Ca^{2+} dependent-NOX5 exaggerates cardiac hypertrophy through ROS production

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Abstract

NADPH oxidase 5 (NOX5) is a homologue of the $gp91^{phox}$ subunit of the phagocyte NADPH oxidase, which generates reactive oxygen species (ROS). NOX5 is involved in sperm motility and vascular contraction and has been implicated in diabetic nephropathy, atherosclerosis and stroke. The function of NOX5 in the heart is unknown. Since NOX5 is a Ca^{2+}-sensitive, pro-contractile NOX isoform, we questioned whether it plays a role in cardiac pathophysiology. Studies were performed in i) cardiac tissue from patients undergoing heart transplant for cardiomyopathy and heart failure, ii) NOX5-expressing rat cardiomyocytes, and iii) mice expressing human NOX5 in a cardiomyocyte-specific manner. Cardiac hypertrophy was induced in mice by transverse aorta coarctation (TAC) and Ang II infusion. NOX5 expression was increased in human failing hearts. Rat cardiomyocytes infected with AdNOX5 exhibited elevated ROS levels with significant hypertrophy and associated increased expression of ANP and β-MHC and pro-hypertrophic genes (*Nppa*, *Nppb*, *Myh7)*. These effects were amplified by Ang II and reduced by NAC and diltiazem. Pressure overload and Ang II infusion induced left ventricular hypertrophy, interstitial fibrosis, and contractile dysfunction, responses that were exaggerated in cardiac-specific NOX5 knock in mice. These phenomena were associated with increased ROS levels and activation of redox-sensitive MAP kinases. NAC treatment reduced cardiac oxidative stress and attenuated cardiac hypertrophy in NOX5 mice. Our study defines Ca^{2+}-regulated NOX5 as an important NOX isoform involved in oxidative stress- and MAPK- mediated cardiac hypertrophy and contractile dysfunction.

Keywords: NOX5, reactive oxidative species (ROS), cardiac hypertrophy, MAPK.

Introduction

Heart failure, which is among the most common causes of mortality, is increasing in prevalence worldwide[1]. A major risk factor for heart failure is cardiac hypertrophy. Cardiac hypertrophy is initially a compensatory mechanism in response to various mechanical and neurohormonal stimuli, such as hypertension, chronic pressure overload, valvular heart disease, ischemic events and endocrine disorders[2,3]. However, sustained or excessive cardiac hypertrophy can progress to decompensation, ventricular contractile dysfunction, heart failure and subsequent death[2,3]. While there has been some development in new drugs for heart failure[4], effective treatments for preventing or reversing the progression of cardiac hypertrophy still remain inadequate. This relates in large part to the lack of understanding of underlying molecular mechanisms[5].

Although there are many causes of cardiac hypertrophy and heart failure, most share a critical common pathological mechanism: oxidative stress[6]. Oxidative stress is an imbalance between the production and clearance of reactive oxygen species (ROS)[7]. In patients with heart failure, production of ROS is increased in the myocardium and plasma, and is associated with cardiac dysfunction[8]. Reactive oxygen species contribute to cardiac remodeling, apoptosis, necrosis, and myocardial dysfunction[8,9]. Enzymatic sources for ROS, such as the mitochondria, cytochrome P450, xanthine oxidases, uncoupled nitric oxide synthases, and NADPH oxidases (NOXs) are all considered relevant sources of ROS in cardiac hypertrophy[10]. Among these sources, only NOXs are considered to produce ROS specifically (professional oxidases), and increasing evidence indicates that NOXs are critical determinants of myocardial ROS generation[11]. Seven NOX isoforms have been identified, including NOX1~5 and dual domain oxidases (DUOX) 1~2[12]. All NOXs possess six transmembrane domains with two haem-binding regions and a NADPH-binding region on the intracellular C-terminus, which catalyze the reduction of O_2 to produce superoxide (O_2^-), which leads to generation of secondary ROS, including hydrogen peroxide (H_2O_2), hydroxyl radical, peroxynitrite, and hypochlorous acid[13,14]. Of the seven known Nox isoforms, Nox2 and Nox4 are the predominant proteins expressed in the heart, at least in rodent models [15,16]. Nox2 is involved in the development of cardiac hypertrophy and remodeling through increasing generation of ROS[17]. However, the role of Nox4 in cardiac hypertrophy is controversial. Nox4 has ben shown to promote cardiac hypertrophy by activation of redox signaling pathways in response to hypertrophic stimuli[18]. On the other hand, Nox4 was also found to have protective effects against chronic pressure overload-induced remodeling in mice[19-20]. These cardioprotective effects of Nox4 involve multiple mechanisms including paracrine effects on myocardial capillary density and activation of the cytoprotective transcription factor Nuclear factor erythroid-derived 2-like 2 (Nrf2)[19-20].

