Chronic β-Adrenergic Receptor Stimulation Induces Cardiac Apoptosis and Aggravates Myocardial Ischemia/Reperfusion Injury by Provoking Inducible Nitric-Oxide Synthase-Mediated Nitrative Stress


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ABSTRACT

The present study provides evidence that inducible nitric-oxide synthase (iNOS)-mediated nitrative stress plays a pivotal role in chronic β-adrenergic receptor (AR) stimulation-induced cardiac damage. In mice, 14 days of isoproterenol (ISO) stimulation via an osmotic minipump induced an up-regulation of iNOS as evidenced by increases in mRNA, protein expression, and immunohistochemical staining of myocardial iNOS. Serum level of C-reactive protein, an inflammatory mediator, was also markedly increased. Under chronic ISO stimulation, the up-regulated iNOS produced a significantly increased amount of nitric oxide (NO) and its byproduct, peroxynitrite, in the circulation and heart and subsequently resulted in an accelerated myocardial apoptosis. Forty-minute myocardial ischemia (MI) and 24-h reperfusion (R) further increased NO production and peroxynitrite formation and resulted in an enlarged infarct size in mice receiving chronic ISO stimulation. However, the treatment with a selective iNOS inhibitor [N-(3-(aminomethyl) benzyl)acetamidine] (1400W) or the use of a genetic modified animal (iNOS-knockout mice) markedly reduced iNOS-mediated production of NO and formation of peroxynitrite and consequently significantly decreased myocardial apoptosis and infarct size, showing a crucial link between iNOS-mediated nitrative stress and myocardial injury. In conclusion, chronic β-AR stimulation up-regulates iNOS expression and increases NO production in the heart, which subsequently markedly enhances formation of reactive nitrogen species/peroxynitrite in the heart, thereby eliciting myocardial apoptosis and potentiating MI/R injury.

Catecholamine release secondary to activation of the sympathetic nervous system is a key neuromodulator of cardiovascular, metabolic, and other physiological functions in humans. However, a prolonged release or sudden surge of catecholamine, as seen in conditions such as major surgery, trauma, sepsis, and congestive heart failure, can profoundly increase the patient’s risk for cardiac complications, including cardiac arrhythmias, myocardial ischemia (MI) or/and infarction, and sudden cardiac death (Bristow, 2000; Lefkowitz et al., 2000). Both apoptotic and necrotic modes of cell death have been proposed as the mechanism underlying these cardiac complications. Many studies have addressed how catecholamine, especially β-adrenergic receptor (AR) stimulation, induces cardiac apoptosis or/and necrosis (Colucci et al., 2000; Remondion et al., 2003). Recent studies also found that in failing myocardium, increased inducible nitric-oxide synthase (iNOS) activity contributes to attenuate β-AR-mediated inotropic effect (Funakoshi et al., 2002; Gealekman et al., 2002). However, the role of iNOS in chronic β-AR stimulation-induced cardiac damage remains to be investigated.

Nitric oxide (NO), a reactive nitrogen species (RNS), is produced by three isoforms of NOS: endothelial NOS (eNOS), neuronal NOS (nNOS), and iNOS. All of them are present in...
the heart (Kelly et al., 1996). Unlike eNOS and nNOS, iNOS has not been found to be constitutively present in the normal, nonaged, adult heart, but it can be induced by proinflammatory substances such as cytokines and lipopolysaccharide or conditions such as stroke, trauma, infection, and a variety of cardiovascular diseases (Haywood et al., 1996; Kelly et al., 1996). Once induced, iNOS isoform is capable of producing large amounts of NO at 100 times greater than normal (≥1 μM versus ~10 nM) by resident cardiac cells and activated immune cells that infiltrate the injured heart (Nathan, 1997; Mungre et al., 2002; Ferdinandy and Schulz, 2003). Such high levels of NO are recognized as a mediator and regulator of immune cells that infiltrate the injured heart (Nathan, 1997; Mungre et al., 2002; Ferdinandy and Schulz, 2003). Thus, high levels of NO are recognized as a mediator and regulator of inflammatory responses. Recently, our studies found that acute β-AR stimulation in aging ischemic heart triggered a marked increase in NO production, generated toxic peroxynitrite, activated apoptosis, and eventually caused cardiac dysfunction and myocardial injury (Li et al., 2006). However, whether the induction or/and expression of iNOS is beneficial or deleterious over the long term, particularly its effects on myocardial apoptosis and MI/reperfusion (R) injury, remains enigmatic.

