Deletion of Inducible Nitric Oxide Synthase Provides Cardioprotection in Mice With 2-Kidney, 1-Clip Hypertension

Ying Sun, Oscar A. Carretero, Jiang Xu, Nour-Eddine Rhaleb, James J. Yang, Patrick J. Pagano, Xiao-Ping Yang

Abstract—Inducible NO synthase (iNOS) has been implicated in the pathogenesis of hypertension and target organ damage. We hypothesized that induction of iNOS contributes to left ventricular (LV) hypertrophy and dysfunction in mice with 2-kidney, 1-clip hypertension. Deletion of iNOS diminishes oxidative stress, thereby attenuating LV hypertrophy and enhancing cardiac performance. 2-Kidney, 1-clip hypertension was induced in mice lacking iNOS and wild-type controls (C57BL/6J). Sham-clipped mice served as controls. Systolic blood pressure was measured weekly by tail cuff. Left ventricular ejection fraction (by echocardiography) and cardiac response (maximum and minimum dP/dt, as well as an indicator of isovolumic contraction) to isoproterenol (50 ng per mouse, IV) were studied at the end of the experiment. 4-Hydroxy-2-nonenal (a byproduct of lipid peroxidation and an indicator of oxidative stress) was measured by immunohistochemical staining. gp91phox, endothelial NO synthase, and iNOS protein expression were determined by Western blot. We found that systolic blood pressure, LV weight, myocyte cross-sectional area, interstitial collagen fraction, ejection fraction, and cardiac response to isoproterenol did not differ between strains with sham clipping. 2-Kidney, 1-clip hypertension increased systolic blood pressure, LV weight, myocyte cross-sectional area, and interstitial collagen fraction similarly in both strains. However, in mice lacking iNOS, maximum and minimum dP/dt, as well as an indicator of isovolumic contraction, markedly increased in response to isoproterenol, associated with decreased cardiac 4-hydroxy-2-nonenal expression and urinary nitrate/nitrite. We concluded that deletion of iNOS does not seem to play a significant role in preventing 2-kidney, 1-clip hypertension–induced hypertension and cardiac hypertrophy; however, it does enhance preservation of cardiac function, probably because of a reduction of iNOS-induced oxidative stress. (Hypertension. 2009;53:49-56.)

Key Words: NO synthase ■ DOCA-salt hypertension ■ cardiac function ■ oxidative stress

Inducible NO synthase (iNOS) is normally expressed at a low level in the heart and blood vessels but can be induced by numerous stimuli, including inflammatory mediators, cytokines, growth factors, and tissue ischemia. In patients and experimental animals with hypertension, cardiac hypertrophy, or heart failure, iNOS expression is reportedly up-regulated, which correlates positively with the severity of cardiac dysfunction and cytokine expression. Induction of iNOS produces excessive amounts of NO, which reacts with superoxide (O$_2^-$) to form the highly cytotoxic oxidant peroxynitrite. In addition, iNOS itself is capable of producing O$_2^-$, which is transformed to hydrogen peroxide either spontaneously or via a catalytic reaction with superoxide dismutase. Both peroxynitrite and hydrogen peroxide have been implicated in tissue injury and organ dysfunction, including the heart.

Inhibition of iNOS reportedly suppressed development of hypertension in spontaneously hypertensive rats and regressed cerebral edema because of severe hypertension in stroke-prone spontaneously hypertensive rats, suggesting that activation of iNOS contributes to the pathogenesis of spontaneous hypertension. Using a mouse model of deoxycorticosterone acetate (DOCA)-salt–induced hypertension (renin-independent hypertension), we found that deletion of iNOS had no effect on systolic blood pressure or left ventricular (LV) hypertrophy; however, it did enhance preservation of cardiac function, probably because of a reduction of iNOS-induced oxidative stress. Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.108.121822
possibly mediated by enhanced oxidative stress. However, the role of upregulated iNOS expression in the development of renin-dependent hypertension and the response of LV contractile function have not been fully explored. It is well documented that angiotensin (Ang) II can induce iNOS expression in cultured endothelial cells and blood vessels, as well as the kidney and heart, associated with increased oxidative stress. Using 2-kidney, 1-clip hypertension (2K1C), a renin-dependent experimental model characterized by increased circulating Ang II that, in many respects, resembles human renovascular hypertension, we tested the hypothesis that induction of iNOS contributes to LV hypertrophy and dysfunction after 2K1C. Deletion of iNOS diminishes oxidative stress, thereby attenuating LV hypertrophy and enhancing performance.