Among the NOX family, NOX5 appears to be especially important in human cardiovascular cells[21]. Unlike other NOXs, NOX5 gene is absent in rodents, it does not require NADPH oxidase subunits for its activation and it is highly sensitive to changes in intracellular Ca^{2+} concentration[22]. Persistent hypertrophic stimuli are potent inducers of sustained increased intracellular Ca^{2+} concentration[23]. Accordingly, it is reasonable to assume that NOX5-induced ROS generation may be especially important in the development of cardiac hypertrophy. However, there is a paucity of information about the role of NOX5 in the heart and its putative mechanistic role in cardiac hypertrophy and heart failure are unknown. To address this, we generated mice that express human NOX5 in a cardiomyocyte-specific manner and induced cardiac hypertrophy using the transverse aorta coarctation (TAC) and Ang II infusion approach. Molecular mechanisms were interrogated in mice cardiac tissue and rat cardiomyocytes expressing human NOX5.

Materials and methods

A detailed description about the materials and methods that were used in this study is available in the online-only Data Supplement, including Reagents, Human heart samples, Cardiac-Specific NOX5-Knockin Mice, Animal models[24], Echocardiography[25], Histological analysis[26], Cultured neonatal rat cardiac myocytes and Recombinant Adenoviral Vectors[27], Immunofluorescent staining[28], Quantitative Real-time PCR, Western blotting[29], Dihydroethidium (DHE) staining[30], measurement of NADPH oxidase activity and superoxide anion (O_2^-) production[31], measurement of H_2O_2 levels, measurement of intracellular calcium in cardiomyocytes and Statistical analysis. All data were expressed as means ± SEM. Two-tailed student's t-test was used to compare the means of two-group samples. One-way ANOVA with Bonferroni (assuming equal variances) or Tamhane's T2 (equal variances not assumed) tests was used to compare the means of multiple groups. All statistical analyses were performed using SPSS 19.0 (Statistical Package for the Social Sciences) software. A value of $P<0.05$ was considered significant.

Results

NOX5 expression is increased in failing human hearts.

In order to explore the NAPDH oxidase subunits status in the development of pathological cardiac hypertrophy, we first performed quantitative polymerase chain reaction (qPCR) and western blot analyses to investigate expression levels of NADPH

oxidase subunits in mice heart samples. Our qPCR and western blot results indicated that Nox4 levels were significantly increased in response to pressure overload. (Figure S1B and S1C). In order to better understand the status of Noxs in the clinical situation, we also checked expression of Nox isoforms in failing human hearts. Our results showed that expression levels of both NOX5 and NOX4 in failing human hearts were increased compared with normal donor hearts, moreover, the expression of NOX5 gene is higher both in mRNA and protein levels than that of NOX4 (Figure 1A and 1B). Other Nox isoforms, NOX1 and NOX2 and NADPH oxidase subunits $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, and $p7^{phox}$ did not differ significantly between the two groups (Figure 1A and 1B). Given that there are 6 isoforms of NOX5, i.e., NOX5(V1)-NOX5(V6). In order to clarify which splicing isoform of NOX5 is up-regulated in human failing hearts, we examined the mRNA expression of all 6 NOX5 isoforms in human failing hearts. Our data demonstrate that mRNA expression of V1 and V2 was increased compared with normal donor hearts. V2 was especially abundant (Figure 1C). Thus, the NOX5 V2 was applied in our study.

Immunohistochemistry staining also showed that failing hearts expressed higher NOX5 protein levels than nonfailing hearts (Figure S1D). Moreover, high magnification images showed that NOX5 was present in some endothelial cells of intramyocardial blood vessels and expressed primarily in cardiomyocytes. Furthermore, the increased NOX5 is predominantly localized in the cytoplasm and to a lesser extent in the plasma membrane. Additionally, we performed immunofluorescence staining to clarify the cellular expression patterns of NOX5 in human heart tissue. As shown in Figure 1E, NOX5 was specifically and significantly increased in cardiomyocytes versus other cell types in failing hearts compared with the nonfailing hearts.

NOX5 exacerbates Ang II-induced cardiomyocyte hypertrophy in vitro.

Cardiomyocyte enlargement is the most defining characteristic of cardiac hypertrophy. To evaluate the impact of NOX5 on cardiac hypertrophy, a gain of function assay was performed by infecting cultured NRVMs with adenoviral vector encoding human NOX5 cDNA (Ad*NOX5*). The efficiency of lentivirus-mediated gene expression was validated by Western blotting in NRVMs (Figure 2A). Isolated NRVMs were infected with adenoviruses expressing either NOX5 (Ad*NOX5*) or the empty vector (Ad*Vector*) as a control, then, these cells were treated with either Ang II or PBS as a control for 48 h before immunostaining for α-actinin to determine cardiomyocyte surface area. Under basal conditions (PBS administration), there was no significant difference in cardiomyocyte surface area between groups. Compared with Ad*Vector*-infected controls, Ad*NOX5* infection-mediated NOX5 expression significantly increased AngII-induced cardiomyocyte hypertrophy, as evidenced by the increase in