Therefore, the aims of this study were 1) to investigate whether chronic β-AR stimulation induces expression change of iNOS in the heart, and if so, 2) to determine whether iNOS-mediated nitrative stress results in myocardial apoptosis and contributes to MI/R injury. The results of this study show a pivotal role of iNOS in chronic β-AR stimulation-induced cardiac damages, including myocardial apoptosis and MI/R injury.

**Materials and Methods**

**Experimental Protocol.** The animal experiments involved in this study have been carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1996). C57B1/6 mice (3 months old, n = 12/group) were randomized to receive vehicle, isoproterenol (ISO, 30 μg/g/day), or ISO plus a selective iNOS inhibitor, 1400W (ISO + 1400W, 2 mg/kg/day started 24 h before chronic ISO infusion), via an osmotic minipump (model 2002; Alza Corp., Palo Alto, CA) implanted s.c. via a small interscapular incision and using sterile surgical technique. Fourteen days after the treatment, animals were then subjected to 40-min MI and 24-h R (MI/R). As described previously, the animals were anesthetized by 2% isoflurane for the surgery. MI was produced by temporarily exteriorizing the heart by means of a left thoracic incision and placing a 6-0 silk suture slipknot around the left descending coronary artery (Tao et al., 2004). After 40 min of MI, the slipknot was released, and the myocardium was reperfused for 24 h. After the surgery, the animals were allowed to recover and then individually housed and maintained on an ad libitum diet with free access to water. Another group of iNOS-knockout (KO) mice (Q57/BL6; The Jackson Laboratory, Bar Harbor, ME) was also chosen to receive 14-day ISO infusion without MI/R.

**ISO bitartrate.** (Sigma, St. Louis, MO) and 1400W were prepared in 0.9% sodium chloride at a concentration calculated to deliver a final dose of 30 μg/g/day for ISO and 2 mg/kg/day for 1400W, respectively, during the 14-day infusion period. 1400W (Cayman Chemical, Ann Arbor, MI) is a highly selective iNOS inhibitor (approximately 5000- and 200-fold more potent against purified human iNOS than eNOS and nNOS, respectively) (Garvey et al., 1997). The dosage of 1400W was chosen based on our previous study (Li et al., 2006) and other reports (Egi et al., 2001; Zhang et al., 2003). For the vehicle group, the mice were subjected to the same surgery for implanting the pump, and 0.9% sodium chloride was infused for 14 days in the same fashion as the treatment groups.

**RNA Isolation and Analysis.** A quantitative reverse transcription-polymerase chain reaction (RT-PCR) technique was used to determine expression levels of iNOS mRNA in myocardium. Total myocardial RNA was isolated and was reverse-transcribed. PCR reactions were performed using a real-time fluorescent determination (ABI Prism 7700 sequence; Applied Biosystems, Foster City, CA). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used for internal normalization.

**Western Blot Analyses of iNOS.** Left ventricular (LV) tissues were homogenized and lysed in 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid lysis buffer. An equal amount of protein (60 μg/lane) determined by the BCA method was loaded. Western blot analysis was performed with a monoclonal antibody against iNOS (1:500, respectively; Transduction Laboratories). Nitrocellulose membranes were then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG antibody (1:2000; Cell Signaling Technology Inc., Beverly, MA) and visualized using Supersignal chemiluminescent detection kit (Pierce, Rockford, IL). Imaging was performed, and whole band densities were analyzed using KODAK Image Station (PerkinElmer, Boston, MA).

**Immunohistochemistry.** LV tissues were removed and processed for immunohistochemical studies as described previously (Ma et al., 2001). Rabbit polyclonal anti-iNOS and anti-3-nitrotyrosine antibodies were purchased from Upstate Biotech (Lake Placid, NY). Immunological staining was developed with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

**Nitrite and Nitrate Measurements.** Blood was collected in the aorta immediately before removal of the heart in each group of mice. Nitrate concentrations were determined based on the previously reported vanadium reduction method (Gao et al., 2000).

**3-Nitrotyrosine Assay.** Quantification of myocardial tissue 3-nitrotyrosine levels was performed by using an enzyme-linked immunosorbent assay (ELISA) procedure as reported previously (Li et al., 2006).

**Determination of Myocardial Apoptosis.** Myocardial apoptosis was quantitatively analyzed by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining and caspase-3 activity assay as described previously (Gao et al., 2001). The number of TUNEL-positive cardiomyocytes was counted in 60 randomly selected high-power fields on the LV free wall at the mid-LV level from the endocardial to the epicardial portion. The percentage of TUNEL-positive cardiomyocytes was calculated by dividing the number of TUNEL-positive cardiomyocytes by the total number of cardiomyocytes in 60 microscopic fields.

