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Original Contribution

Inhibition of nuclear factor κB regresses cardiac hypertrophy by modulating the expression of extracellular matrix and adhesion molecules

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ABSTRACT

Myocardial remodeling denotes a chronic pathological condition of dysfunctional myocardium that occurs in cardiac hypertrophy (CH) and heart failure (HF). Reactive oxygen species (ROS) are major initiators of excessive collagen and fibronectin deposition in cardiac fibrosis. Increased production of ROS and nuclear factor κB (NF-κB) activation provide a strong link between oxidative stress and extracellular matrix (ECM) remodeling in cardiac hypertrophy. The protective inhibitory actions of pyrrolidine dithiocarbamate (PDTC), a pharmacological inhibitor of NF-κB and a potent antioxidant, make this a good agent to evaluate the role of inhibition of NF-κB and prevention of excessive ECM deposition in maladaptive cardiac remodeling during HF. In this report, we used a transgenic mouse model (Myo-Tg) that has cardiac-specific overexpression of myotrophin. This overexpression of myotrophin in the Myo-Tg model directs ECM deposition and increased NF-κB activity, which result in CH and ultimately HF. Using the Myo-Tg model, our data showed upregulation of profibrotic genes (including collagen types I and III, connective tissue growth factor, and fibronectin) in Myo-Tg mice, compared to wild-type mice, during the progression of CH. Pharmacological inhibition of NF-κB by PDTC in the Myo-Tg mice resulted in a significant reduction in cardiac mass, NF-κB activity, and profibrotic gene expression and improved cardiac function. To the best of our knowledge, this is the first report of ECM regulation by inhibition of NF-κB activation by PDTC. The study highlights the importance of the NF-κB signaling pathway and therapeutic benefits of PDTC treatment in cardiac remodeling.

Cardiac remodeling is a complex geometric alteration that involves ultrastructural and microscopic changes in the muscle fibers of the heart. This process occurs in various stages of cardiac hypertrophy that ultimately transitions to heart failure. It is well known that collagen type I (Col I) and collagen type III (Col III) interconnect the cellular components of the heart, maintaining structural and functional integrity. In normal heart, collagen turnover is regulated by matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs) [1]. A fine balance between MMPs and TIMPs maintains the integrity of the extracellular matrix (ECM). An imbalance between MMPs and TIMPs alters the collagen turnover and leads to collagen deposition in left ventricle [1–3]. Cardiac fibrosis occurs after the abnormal accumulation of collagen within the ventricle, which ultimately leads to cardiac dysfunction. Collagen deposition is a fundamental step in cardiac fibrosis, providing the matrix for cardiac tissue remodeling, leading to abnormal accumulation of collagen within the ventricle. There are several posttranslational steps in collagen synthesis that are sensitive to reactive oxygen species (ROS), especially the activity of the enzymes prolyl hydroxylase (PHD), lysyl hydroxylase, and lysyl oxidase (LOX). PHD is required to convert proline residues to hydroxyproline, which allows the procollagen peptide chains to assume the triple-helix configuration [4]. A recent report suggests that ROS influence cofactor concentrations required for the function of PHD [6]. Once the procollagen has assumed the triple-helix conformation and been excreted, the individual collagen fibers are arranged into linear fibrils via cross-linking of lysyl hydroxylase and finally through cross-linking between large fibrils by LOX [5]. These extracellular cross-linkages are ultimately responsible for cardiac fibrosis. Furthermore, it has been reported that an increased level of ROS triggers the excessive accumulation of ECM, including collagen and fibronectin that lead to the progression of cardiac fibrosis.

Therefore, inhibition of myocardial collagen deposition and fibrosis may result in improvement of cardiac function and attenuate cardiac hypertrophy. It has been reported recently that inhibition of PHD prevents left ventricular remodeling in the thoracic aortic-

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banded rat model [2]. Also, using explanted human hearts, it has been shown recently that upregulation of LOX plays an important role in human dilated cardiomyopathy [7]. Correspondingly, a number of studies have shown that inhibition or knockout of MMPs suppresses ventricular remodeling, cardiac dysfunction, and progression of heart failure [1,8–11]. However, there is a lack of information about the systemic changes in the expression of collagen, MMPs and TIMPs, PHD, and LOX during progression of cardiac hypertrophy or compensated hypertrophy to decompensation and heart failure. This study demonstrates a relationship between NF-κB inhibition by the antioxidant pyrrolidine dithiocarbamate (PDTC) and its effects on the ECM and ECM regulatory enzymes LOX and PHD during cardiac hypertrophy (CH).

In humans, it is not possible to study the molecular changes occurring in the heart during CH; therefore, a myotrophin-overexpressing transgenic mouse model (Myo-Tg) has been suggested for studying various stages of CH in vivo [12]. This experimental mouse model has heart-specific myotrophin overexpression through the use of an α-myosin heavy chain promoter. The overexpression of myotrophin in the myocardium of the Myo-Tg mouse initiates hypertrophy that gradually progresses from chronic hypertrophy to heart failure (HF) over a span of 36 weeks. Myo-Tg mice exhibit left ventricular (LV) hypertrophy, multiple focal fibrosis, myocyte necrosis, pleural effusion, and compromised cardiac function, which closely mimic human heart failure [12]. Therefore, this model was used to study the molecular events during the progression of hypertrophy and its transition to HF.