cardiomyocyte surface area and mRNA and protein levels of hypertrophy markers (ANP, BNP and β-MHC) (Figure 2B-2D). To confirm the pro-hypertrophy action of NOX5 in human cardiomyocytes, we also explored effects of NOX5 on cardiomyocyte hypertrophy using human AC16 cardiomyocytes. Consistent with the results in rat cardiomyocytes, under basal conditions (PBS administration), there was no significant difference in cardiomyocyte surface area between groups. However, compared with Ad*Vector*-infected controls, Ad*NOX5* infection-mediated NOX5 expression significantly increased AngII-induced cardiomyocyte hypertrophy, as evidenced by the increase in cardiomyocyte surface area and levels of hypertrophy markers (ANP and β-MHC), (Figure 2E-2G).

Together, these results indicate that NOX5 promotes cardiomyocyte hypertrophy in vitro. Mechanical stress and hormonal stimuli are common triggers in cardiac hypertrophy. Stimulating myocytes with Ang II or mechanical stretch directly reflects how myocytes respond to hypertrophic stimuli at the cellular level. However, the pathophysiology of hypertrophy is complex and multifactorial, as it influences several cellular and molecular systems. Since *in vitro* conditions do not fully represent the features observed *in vivo*, and to gain insights on the pathophysiological role of NOX5 in the whole organism, we generated cardiomyocyte specific NOX5 transgenic mice and focus mainly on the *in vivo* experiments.

TAC and Ang II -induced cardiac hypertrophy are exaggerated in NOX5-expressing mice.

To determine whether cardiac NOX5 influences cardiac status in vivo we generated cardiac-specific NOX5 transgenic (NOX5-Tg) mice that express human NOX5 using the a-myosin heavy chain promoter (Figure 3A). Two independent lines of NOX5-Tg mice (Tg1 and Tg2) were successfully established and western blotting results showed that the Tg2 line had the highest level of NOX5 expression in the heart, thus, the Tg2 was selected for the following experiments (Figure 3B and 3C). Our western blotting results showed Nox5 transgene does not influence endogenous Noxs (NOX1, NOX2, NOX4) expression (Figure S2A).

NOX5-Tg and the wild type (WT) control mice were challenged with TAC surgery or sham operation for 4 weeks. Under basal conditions (Sham), the NOX5-Tg mice did not show any alterations in cardiac structure or function compared with the WT control group (Figure 3D and 3E). However, compared to the TAC-treated WT mice, the myocardial contractile function was significantly reduced in NOX5-Tg mice, as shown by echocardiography measurements of left ventricular end-diastolic diameter (LVEDd), LV end-systolic diameter (LVESd), LV ejection fraction (LVEF) and LV shortening fraction (LVFS) (Figure 3D and 3E). We subsequently sought to explore the

effect of NOX5 on cardiac remodeling. After four weeks of TAC, NOX5-Tg mice showed an enhanced hypertrophic response compared with the WT group, as indicated by increased HW/BW and HW/TL ratios (Figure 3F). In addition, NOX5-Tg mice had significantly increased cardiomyocyte size after 4 weeks of TAC, which was identified by H&E staining of heart sections and quantified based on the cardiomyocyte cross-sectional area in NOX5-Tg and control mice (Figure 3G and 3H). These changes were accompanied by elevated levels of cardiac fetal and pro-hypertrophic genes in TAC-treated NOX5-Tg mice (Figure 3I and 3J). Furthermore, picrosirius red (PSR)-stained heart sections exhibited more prominent interstitial fibrosis in NOX5-Tg mice compared with controls (Figure 3G and 3H). Indices of collagen synthesis, including collagen I (COL1A1) and collagen III (COL3A1) were also markedly increased in NOX5-Tg hearts compared with controls (Figure 3I and 3J). Together these data demonstrate that NOX5 exaggerates pressure overload-induced cardiac remodeling and dysfunction.

We next examined the potential effect of *NOX5* on hypertrophy mediated by Ang II infusion to exclude the possibility that these effects of *NOX5* were specific to pressure overload-induced cardiac hypertrophy. As shown in Figure S2, 4 weeks of Ang II infusion in wild-type (WT) mice led to significantly increased HW/BW and HW/TL, impaired contractile function and enlarged myocytes compared with saline controls. Similar to the results that observed with TAC treatment, the structural remodeling and functional changes of the heart induced by Ang II infusion were significantly exacerbated by NOX5 over expression. These data indicate that NOX5 is an important molecular mediator underlying pathological cardiac hypertrophy induced by both pressure overload and hormonal stresses.

NOX5 promotes ROS production in response to hypertrophic stress.