Materials and Methods

Animals

Eight-week-old male iNOS knockout mice (iNOS−/−) on a C57BL/6J background were purchased from Jackson Laboratory (Bar Harbor, Maine). Age-matched C57BL/6J mice served as wild-type (WT) controls. Mice were housed in an air-conditioned room with a 12-hour light/dark cycle and given standard chow and tap water. This study was approved by the Henry Ford Hospital Institutional Animal Care and Use Committee. All of the experiments in animals were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Induction of 2K1C Hypertension

One week after adapting to their new environment, mice were anesthetized and the left kidney exposed through a flank incision. After separating the renal artery and vein, a silver clip with an ID of 127 μm was placed around the renal artery. With sham operation, mice had the same surgery, but the artery was not clipped. The experiment was continued for 12 weeks.

Systolic Blood Pressure

Systolic blood pressure (SBP) was measured weekly in conscious mice using a noninvasive computerized tail-cuff system (BP-2000, Visitech). Each SBP was composed of 3 sets of 10 measurements, with each set including 6 of 10 successful measurements. Weekly SBP was averaged every 4 weeks.

Echocardiographic Evaluation of LV Morphology and Function

After 12 weeks, LV dimensions and ejection fraction were evaluated with a Doppler echocardiographic system equipped with a 15-MHz linear transducer (Acuson c256) in awake mice as described previously.

LV Function Response to Isoproterenol

After echocardiography, mice were anesthetized, and LV function was measured both at baseline and after injecting isoproterenol (ISO; a β-adrenergic agonist; 50 ng per mouse, IV) using a Millar pressure system. Parameters included heart rate, LV systolic pressure, maximum and minimum dp/dt (dp/dtmax and dp/dtmin, respectively), instantaneous pressure (LV pressure at maximum dp/dt), and dp/dt/Δp (an indicator of isovolumic contraction), which is the maximum rise in ventricular pressure divided by the pressure at the moment that dp/dt reaches a maximum, expressed as a function of seconds−1. Data were acquired using a Biobench system and analyzed using PVAN analysis software (Millar).

Plasma Renin Concentration and Urinary Nitrate/Nitrite Concentration

Before assessment of LV function, 20 μL of blood were collected in a microhematocrit tube by puncturing the retro-orbital plexus. Plasma was incubated with 250 ng of sheep angiotensinogen (renin substrate) at 37°C for 30 minutes. Ang I concentration was determined with a commercially available radioimmunoassay kit (Dia- sorn) and expressed as nanograms of Ang I per milliliter per hour. Urine samples were collected directly from the bladder. Urinary nitrate/nitrite concentration was determined by colorimetric assay (Griess reagent) after adding nitrate reductase, which converts nitrate to nitrite (Oxford Biomedical Research), and was corrected by urinary creatinine.

Histology and Immunohistochemistry

After evaluating LV function, the heart was dissected and weighed. The left ventricles were sectioned transversely into 3 sections from apex to base, rapidly frozen in liquid nitrogen, and stored at −80°C for assessment of myocyte cross-sectional area (MCSA), interstitial collagen fraction (ICF), and 4-hydroxy-2-nonenal (4-HNE).