NF-κB, a ubiquitous inducible transcription factor, activates a number of genes, including inflammatory cytokines [13–15]. A key role for NF-κB is becoming apparent in the pathophysiology of ischemia–reperfusion injury, ischemia preconditioning, and unstable angina [16–21]. Furthermore, NF-κB regulates MMPs and thus plays an important role in the pathogenesis of cardiac remodeling [22]. However, the most direct mechanism, its regulation in cardiac collagen remodeling, has not been considered to date.

Our previous findings showed that NF-κB activation is implicated in the development of CH in spontaneously hypertensive rats, as well as in the failing human hearts [23,24]. In a separate study, we demonstrated that NF-κB expression increased during the initiation, progression, and culmination of cardiac hypertrophy at 4, 16, and 36 weeks, respectively, in the Myo-Tg model compared to its wild-type counterpart [25]. Furthermore, to establish a cause-and-effect relationship between NF-κB activation and remodeling of cardiac mass, we showed that knocking down the NF-κB p65 gene using lentivirus-mediated siRNA delivery led to attenuation of cardiac mass with improved cardiac functions. As ECM has a major role in cardiac remodeling, we decided to examine the status of ECM protein changes during progression of cardiac hypertrophy in the Myo-Tg model. This interesting correlation between the progression of cardiac hypertrophy associated with NF-κB activation led us to investigate further the role of NF-κB in the ECM remodeling; especially during cardiac regression when NF-κB activity is inhibited.

A previous report suggested that inhibition of NF-κB could be a good therapeutic target for treating CH and HF [25]. It has been shown that PDTC, an antioxidant and inhibitor of NF-κB, was able to attenuate CH in other experimental models [24,26]; but no information was available on the regulation of ECM proteins by inhibition of NF-κB activity.

ROS are the major initiators of myocardial damage during cardiac hypertrophy. The role of ROS in cardiac fibrosis, during the progression of cardiac hypertrophy, remains poorly understood. Therefore, a protective inhibitory action of antioxidant therapy would be considered useful for reversing the process of cardiac hypertrophy via inhibition of NF-κB and ECM proteins.

In this study, we tested our hypothesis that inhibition of NF-κB by an antioxidant, PDTC, will suppress the expression of ECM proteins during the progression of CH in Myo-Tg mouse models. This study was, therefore, aimed at inhibiting NF-κB activation by PDTC and its role in modulation of ECM proteins during the progression of cardiac hypertrophy to heart failure.

In the first part of this report, we elucidate the changes in ECM gene expression including collagen, MMP, TIMP, LOX, and PHD in the initiation and progression of CH and HF that were responsible for maladaptive cardiac remodeling in the Myo-Tg model to establish the cause-and-effect relationship, and in the second part, we compare the ECM gene expression in cardiac reverse remodeling in the same experimental model in which PDTC was used to inhibit the NF-κB activity.

Materials and methods

Generation of transgenic mice overexpressing myotrophin in the heart

The generation of the Myo-Tg mouse model has been described previously [12]. As described previously, the onset of hypertrophy in the Myo-Tg mice is at 4 weeks, followed by progression of hypertrophy until 16 weeks and transition from hypertrophy to HF by 36 weeks. The progression of CH at 4, 16, and 36 weeks was determined based on the cardiac mass changes and cardiac function data as described previously [25]. The NF-κB activity also increased with the progression of cardiac hypertrophy at 4, 16, and 36 weeks in the Myo-Tg model compared to wild type (WT) as shown previously [25]. Considering this, all the experiments in this study were carried out at 4, 16, and 36 weeks. Age- and sex-matched WT mice were used for comparison with the Myo-Tg mice.

Electrophoretic mobility-shift assay (EMSA)

An EMSA was performed using a double-stranded NF-κB binding site oligonucleotide as a probe, as described previously [27].

Western blot analysis

Hearts of WT, Myo-Tg, and PDTC–Myo-Tg mice were excised and washed with cold phosphate-buffered saline (PBS) to remove blood. Western blot analysis was performed as described previously [23]. Membranes were probed with primary antibodies for IκBα (Cell Signaling Technology; at 1:1000 dilution), Col I (Rockland Immunochemical; at 1:5000), Col III (EMD Biosciences; at 1:1000), and connective tissue growth factor (CTGF; ABCAM; at 1:5000); washed three times in Tris-Buffered Saline Tween-20 (TBS-T); and then detected using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (NEN). For quantification of LOX, mouse hearts were homogenized in cold T-PER buffer (Pierce) and LOX level was determined using its specific antibody as described previously [7]. The antibody detects the mature enzyme at the 32 kDa position.

For quantification of MMP-2 and MMP-9, WT and Myo-Tg mouse hearts were homogenized in cold cacodylic extraction buffer and Western blot was performed with anti-MMP-2 and anti-MMP-9 monoclonal antibodies (1:5000; Millipore) as described previously [7]. The images were digitized and band densities were analyzed by ImagePro software. For normalization, we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody as a probe.

RNA extraction and Northern blot analysis

Total RNA was isolated from the heart of WT and Myo-Tg mice and PDTC–Myo-Tg according to the protocol of Chomczynski and Sacchi [28]. RNA was resuspended in DEPC water, quantitated by optical density at 260 nm. Transcript levels of Col I and Col III were determined by Northern blot analysis. Northern blotting and hybridization were performed as described previously [27]. cDNA probes were used for IκBα and p65 mRNA expression studies.