It is well known that oxidative stress plays a crucial role in cardiac hypertrophy. Furthermore, in an NADPH-dependent manner, NOX5 catalyzes the reduction of O_2 to produce O_2^-, which in turn dismutates to generate H_2O_2. Thus, we questioned whether NOX5 upregulation causes increased ROS generation in the presence of hypertrophic stimuli. To address this, we assessed ROS levels in the hearts of NOX5-Tg mice and cultured NRVMs by DHE staining. Results showed that TAC induced a significant increase in ROS levels in the hearts of WT and NOX5-Tg mice, with significantly greater responses in NOX5-expressing mice (Figure 4A). Additionally, we performed the lucigenin-enhanced chemiluminescence assay to determine the NADPH oxidase activity and O_2^- production in cardiac tissue. As a stable ROS, H_2O_2 was also measured. As shown in Figures 4B and 4C, *NOX5*-Tg mice exhibited increased NADPH-induced O_2^- production and H_2O_2 levels under the stress condition, which was concomitant with

development of cardiac hypertrophy. Consistent with the in vivo results, NOX5 expression also exhibited higher levels of ROS after Ang II treatment in NRVMs compared with the control group (Figure 4D-4F). Together these findings suggest that NOX5 promotes ROS production in response to hypertrophic stress. Since ROS can alter activation of multiple signaling cascades that participate in regulating cardiac hypertrophy, we assessed phosphorylation levels of redox-sensitive mitogen-activated protein kinases (MAPK): JNK, ERK, and p38 MAPK, which are downstream signaling kinases of ROS and master regulators of cell growth. Our results showed that *NOX5*-Tg mice exhibited higher phosphorylation levels of JNK, ERK, and p38 MAPK compared with WT controls (Figure 4G). Similar effects were also observed in Ang II-treated NRVMs (Figure 4H). These data indicate that *NOX5* could promote accumulation of ROS in the presence of hypertrophic stimuli, which induces activation of downstream MAPK signaling pathways.

Inhibition of ROS abrogates the NOX5-related effect on the hypertrophic response.

Based on the fact that ROS production significantly promotes cardiac hypertrophy development, and NOX5 is capable of increasing ROS levels during cardiac hypertrophy, we next questioned whether ROS scavenging could prevent the pro-hypertrophic effect of NOX5. To address this, we performed in vitro experiments by treating cultured NRVMs with NAC or saline as control. As shown in Figures S3A-3C, compared with the saline group, NAC treatment significantly reduced Ang II-induced cardiomyocyte hypertrophy, as evidenced by the increase in cardiomyocyte surface area and mRNA and protein levels of hypertrophy markers (ANP and β-MHC). We also treated TAC mice with NAC for 4 weeks. As shown in Figure 5A, NAC significantly increased the TAC-induced decrease in heart function, as evidenced by their increased EF, FS and decreased LVEDd and LVESd (Figure 5E). Furthermore, NAC significantly attenuated the increased heart weight induced by pressure overload in both WT and NOX5-Tg mice, as evidenced by their decreased HW/BW and HW/TL (Figure 5B). Moreover, treatment with NAC abolished the NOX5-induced increase in cardiomyocyte enlargement and interstitial fibrosis as identified by HE and PSR staining (Figure 5C and 5D). To further verify this, we used another approach to modulate ROS by genetic manipulation of *NOX*. The S236 domain is critical for ROS production within NOX5[32]. To address if the pro-hypertrophic effect of NOX5 is dependent on ROS generation, we generated a NOX5 enzyme-dead mutant NOX5(M) by substituting the amino acid 236 from serine to arginine which had no ROS producing capacity. As shown in Figure 5E, the S236R NOX5 mutant were almost completely inactive under AngII stimulation. Compared with the NOX5(M)-infected group, the NOX5(WT)-infected group exhibited significantly increased Ang II-induced cardiomyocyte hypertrophy, as evidenced by the increase in cardiomyocyte surface area

and mRNA and protein levels of hypertrophy markers (*Nppa, Myh7*), (Figure 5F-5H). Together, our data suggest that NOX5-mediated pro-hypertrophic effects are mediated in large part through increased production of ROS.

Ca^{2+} is required for NOX5 activation.

It is well established that intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increases in response to sustained hypertrophic stimuli. Furthermore, NOX5 activation is dependent on $[Ca^{2+}]_i$. Thus, we hypothesized that reducing intracellular Ca^{2+} concentration will affect activity of NOX5 and its effect on cardiac hypertrophy. To address this, cultured NRVMs infected with either Ad*NOX5* or Ad*Vector* were treated with the L-type Ca^{2+} channel blocker diltiazem before the cells were stimulated with Ang II or PBS. Our results showed that Ang II-induced a significant increase in intracellular Ca^{2+} concentration, effects that were amplified by increased NOX5 and abrogated by diltiazem treatment, (Figure 6A).