Endothelial NOS, iNOS, and gp91phox Protein Expression

Heart tissue was homogenized in lysis buffer and centrifuged. The supernatant was collected and protein content measured with a Bio-Rad assay kit. Protein was separated out in 8% SDS-PAGE and electrotransferred to a nitrocellulose membrane (Amersham BioSciences). Membranes were blocked with 5% nonfat milk and incubated with a primary antibody (anti-endothelial NO synthase [eNOS] or -iNOS, Transduction Laboratories; anti-gp91phox, generously supplied by Dr Mark Quinn, Montana State University; anti-β-actin, Santa Cruz Biotechnology) overnight at 4°C. They were then washed and incubated with horseradish peroxidase–conjugated secondary antibodies (Amersham Biosciences) for 1 hour at room temperature. Immunoreactive bands were detected by a chemiluminescent reaction (ECL kit, Amersham Pharmacia) and semiquantified by densitometry. Results were expressed as the ratio of the density of specific bands with the corresponding β-actin.

Data Analysis

All of the data are expressed as means±SEs. For SBP, 1-way ANOVA and linear models were used to test the time effect of treatment within each strain, as well as the time effect between strains per treatment. For all of the other parameters, Student 2-sample t test was used to compare differences between strains within treatments (2K1C) or between treatments within each strain. The type 1 error rate was set at α=0.05, considering P<α as
Table 1. Effects of iNOS Deletion on Cardiac Hypertrophy and Fibrosis in Mice With 2K1C

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>inOS−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham (n=11)</td>
<td>2K1C (n=17)</td>
</tr>
<tr>
<td>BW, g</td>
<td>30±1</td>
<td>31±1</td>
</tr>
<tr>
<td>THW, mg/10 g</td>
<td>40.2±1.0</td>
<td>49.6±1.2*</td>
</tr>
<tr>
<td>RWV, mg/10 g</td>
<td>6.9±0.3</td>
<td>8.0±0.3*</td>
</tr>
<tr>
<td>LWV, mg/10 g</td>
<td>30.4±0.8</td>
<td>38.4±1.2*</td>
</tr>
<tr>
<td>LVDd, mm</td>
<td>2.68±0.07</td>
<td>2.78±0.11</td>
</tr>
<tr>
<td>LVDs, mm</td>
<td>1.10±0.03</td>
<td>1.14±0.06</td>
</tr>
<tr>
<td>PWT, mm</td>
<td>0.88±0.02</td>
<td>1.08±0.04*</td>
</tr>
<tr>
<td>EF, %</td>
<td>76.0±1.3</td>
<td>72.8±0.8*</td>
</tr>
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</table>

BW indicates body weight; THW, total heart weight corrected by body weight; RWV, right ventricular weight corrected by body weight; LWV, LV weight corrected by body weight; LVDs and LVDd, LV systolic and diastolic dimensions; PWT, posterior wall thickness; EF, ejection fraction.

*p<0.05, 2K1C vs sham within strains.
†p<0.05, iNOS vs WT with 2K1C.

Table 2. Effects of iNOS Deletion on Cardiac Functional Response to ISO in Mice With 2K1C

<table>
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<tr>
<th>Parameters</th>
<th>WT, ISO</th>
<th>inOS−/−, ISO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>369±20</td>
<td>516±19*</td>
</tr>
<tr>
<td>LVSP, mm Hg</td>
<td>64.2±3.1</td>
<td>123.1±8.5*</td>
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<td>dP/dt max, mm Hg/s</td>
<td>4750±577</td>
<td>10229±847*</td>
</tr>
<tr>
<td>dP/dt/ν, s⁻¹</td>
<td>120.9±9.3</td>
<td>254.2±10.0*</td>
</tr>
<tr>
<td>dP/dt min, mm Hg/s</td>
<td>-3933±455</td>
<td>-6455±378*</td>
</tr>
</tbody>
</table>

HR indicates heart rate; LVSP, LV systolic pressure.
*Data are after vs before ISO within treatment and within strains.
†p<0.05, 2K1C vs sham after ISO.
‡p<0.05, iNOS vs WT sham after ISO.
§p<0.05, iNOS vs WT 2K1C after ISO.