**Measurement of Serum C-Reactive Protein.** Serum level of CRP was determined by using ELISA commercial kits (Immunology Consultants Lab Inc., Newberg, OR) according to the manufacturer’s instructions.

**Measurement of Infarct Size.** Twenty-four hours after R, the mice were anesthetized, and the hearts were excised. Myocardial infarct size was determined using 2,3,5- triphenyltetrazolium chloride (TTC) as described previously (Gao et al., 2002) with the following modifications. At the end of reperfusion, the left anterior descending coronary artery was occluded again, and 1.0% Evans blue dye (1.5 ml) was infused into the carotid artery catheter in a retrograde manner to delineate the ischemic zone [area at risk (AAR)] from the nonischemic zone. The heart was excised and stored at −70°C. For infarct size determination, frozen hearts were sliced perpendicularly to the long axis from apex to base in 1.0-mm-thick sections. Sections were stained by a 1% (w/v) solution of TTC in phosphate buffer (88 mM Na2HPO4 and 1.8 mM NaH2PO4) for 30 min at 37°C. After fixation in a 10% formalin solution, slices were placed between two microscope slides and digitally photographed. Evans blue dye-stained area, AAR (TTC-stained), and infarction area (negative TTC staining) for each slice were then determined by
the computer. The infarct area was compared with the entire AAR, and the infarct size was expressed as a percentage of the AAR.

**Statistical Analysis.** Data are reported as mean ± S.E.M. All the data were analyzed by analysis of variance, followed by Bonferroni post hoc tests. All the statistical analyses were performed using GraphPad Prism Software (San Diego, CA). Probabilities of $P < 0.05$ were considered to be statistically significant.

**Results**

ISO Stimulation Increases Myocardial iNOS Expression. As illustrated in Fig. 1, 14 days of ISO stimulation markedly increased mRNA and protein expressions of myocardial iNOS, which was associated with increasing level of iNOS immunohistochemical staining in myocardial tissues (Fig. 1). These results confirm that chronic β-AR stimulation induces both increases in mRNA and protein expression of iNOS.

ISO Stimulation Increases Plasma NO and Myocardial Nitrotyrosine Formation. To determine whether increased myocardial iNOS expression also increases NO production, plasma NO levels were measured after 14 days of ISO infusion. The toxic byproduct of NO-ONOO$^-$ was also measured in the myocardium. As shown in Fig. 2, plasma NO level and myocardial nitrotyrosine formation, a footprint of ONOO$^-$ formation, were significantly increased in the group of mice that received ISO compared with the vehicle group. The increase of NO production was significantly reduced by blocking iNOS pharmacologically (1400W treatment) or genetically (iNOS-KO).

iNOS-Mediated Nitrative Stress Significantly Contributes to Chronic β-AR Stimulation-Induced Myocardial Apoptosis. Figure 3 showed that 14 days of ISO infusion resulted in significant myocardial apoptosis as evidenced by significant increases in TUNEL staining and caspase-3 activity in the cardiomyocytes. The treatment with 1400W markedly reduced iNOS-mediated nitrative stress as evidenced by decreasing production of plasma NO and formation of myocardial nitrotyrosine (Fig. 2) ($P < 0.05$ versus ISO group). Moreover, myocardial apoptotic process (caspase-3 activation and TUNEL staining) was also significantly reduced (Fig. 3) ($P < 0.05$ versus ISO group). To obtain further evidence that iNOS expression contributes to ISO-induced nitrative stress and cardiomyocyte apoptosis, iNOS-KO mice were stimulated with ISO in an identical fashion as in wild-type mice. As illustrated in Fig. 2 and Fig. 3, ISO-induced nitrative stress and cardiomyocyte apoptosis were markedly reduced in iNOS-KO mice. These results clearly showed a causal relationship between iNOS-mediated overproduction of RNS and myocardial cell apoptosis.

Inflammatory Mediator CRP Was Increased Significantly after Chronic β-AR Stimulation. How β-AR stimulation increases iNOS expression and NO production must still be investigated. This experiment provides the evidence that plasma CRP, which is an inflammatory mediator and marker (Yeh, 2004), was markedly increased after 14 days of ISO stimulation. iNOS-KO or 1400W treatment failed to decrease CRP formation (Fig. 4A), indicating that β-AR stimulation increases CRP formation via a non-iNOS-mediated process.

