydrolase I mRNA and accelerated expression of inducible nitric oxide synthase mRNA in endomyocardial biopsy specimens from patients with dilated cardiomyopathy

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Abstract

\textbf{Background:} Tetrahydrobiopterin (BH\textsubscript{4}) is an essential cofactor of nitric oxide synthase, and GTP cyclohydrolase I (GCHI) is a rate-limiting enzyme in the biosynthesis of BH\textsubscript{4}. The expression of inducible nitric oxide synthase (iNOS) was earlier demonstrated in the ventricles of patients with dilated cardiomyopathy (DCM) although that of GCHI was not clarified. The present study was designed to determine the GCHI mRNA expression as well as to confirm iNOS mRNA expression in endomyocardial biopsy specimens from patients with DCM.

\textbf{Methods:} Clinical details were assessed in 19 patients with DCM and in 9 control subjects. The real-time reverse transcription polymerase chain reaction (PCR) was performed on total RNA extracted from endomyocardial biopsy specimens. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was quantified for use as an internal control.

\textbf{Results:} iNOS/GAPDH for the DCM samples was 4.8-fold greater than that for the control ones (\(P<0.01\)), whereas the GCHI/GAPDH for the DCM samples was reduced to 31.1\% of the control (\(P<0.05\)).
Conclusions: The increased expression of iNOS mRNA was confirmed in endomyocardial biopsy specimens from patients with DCM. The GCHI mRNA level was suppressed in these specimens. © 2004 Elsevier B.V. All rights reserved.

Keywords: Inducible nitric oxide synthase; GTP cyclohydrolase I; Endomyocardial biopsy; Dilated cardiomyopathy; Real-time reverse transcription polymerase chain reaction

1. Introduction

Cytokine-inducible, calcium-insensitive nitric oxide synthase isoform (iNOS, EC 1.14.13.39) expression was earlier demonstrated in the ventricles of patients with dilated cardiomyopathy (DCM) [1,2]. Moreover, expression of iNOS was reported in ventricular myocardium of failing hearts regardless of the underlying cause [3]. (6R)-(L-erythro-1’, 2-dihydroxypropyl)-2-amino-4-hydroxy-5, 6, 7, 8-tetrahydro-L-biopeterin (tetrahydrobiopeterin, BH₄) is an essential cofactor for the generation of nitric oxide [4]. It is synthesized de novo from GTP, and the rate-limiting step in the biosynthetic pathway is catalyzed by GTP cyclohydrolase I (GCHI, EC 3.5.4.16). Proinflammatory cytokines were reported to stimulate the de novo synthesis of BH₄ in humans [5]. This increased production of BH₄ is thought to be required to ensure a sufficient supply of the cofactor for massive production of nitric oxide by iNOS [6]. Hattori et al. [7] reported that proinflammatory cytokines stimulated not only the induction iNOS mRNA but also that of GCHI mRNA in cultured myocardium. At present, whether or not the expression level of GCHI mRNA is altered in the myocardium of patients with DCM is unclear. Thus, the present study was designed to determine the level of GCHI mRNA expression as well as to confirm the increased iNOS mRNA expression in biopsy specimens from patients with DCM by using the quantitative reverse transcription (RT)–polymerase chain reaction (PCR) method and to compare these levels with those from normal subjects.

2. Materials and methods

2.1. Patients

Nineteen patients with DCM (left ventricular ejection fraction <45%) were selected based on clinical, echocardiographic, hemodynamic, angiographic, and histological findings (14 males and 5 females ranging in age from 28 to 69 years old). One patient was classified as functional class I according to the standard of the New York Heart Association (NYHA), 11 patients as functional class II, and 7 patients as functional class III. The mean left ventricular ejection fraction of the 19 patients was 31.2 ±2.1%. Left ventricular ejection fraction was calculated by echocardiography using a modified Simpson method. For ethical reasons, the therapy for heart failure was maintained during the study. Nine patients with arrhythmia and whose biopsy samples were histologically normal and free from organic cardiovascular findings (left ventricular ejection fraction >55%) were used as the control (six males and three females ranging in age from 21 to 76 years old). Their mean left ventricular ejection fraction was 61.6 ±1.3%. All control subjects were classified as functional class I. One or two endomyocardial biopsy samples (0.5–3 mg) obtained from the right ventricle were immediately frozen in liquid nitrogen and stored at −80 °C. Informed written consent was obtained from all patients, and the study protocol was approved by the institutional review board of Fujita Health University.