We also tested whether NOX5-based NAPDH oxidase contributes to ROS production in NRVMs. As shown in figure 6B, Ang II induced a significant increase in NAPDH oxidase activity, an increase that was further exaggerated by infection with NOX5. Moreover, the increased ROS production was abrogated by diltiazem, similar to what we observed for $[Ca^{2+}]_i$. To elucidate the functional significance of Nox5, we next measured the hypertrophic responses induced by Ang II in NRVMs expressing NOX5 that pretreatment with diltiazem or PBS. As shown in figure X, effects of NOX5 on the hypertrophic response, assessed as cardiomyocyte surface area and expression of pro-hypertrophic genes, *Nppa* and *Myh7* were virtually abrogated by diltiazem (Figure 6C and 6D).

Discussion

Pathological cardiac hypertrophy is driven by multiple neuroendocrine hormones, such as Ang II, and endothelin 1, as well as mechanical forces, such as chronic pressure overload. These factors can directly or indirectly increase ROS production and myocardial accumulation of metabolic intermediates, which induce cell death, fibrosis, and mitochondrial dysfunction[3]. Thus, understanding sources of cardiac ROS so that disease-specific mechanisms can be targeted to prevent oxidative stress in the development of cardiac hypertrophy represents an attractive therapeutic strategy. However current pharmacological treatment to prevent or regress cardiac hypertrophy is sub-optimal with many patients still dying prematurely from heart failure[33]. In this study, we demonstrate that NOX5, activated by intracellular Ca^{2+}, contributes to the development of cardiac hypertrophy through ROS production and activation of redox-

sensitive MAP kinases.

Previous studies have shown that increased expression and activity of cardiac NOX1 and NOX2 participate in the process of pathological cardiac hypertrophy[34,35]. NOX2 aggravates the hypertrophic response in a model of experimental cardiac hypertrophy[17]. On the other hand, NOX4 seems to be cardioprotective[36]. Reasons for the distinct pathophysiological roles of different NOXs may relate to differential patterns of activation, subcellular localization, and type of ROS produced[37]. While NOX5 has been implicated in atherosclerosis, diabetic nephropathy, stroke and coronary artery disease[36-43], there is a paucity of information about NOX5 in the heart and pathogenesis of cardiac disease. NOX5 seems to be important in cardiac development since human fetuses with ventricular septal defects, the most common form of congenital heart disease, have significantly increased methylation of *NOX5*[44]. A clinical study in patients with acute myocardial infarction showed increased NOX5 expression in intramyocardial arteries and cardiomyocytes in infarcted tissue[45]. We demonstrate here that NOX5 is present in human heart, that it is important in ROS production in cardiomyocytes and that it is abundantly expressed in human heart failure.

Although it is clear that cardiac NOX5 is upregulated in human heart pathology, the functional significance during development of disease remains unclear. It has been impossible to investigate this in rats and mice because rodents lack *NOX5*. To address this we generated cardiomyocyte-specific *NOX5* knockin mice, which provided a unique opportunity to study human NOX5 in an intact system. While there are limitations in such transgenic approaches, humanized models are used extensively in pre-clinical research to study mechanisms and new therapies of human disease[46]. In our NOX5 knockin mice, cardiac hypertrophy and myocardial dysfunction were markedly exaggerated in TAC-induced pressure overload and in Ang II-infused mice. These findings indicate that NOX5 is a key player in pathological cardiac remodeling and dysfunction due to both mechanical and humoral factors.

NOXs are professional ROS-producing enzymes[47-48]. Consistent with previous studies, we observed that NOX5 promotes cellular ROS production[49], which is increased in the myocardium upon hypertrophic stress. Although a large body of evidence has demonstrated that ROS influence the pathophysiology of cardiac hypertrophy through several mechanisms, including alterations in myofilament calcium responsiveness and alterations in cellular energetics[50-52], the pro-hypertrophic response triggered by ROS in cardiomyocytes remains to be fully defined[50]. Activation of MAPKs may be especially relevant because they are highly redox-sensitive and could be activated through an increase in intracellular ROS[50-51,53]. Activated p38MAPK, JNK, and ERK phosphorylate numerous contractile, mitogenic and pro-inflammatory

intracellular targets as well as transcription factors, and thus play an important role in regulating cardiac contraction and hypertrophy[50-51,53]. Therefore, mechanisms by which NOX5 aggravates cardiac hypertrophy and contractile dysfunction are likely mediated by activation of ROS-mediated downstream MAPK cascades[31]. ERK1/2 itself has been shown to regulate NOX5 activity, in part through a Ca^{2+}-independent mechanism[54]. Accordingly, it may be possible that in the context of cardiac disease with increased NOX5 activity, redox-sensitive ERK1/2 is activated, which in turn further activates NOX5. This feedforward phenomenon may contribute to exaggerated cardiac damage. MAPKs are master regulators of cell growth and cardiomyocyte function and while our study identifies ERK1/2 as being especially important in NOX5-mediated cardiomyocyte growth and cardiac hypertrophy, we can not exclude the role of other redox-sensitive signaling molecules as well.