Results

Systolic Blood Pressure
Basal SBP remained unchanged in sham groups of both strains. 2K1C caused a significant increase in SBP; however, there was no difference between strains (Figure 1).

Heart Weight and Morphometry
Heart weight, LV chamber dimensions, and wall thickness were similar between strains before sham operation. 2K1C increased heart weight and posterior wall thickness to a similar extent in both strains, but diastolic LV chamber dimension was smaller in iNOS−/− (Table 1).

LV Function
Basal ejection fraction did not differ between wild-type (WT) and iNOS−/−. 2K1C caused a slight but significant decrease in ejection fraction in WT but not in iNOS (Table 1). In anesthetized mice with sham clamping, heart rate, LV systolic pressure, dP/dt max, dP/dt/ν, and dP/dt min were similar between strains both before and after ISO (Table 2). In mice with 2K1C, basal heart rate, dP/dt max, dP/dt/ν, and dP/dt min (before ISO) did not differ between strains; however, contraction and relaxation responses to ISO were significantly enhanced in iNOS−/− (Table 2 and Figure 2).

Myocyte Hypertrophy and Interstitial Fibrosis
MCSA and ICF were similar in both sham groups. 2K1C increased MCSA and ICF in both WT and iNOS−/− mice, and there was no difference between strains (Figure 3).

Markers for Oxidative Stress
4-HNE, a byproduct of lipid peroxidation and an indicator of oxidative stress, was weakly expressed in the heart in both sham groups. 2K1C enhanced 4-HNE expression in WT mice, and this effect was blunted in iNOS−/− mice (Figure 4). gp91phox (Nox2), a major subunit of NADPH oxidase expressed in the heart and vasculature, was also upregulated by 2K1C; however, the increase in Nox2 protein expression was the same in both WT and iNOS−/− mice (Figure 5).
**eNOS and iNOS Protein Expression in the Heart**

Of the sham-operated groups, eNOS expression tended to be higher in iNOS−/− mice, although the difference was not significant. In 2K1C hypertension, eNOS protein was upregulated in both strains, although it tended to be higher in iNOS−/− mice (Figure 6, left). In WT mice, iNOS protein was detected in the heart and upregulated by 2K1C. iNOS protein was undetectable in iNOS−/− mice with or without 2K1C (Figure 6, right).

**Urinary Nitrate/Nitrite and Plasma Renin Concentration**

Urinary nitrate/nitrite (NOX) was significantly lower in iNOS−/− under basal conditions (sham-operated group). After 2K1C, it increased significantly in both strains, although it was still significantly lower in iNOS−/− (Figure 7, left). Basal plasma renin concentration was similar in WT and iNOS−/−. As expected, 2K1C significantly increased plasma renin concentration in both strains; it tended to be higher in iNOS−/− mice, but the difference was not significant (Figure 7, right).

**Discussion**

Using a renin-Ang II–dependent hypertension model, we found that lack of iNOS did not affect SBP, myocyte size, or interstitial collagen deposition under basal conditions, nor did it influence the development of hypertension, LV hypertrophy, and interstitial fibrosis. However, cardiac function (as indicated by ejection fraction) and cardiac contraction and relaxation responses to ISO were greater in iNOS−/− than in WT mice with 2K1C hypertension. 4-HNE expression, a marker for the oxidant peroxynitrite and an indicator of oxidative stress, was significantly upregulated in WT mice with 2K1C, but this effect was blunted in iNOS−/− mice. These data are similar to our previous findings in mice with DOCA-salt hypertension, a renin-independent model, suggesting that, although iNOS does not play an important role in the development of hypertension, cardiac hypertrophy, and fibrosis in either renin-dependent or -independent hypertension, induction of iNOS could adversely affect cardiac responsiveness to adrenergic stimulation. Furthermore, these adverse effects may be mediated by increased oxidative stress brought on by excessive production of reactive oxygen species.