2.2. Real-time RT–PCR

iNOS and GCHI mRNA levels were determined by RT–PCR amplification and quantified by the 5’ nuclease assay with fluorescent TaqMan probes analyzed with the use of real-time PCR as follows. Total RNA was extracted by the Qiagen RNeasy minicolumn procedure. cDNA was synthesized from 150 ng of total RNA with an RT kit (Qiagen) using random hexamer primers. Primers and TaqMan probe for human INOS were designed with the use of Primer Express Software (ABI, Perkin–Elmer) from the published mRNA sequence [8] (EMBL/GenBank accession No. NM 153292) with flanking primers located on separate exons to give an amplicon size of
118 bp and with the TaqMan probe straddling the exon–exon junction: forward primer, 5′-TCTGCAAGACGCTGTTAATGCACT-3′; reverse primer, 5′-CTGCAAGCTGAGCATCTT-3′; TaqMan probe, 5′-CAATGGCAACATCAGG-3′. Primers and TaqMan probe for human GCHI were designed from published mRNA sequence [9] (EMBL/GenBank accession No. U19523) with flanking primers located on separate exons, giving an amplicon size of 83 bp, and with the TaqMan MBG probe straddling the exon–exon junction: forward primer, 5′-CAAGGGCTACCAGGAGACCAT-3′; reverse primer, 5′-CCTTCACAATCACCATCTCATCA-3′; TaqMan MBG probe, 5′-CAGATGTCCTAAACGATGC-3′. Primers and TaqMan probe of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as the internal control were provided in a preoptimized kit (ABI, Perkin–Elmer). PCR reactions were performed with the use of an ABI-prism 7700 sequence detector (ABI, Perkin–Elmer). PCR amplifications were performed in a 25-μL volume in 2× PCR Master Mix (ABI, Perkin–Elmer) at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each sample was analyzed in duplicate. Results were analyzed with Sequence Detection Software (ABI, Perkin–Elmer), and the levels of expression of iNOS and GCHI mRNA were normalized to the levels of GAPDH mRNA.

2.3. Immunohistochemistry

To determine cellular localization of nitrotyrosine, the fingerprint of peroxynitrite, in the myocardium, immunohistochemical staining was conducted in paraffin-embedded sections of the heart by use of mouse anti-nitrotyrosine monoclonal antibody (Chemicon International). Experimental controls were performed by omission of the primary antibody layer. Staining intensity in endomyocardial biopsy specimens was visually graded to give a semiquantitative score. Intensity was graded between 0 and 2+ (where 0=nonspecific reactivity, 1+=weak staining, and 2+=strong staining).

2.4. Statistical analysis

All results were expressed as the mean±S.E.M. Univariate analysis was performed by using the Student’s t test for nonpaired data. A P<0.05 was regarded as significant.

3. Results and discussion

GCHI mRNA levels in endomyocardial biopsy specimens from the patients with DCM were reduced to 31.1% of control (P<0.05, Fig. 1). On the other hand, iNOS mRNA levels in endomyocardial biopsy specimens from these patients were 4.8-fold greater than those in the control subjects (P<0.01, Fig. 2).

In this study, mRNA levels of GCHI and iNOS in endomyocardial biopsy specimens were within the detectable limit when determined by the real-time RT–PCR. We show here, for the first time, that GCHI mRNA levels in endomyocardial biopsy specimens from patients with DCM were suppressed compared with those from the control subjects. In contrast, iNOS mRNA levels were confirmed to be increased significantly compared with those of the control subjects.

It was previously reported that circulating tumor necrosis factor α levels were increased in patients with heart failure [10]. Proinflammatory cytokines were shown to stimulate the expression of iNOS mRNA in the myocardium [7]. In fact, both tumor necrosis factor α and iNOS protein were demonstrated in cardiac
tissue of patients with DCM [11–13]. Our present finding of elevated iNOS mRNA expression in the DCM heart is in accordance with and confirms the report by Haywood et al. [3]. Does nitric oxide generated by iNOS lead to the development of heart failure? Nitric oxide was found to cause myocardial damage and deterioration of left ventricular function [3]. However, Heger et al. [14] documented that a cardiосpecific overproduction of nitric oxide in iNOS transgenic mice did not result in heart failure. Moreover, a linear correlation between endomyocardial expression of iNOS mRNA and left ventricular stroke volume was found in patients with dilated nonischemic cardiomyopathy [15]. In addition, myocardial iNOS gene expression was reportedly up-regulated in NYHA class II patients compared with class IV patients. We failed to find any correlation between iNOS mRNA levels in the right ventricular endomyocardial biopsy specimens and left ventricular ejection fraction in our DCM patients (data not shown). Thoenes et al. [16] reported that no iNOS protein expression was detected in failing hearts. Taken together, the data suggest that iNOS expression or nitric oxide production per se does not explain the deterioration of cardiac function. Our present new finding is that GCHI mRNA expression in the endomyocardial biopsy specimens was markedly suppressed in DCM patients. In the present study, BH₄ levels in endomyocardial biopsy specimens were not determined because of limitations of the amount of the sample. However, it is reasonable to assume that BH₄ production in the DCM heart would be markedly reduced under the condition of decreased GCHI mRNA expression in the tissue. iNOS has been shown to generate superoxide anions instead of nitric oxide in BH₄ deficiency [17]. It is thus likely that myocardial cells in DCM are exposed to superoxide anions and/or peroxynitrite, the latter of which is known to generate nitrotyrosine in the myocardium and to exert damage to cardiac tissues [18]. In the present study, the enhanced nitrotyrosine formation in histological sections obtained from DCM heart has been demonstrated [staining intensity: control (n=8); 0.38±0.10, DCM (n=19); 0.95±0.14, P<0.05]. It is known that proinflammatory cytokines induce GCHI mRNA, as well as iNOS mRNA in various tissues. Indeed, Luss et al. [19] reported an increased GCHI mRNA and protein levels in human cardiomyocytes stimulated with cytokines. Why there is a reduced GCHI mRNA expression in the present study is a question that also remains to be answered.

Our study was limited by the small amount of tissue available from diagnostic endomyocardial biopsies, which was insufficient to allow the determination of enzyme activities, such as those of iNOS and GCHI and of the levels of BH₄, nitric oxide, and superoxide anions.

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References