With regard to cardiac fibrosis, the mechanism through which ROS accelerates cardiac fibrosis progression has been well studied. ROS mediates pathological fibrosis by stimulating fibroblast proliferation, collagen deposition and activation of matrix metalloproteinases[50-52]. Moreover, pharmacological inhibition of redox-sensitive signaling pathways could have beneficial effects on cardiac function by decreasing proliferation of myocardial fibroblasts[50]. This was supported by our findings that the ROS scavenger NAC ameliorated the NOX5-mediated hypertrophic response, indicating that NOX5-mediated pro-hypertrophic effects are mainly dependent on ROS generation. Thus, inhibition of NOX5 may represent a promising therapeutic strategy to reduce cardiac remodeling in heart disease.

In cell-free systems and other experimental models, it has been clearly established that NOX5 can be directly activated by increased intracellular Ca^{2+} concentration[12,55]. NOX5 has a unique N terminus, which contains 3-4 $Ca2^{+}$-binding helix-loop-helix structure domains (EF hand)[22], which behave like a calmodulin-like activator module[12]. In the absence of Ca^{2+}, the calmodulin-like regulatory EF-domain is partially unfolded and detached from the rest of the protein[54]. When intracellular Ca^{2+} increases, the Ca^{2+} directly binds to the EF hand. This causes the regulatory domain to binds to the catalytic domain causing its activation, and this is why NOX5, unlike other NOXs, does not require any NADPH oxidase subunits for its activation. While Ca^{2+} is thus upstream of the NOX5-ROS pathway, intracellular Ca^{2+} is also downstream of ROS because Ca^{2+} channels and exchangers (L-type Ca^{2+} channels, sarcoplasmic reticulum (SR) ATPase (SERCA), Na^{+}/ Ca^{2+}exchanger, ryanodine receptor and transient receptor potential melastatin 2 cation channel), are regulated by ROS[56-57]. These findings suggest that Ca^{2+} and ROS exert a reciprocal regulation loop on each other: increased intracellular Ca^{2+} induces ROS production, meanwhile increased ROS promotes cytoplasmic Ca^{2+} mobilization. This is particularly significant in the heart, where increased intracellular

Ca^{2+} and ROS interact in a bidirectional manner to amplify the hypertrophic signal promoting pathological cardiac hypertrophy[58]. In our study, Ca^{2+} channel blockade with diltiazem abrogated the NOX5-related effect on the hypertrophic response in cardiomyocytes. Together our data suggest that NOX5 acts as an important point of cross-talk between ROS and Ca^{2+} linking signaling pathways such as MAPKs that are both redox- and Ca^{2+}-sensitive. These systems are critically involved in the regulation of cardiac cell growth, inflammation, fibrosis and contraction and when dysregulated lead to pathological cardiac remodeling and contractile dysfunction[59]. Targeting inhibition of the activity of NOX5 may inhibit the reciprocal regulation of Ca^{2+} and ROS, and thus play an important role in certain oxidative stress-related cardiac diseases.

In conclusion, our multidisciplinary approach from clinically-relevant human tissue to transgenic mice clearly delineates a mechanistic role for NOX5 in pathological cardiac hypertrophy. In particular we identify Ca^{2+}-sensitive NOX5 and redox-regulated ERK1/2 as important molecular mechanisms contributing to the development of LV hypertrophy, contractile dysfunction and myocardial fibrosis in response to pressure and humoral hypertrophic stresses. Our novel findings suggest that NOX5 may be a putative target for therapeutic intervention to manage cardiac hypertrophy and heart failure.

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Disclosures

None.

Supplemental Materials

Expanded Materials and Methods

Supplementary Figures

Supplementary Table 1

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Novelty and Significance

What is new?

1. NOX5 expression is significantly induced in human failing hearts.
2. NOX5 exacerbates LV hypertrophy, fibrosis and dysfunction in different preclinical mouse cardiac disease models.
3. NOX5 modulates the development of cardiac hypertrophy by promoting ROS production in response to hypertrophic stress.
4. NOX5 is a point of cross-talk between intracellular calcium and ROS production.

What is relevant?

In this study, our findings indicate an important role for NOX5 in cardiomyocyte dysfunction in cardiac hypertrophy. Molecular mechanisms underlying NOX5 actions involve ROS and MAPK. Our study defines Ca^{2+}-regulated NOX5 as a novel NOX

isoform involved in oxidative stress-mediated cardiac hypertrophy and contractile dysfunction. Modulating NOX5 may have therapeutic potential by targeting the source of ROS.

Summary

Our studies in human hearts and NOX5-expressing rodent models identify Ca^{2+}-regulated NOX5-mediated ROS generation as an important pathway contributing to the development of LV hypertrophy, contractile dysfunction and myocardial fibrosis in response to hypertrophic stimuli. Our novel findings suggest that NOX5 might be an attractive target for therapeutic intervention to treat cardiac hypertrophy and heart failure.