Unlike eNOS and neuronal NO synthase, iNOS is expressed at low levels in normal tissue but could be induced in response to stimuli such as tissue injury, inflammation, cytokines, and growth factors, as well as cardiovascular disease. The pathophysiological significance of iNOS induction/activation in the heart remains controversial. Bolli and colleagues reported that iNOS protects the heart against ischemia/reperfusion injury and plays a fundamental role in ischemic preconditioning; however, Mungre et al. showed that overexpression of iNOS causes cardiomyopathy,
heart block, and sudden death. The detrimental cardiac effects of iNOS have been well documented by many other investigators, including our own work. We reported that blockade of iNOS or targeted deletion of the iNOS gene reduces infarct size, improves cardiac remodeling and function after myocardial infarction, and affords better preservation of LV contraction and relaxation in mice with DOCA-salt hypertension (which is renin independent). However, iNOS does not seem to play an important role in the development of hypertension and/or cardiac hypertrophy. In our DOCA-salt mouse model, the degree of hypertension in iNOS−/− mice did not differ from WT mice. In the present study of mice with 2K1C hypertension (a model of renin-dependent hypertension that mimics human renovascular hypertension), we again showed that lack of iNOS had no effect on the development of hypertension and LV hypertrophy. Our data agree with the finding by Theuer et al that L-N(6)-(1-iminoethyl) lysine, an inhibitor of iNOS, could not suppress hypertension and cardiac hypertrophy in rats overexpressing both human renin and angiotensinogen genes (double-transgenic rats).

However, we did find that iNOS−/− had enhanced LV contractile and relaxation responses to ISO. Sam et al showed that peak LV-developed pressure was higher in iNOS−/− than WT mice after myocardial infarction, suggesting that contractile function was improved in iNOS−/− mice with myocardial infarction. Funakoshi et al demonstrated that disruption of the iNOS gene improved the β-adrenergic inotropic response in mice with cytokine-induced cardiomyopathy. These results were similar to the observation of Ullrich et al that iNOS−/− had a greater shortening fraction, dP/dt max, and dP/dt min after 7 hours of endotoxin challenge. Kobayashi et al reported that cardiac contractile function was protected when the iNOS inhibitor aminoguanidine was given to Dahl salt-sensitive rats on high salt. Taken together, these data support the hypothesis that, although iNOS may not play a significant role in the pathogenesis of hypertension and LV hypertrophy, it does adversely affect contractile and relaxation properties, especially in the hypertrophic and/or ischemic heart.

The underlying mechanisms responsible for iNOS-induced impairment of contractility are not yet fully understood. One possibility is that activation of iNOS produces large quantities of NO, which, in turn, augments the second messenger cGMP. cGMP accumulation has been shown to decrease cardiac contractility by reducing myofilament sensitivity to calcium. Studies have also suggested that activation of iNOS and increased reactive oxygen species generation may alter intracellular Ca2+ handling. Hydrogen radicals denature sarcoplasmic reticulum Ca2+-ATPase and impair Ca2+ uptake and release from the sarcoplasmic reticulum, whereas overexpression of sarcoplasmic reticulum Ca2+-ATPase preserves Ca2+ handling and reduces hydrogen radical–induced

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**Figure 4.** Left, Representative cardiac immunohistochemical staining with 4-HNE (red staining in the interstitial space and myocytes) in WT and iNOS−/− mice with and without 2K1C. Right, Semiquantitative data on cardiac 4-HNE expression in WT and iNOS−/− mice with and without 2K1C. **P<0.01, 2K1C vs sham within strains; ##P<0.01, iNOS−/− vs WT with 2K1C.

**Figure 5.** Cardiac Nox2 protein expression in WT and iNOS−/− mice with and without 2K1C. *P<0.05, 2K1C vs sham within strains.
contractile dysfunction. Gealekman et al demonstrated that selective iNOS blockade improved intracellular Ca\(^{2+}\) kinetics and enhanced β-adrenergic responsiveness in rats with volume overload–induced heart failure. In addition, inhibition or deletion of iNOS may upregulate neuronal NO synthase localized to the sarcoplasmic reticulum of cardiomyocytes, where it modulates the uptake and release of calcium and the pumping ability of the heart.

More importantly, induction of iNOS increased production of cytotoxic reactive oxygen species. NO reacts with the superoxide anion (O\(_2^-\)) generating the highly reactive oxidant peroxynitrite. iNOS is also capable of generating O\(_2^-\) independent of NO production. Zhang et al recently reported that iNOS is the major source of O\(_2^-\) in the heart, because lack of iNOS or iNOS inhibition attenuated myocardial O\(_2^-\) production. O\(_2^-\) can also dismutate, yielding hydrogen peroxide, which can be reduced to highly reactive hydrogen radicals. These pro-oxidants, together with their oxidizing metabolites, promote lipid peroxidation, damaging the heart and blood vessels.

In the present study, 2K1C hypertension significantly increased iNOS protein expression, urinary NO excretion, and cardiac levels of 4-HNE (a marker of lipid peroxidation) in WT controls, but these effects were diminished in iNOS\(^{-/-}\) mice, indicating that oxidative stress is reduced in iNOS\(^{-/-}\) with 2K1C. We also measured protein expression of Nox2, a subunit of NADPH oxidase and another major cardiovascular source of O\(_2^-\). We found that 2K1C increased Nox2 expression similarly in both WT and iNOS\(^{-/-}\) mice with and without 2K1C. iNOS protein was undetectable in iNOS\(^{-/-}\) hearts, *P<0.05, 2K1C vs sham within strains.

2K1C hypertension is characterized by high levels of renin and Ang II. The present study confirmed that plasma renin was significantly elevated after 2K1C hypertension, which validates our mouse model. In WT mice, iNOS protein was weakly expressed in the heart under normal conditions but upregulated after 2K1C hypertension. We also measured NOX, because NOX has been used as an indicator of NO production and oxidative stress. We found that NOX was significantly increased in WT mice with 2K1C but blunted in iNOS\(^{-/-}\), indicating that increased iNOS in response to 2K1C could be the major source of NO, leading to accumulated reactive oxidants. Ang II has been reported to induce natively
iNOS expression in the vasculature13–15; however, it remains unclear whether it also upregulates cardiac iNOS expression in Ang II–induced hypertension. Neri Serneri et al16 found that, in patients with unstable angina, Ang II levels in coronary sinus blood were greatly increased, associated with enhanced iNOS gene expression.

The precise mechanism responsible for Ang II–induced upregulation of iNOS is not clear. One could speculate that, because Ang II is known to be a profound proinflammatory stimulator, it might possibly stimulate cytokine release, activate monocyte chemoattractant protein-1, and augment inflammatory cell infiltration via activation of nuclear factor-κB, leading to activation of iNOS.13,46,47 In addition, shear stress has been reported to enhance iNOS expression and NO production46,49 so that endothelial shear stress in response to increased afterload could represent a trigger for the iNOS activation seen in 2K1C renovascular hypertension.

**Perspectives**

Induction of iNOS has been observed in numerous forms of cardiovascular diseases, including hypertension, myocardial infarction, and congestive heart failure. However, the pathophysiological role of iNOS induction remains controversial. Our study shows that, although lack of iNOS had no effect on blood pressure and cardiac hypertrophy caused by 2K1C hypertension, it enhanced cardiac contractile and relaxation responses to ISO while reducing production of peroxynitrite, an indicator of oxidative stress. We conclude that deletion of iNOS reduces reactive oxygen species production and oxidative stress, which may be responsible for the beneficial cardiac effect observed in iNOS−/− mice.

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**Disclosures**

None.

**References**


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