Figure legends

Figure 1. NOX5 expression is increased in failing hearts.

A, Quantitative polymerase chain reaction (qPCR) analyses of the mRNA levels of *NOX1*, *NOX2*, *NOX4*, *NOX5*, and NOX subunits ($p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$) in human heart samples from normal control donors and patients with heart failure, (*n*=6). **B,** Western blot analysis and quantitative results of NOX1, NOX2, NOX4, NOX5, ANP, and β-MHC levels in human heart samples from normal donors and patients with heart failure, (*n*=6). **C,** mRNA levels of NOX5 splice variants in human heart samples, (*n*=6). **D,** Expression and cellular localization of NOX5 on human heart sections of normal control donors or patients with heart failure using immunohistochemistry staining, (EC: endothelial cell; CM: cardiomyocyte; CP: cytoplasm; PM: plasma membrane), (*n*=6). Scale bar, 25μm. **E,** Representative images of immunofluorescence staining of NOX5 in the heart of normal control donors and patients with heart failure, (n = 6). Scale bar, 20μm. Data were shown in mean±SEM; *P < 0.05, **P < 0.01, n.s., not significant.

Figure 2. NOX5 promotes cardiomyocyte hypertrophy in vitro.

A, Immunoblots of NOX5 protein expression in NRVMs infected with Ad*Vector* or Ad*NOX5*. **B, Left**, representative immunofluorescence images of α-actinin staining in NRVMs infected with AdVector or Ad*NOX5* and treated for 48 hours with PBS or Ang II. Scale bar, 60μm. **Right**, relative cell surface area of cultured NRVMs infected with the indicated adenoviruses in response to PBS or Ang II treatment, (*n* ≥50 cells per group). **C,** Real-time quantitative PCR (qPCR) analyses of the mRNA levels of hypertrophic marker genes (*Nppa*, *Nppb* and *Myh7*) in PBS- or Ang II–treated NRVMs infected with AdVector or AdNOX5. (*n*=4 samples per group). **D,** Representative

immunoblots of Anp and β-MHC protein expression in NRVMs infected with Ad*Vector* or Ad*NOX5* treated for 48 hours with PBS or Ang II. **E, Left**, representative immunofluorescence images of α-actinin staining in AC16 cells infected with Ad*Vector* or Ad*NOX5* and treated for 48 hours with PBS or Ang II. Scale bar, 60μm. **Right**, relative cell surface area of AC16 cells infected with the indicated adenoviruses in response to PBS or Ang II treatment, (*n* ≥50 cells per group. **F,** qPCR analyses of the mRNA levels of hypertrophic marker genes (*Nppa* and *Myh7*) in PBS- or Ang II–treated AC16 cells infected with Ad*Vector* or Ad*NOX5*, (*n*=4 samples per group). G, Representative immunoblots of Anp and β-MHC protein expression in AC16 cells infected with Ad*Vector* or Ad*NOX5* treated for 48 hours with PBS or Ang II. Data were shown in mean±SEM; *P < 0.05, **P < 0.01.

Fig3. NOX5 aggravates pressure overload-induced cardiac hypertrophy.

A, Schematic diagram depicting the construction of cardiac-specific *NOX5*-overexpressing experimental mice. **B,** Agarose gel photograph illustrating genotyping results of PCR products from WT or *NOX5*-Tg mice. Sizes are indicated in bp. **C,** Representative immunoblots showing NOX5 expression in heart tissues of WT and NOX5-Tg mice. **D,** The representative B mode and M mode echocardiographic images of left ventricle of WT and *NOX5*-Tg mice at 4 weeks after sham or TAC surgery, (*n*=9-12 mice per group). **E,** Comparison of the echocardiographic parameters left ventricular (LV) end-diastolic dimension (LVEDd), LV end-systolic dimension (LVESd), LV ejection fraction (LVEF), and LV fraction shortening (LVFS) between the indicated groups (*n*=8 mice per group). **F,** Ratios of heart weight (HW)/body weight (BW) and heart weight to tibia length (HW/TL) in the indicated groups, (*n*=8 mice per group). **G,** Histological examinations of hematoxylin and eosin-stained (first and second) and picrosirius red-stained (third) left ventricle cross-sections from the indicated groups, (*n*=6 mice per group). Scale bar, 1mm for the upper most set of panels and scale bar, 25μm for lower panels. **H,** Statistical results of individual cardiomyocyte cross-sectional area and LV collagen volume in the indicated groups. **I,** Representative Western blots showing the protein levels of Anp, β-MHC, Col1a1, and Col3a1 at 4 weeks after sham or TAC surgery in WT and *NOX5*-Tg mice. **J,** Real-time PCR analyses of hypertrophic markers (*Nppa*, *Myh7*) and the fibrotic markers (*collagen I*, *collagen III*) in the indicated mice, (*n*=4 mice per group). Data were shown in mean± SEM; *P < 0.05, **P < 0.01, n.s., not significant.

Fig4. NOX5 increases ROS production and downstream MAPK signaling activation.

A, Left, representative immunofluorescence images of dihydroethidium staining in the hearts of WT and *NOX5*-Tg mice at 4 weeks after sham or TAC surgery. Scale bar, 50μm. **Right**, quantification of reactive oxygen species levels in the indicated groups based on measurement of the intensity of fluorescence, (n **>** 40 fields per group). **B,** NADPH oxidase activity and O_2^- generation in cardiac tissues from WT and *NOX5*-Tg mice assessed by lucigenin chemiluminescence, (*n*=6 mice per group). Relative luminescence units (RLU) were corrected by protein concentration of each sample. **C,** Relative H_2O_2 levels in cardiac tissues from WT and *NOX5*-Tg mice assessed by Elisa, (*n*=6 mice per group). **D, Left**, dihydroethidium staining of reactive oxygen species levels in NRVMs infected with AdVector or Ad*NOX5* and treated for 48 hours with PBS or Ang II. Scale bar, 50μm. **Right**, quantification of reactive oxygen species levels of cultured NRVMs infected with the indicated adenoviruses in response to PBS or Ang II treatment, (n **>** 19 fields per group). **E,** NADPH oxidase activity and O^{2-} generation in NRVMs infected with Ad*Vector* or Ad*NOX5* and treated for 48 hours with PBS or Ang II, (*n*=4 samples per group). Relative luminescence units (RLU) were corrected by protein concentration of each sample. **F,** Relative H_2O_2 levels in NRVMs assessed by Elisa, (*n*=4 samples per group). **G,** Representative western blotting showing the expression of p-Erk, Erk, p-Jnk, Jnk, p-p38, and p38 in heart samples from WT and *NOX5*-Tg mice at 4 weeks after sham or TAC surgery. **H,** Representative western blotting showing the expression of the indicated kinases in NRVMs infected with Ad*Vector* or Ad*NOX5* following PBS or Ang II treatment. Data were shown in mean± SEM; *P < 0.05, **P < 0.01.

Fig5. Inhibition of ROS abolishes the NOX5-mediated pro-hypertrophic effect in vivo.

A, Comparison of the echocardiographic parameters left ventricular (LV) end-diastolic dimension (LVEDd), LV end-systolic dimension (LVESd), LV ejection fraction (LVEF), and LV fraction shortening (LVFS) between the WT and *NOX5*-Tg mice treated with saline or NAC after TAC surgery for 4 weeks, (*n*=11-12 mice per group). **B,** The ratio of HW/BW and HW/TL in the indicated groups, (*n*=8 mice per group). **C-D, Left**, representative images of H&E staining and PSR staining of hearts from the indicated groups, (*n*=6 mice per group). Scale bar, 1mm for the upper panel and scale bar, 25μm for the lower panel. **Right,** statistical results of the cardiomyocyte cross-sectional area and LV collagen volume in the indicated groups. **E,** NADPH oxidase activity and O^{2-} generation in NRVMs infected with Ad*Vector*, Ad*NOX5* or Ad*NOX5*(M) and treated for 48 hours with Ang II. Relative luminescence units (RLU) were corrected by protein

concentration of each sample, (*n*=4 samples per group). **F, Left,** representative immunofluorescence images of α-actinin staining in NRVMs infected with Ad*Vector*, Ad*NOX5* or Ad*NOX5*(M) and treated with Ang II for 48 hours, scale bar, 60μm; **Right,** Relative cell surface area. (*n* ≥50 cells per group). **G,** Real-time quantitative PCR (qPCR) analyses of the mRNA levels of hypertrophic marker genes (*Nppa* and *Myh7*) in the indicated groups, (*n*=4 samples per group). **H,** Representative immunoblots of Anp, β-MHC and NOX5 proteins expression in the indicated groups. Data were shown in mean±SEM; *P < 0.05, **P < 0.01.

Fig6. Activity of NOX5 is Ca^{2+} dependent.

A, Relative intracellular Ca^{2+} concentration detected by Fluo 4-AM in the indicated groups. B, NADPH oxidase activity assessed by enhanced lucigenin assay in the indicated group. Relative luminescence units (RLU) were corrected by protein concentration of each sample, (*n*=4 samples per group). **C, Left,** Representative immunofluorescence images of α-actinin staining in NRVMs infected with AdVector or Ad*NOX5* and treated for 48 hours with PBS or Ang II in the presence or absence of diltiazem, scale bar, 60μm; **Right,** Quantitative analysis of the cell surface area, (n≥50 cells per group). **D,** mRNA levels of the hypertrophic marker genes (*Nppa* and *Myh7*) in the indicated groups. **E**, Schematic figure. Data were shown in mean±SEM; *P < 0.05, **P < 0.01, n.s., not significant.