ISO Stimulation Exacerbated MI/R Injury. Fourteen days of ISO stimulation not only induced myocardial cell

![Fig. 1. A, Western blot analysis of iNOS protein expression in the mice treated with vehicle, ISO, or ISO + 1400W. Anti-β-actin was used to normalize loading variations. B, bar graphs are summary data of normalized densitometric ratio. C, quantification of mRNA expression was performed by real-time PCR with the Applied Biosystems system. D, representative photomicrographs showing iNOS immunoreactivity in myocardium of mice treated with vehicle, ISO, or ISO + 1400W or iNOS-KO mice (KO) + ISO. In all the figures, data are mean ± S.E.M., n = 5–7 mice/group; *, $P < 0.05$ versus vehicle; #, $P < 0.05$ versus ISO; +, $P < 0.05$ versus ISO + MI/R.](image-url)
apoptotic death (Fig. 3) but also exacerbated myocardial cell death when the heart was subjected to MI/R injury as shown in Fig. 5. To determine the mechanisms for the exacerbation of MI/R injury or enlargement of infarct size, the levels of plasma NO and myocardial nitrotyrosine were measured. As illustrated in Fig. 2, 40 min of MI and 24 h of R resulted in further increases in NO production and nitrotyrosine formation (i.e., a marked increase of nitrative stress) and consequently led to an enlarged infarct size in the ISO group; however, 1400W treatment significantly reduced this nitrative stress (Fig. 2) and thereby decreased infarct size (Fig. 5).

Fig. 2. A, serum levels of NO in the mice receiving different treatments: mice received vehicle, ISO, or ISO with 1400W (ISO + 1400W); iNOS-KO mice received ISO (KO + ISO); and mice subjected to MI/R received vehicle, ISO, or ISO with 1400W. B, representative photomicrographs showing immunostaining for 3-nitrotyrosine in myocardium of mice receiving different treatments. C, bar graphs are ELISA analysis of myocardial 3-nitrotyrosine in the mice that received different treatments.

Fig. 3. A, representative photographs of TUNEL staining. Nuclei of normal cells are stained blue, and apoptotic nuclei are stained red. B, summary of TUNEL-positive myocytes from five to seven mice/group. Assays were performed in a blinded fashion. C, summary of caspase-3 activity determined with a colorimetric assay kit.
These results show that iNOS-mediated nitrative stress plays an important role in catecholamine-induced MI/R injury.

**Discussion**

The major hypothesis behind this study was that chronic β-AR stimulation may result in a phenotypic up-regulation of iNOS in the heart and may increase release of proinflammatory mediators. These changes may trigger a significant increase in NO production and subsequent generation of the powerful oxidant molecule peroxynitrite (ONOO\(^-\)). This peroxynitrite-induced nitrative stress may cause an accelerated cardiac apoptosis and ultimately create a vulnerable condition in the heart that contributes to myocardial damage, including MI/R injury. The findings of this study provide firm evidence that iNOS-mediated nitrative stress plays an essential role in chronic β-AR stimulation-induced cardiac damage. First, chronic ISO stimulation induced an up-regulation of iNOS as evidenced by increases in mRNA, protein expression, and immunohistochemical staining of myocardial iNOS. Second, the up-regulated iNOS produced a significant amount of NO and its toxic byproducts (peroxynitrite) in the circulation and heart, respectively. Third, chronic ISO stimulation resulted in myocardial apoptosis as shown by the activation of caspase-3 and an increase in TUNEL staining. Fourth, with the up-regulated iNOS and accelerated apoptotic process, 40-min MI and 24-h R further increased NO production and peroxynitrite formation and eventually resulted in an enlarged infarct size when subjected to MI/R injury in the mice receiving chronic ISO infusion. Fifth, the treatment with a selective iNOS inhibitor (1400W) or the use of a genetic modified animal (iNOS-KO mice) markedly reduced iNOS-mediated production of NO and peroxynitrite and also significantly decreased myocardial apoptosis and infarct size, showing a cause/effect relationship between iNOS-mediated nitrative stress and myocardial injury, induced by chronic ISO stimulation. To delineate potential mechanisms responsible for chronic β-AR stimulation-induced up-regulation of iNOS and iNOS-mediated cardiac damage, we further showed that chronic β-AR stimulation results in an elevation of circulating CRP, indicating release of proinflammatory mediators in the circulation. By selectively blocking iNOS pharmacologically with 1400W or genetically with iNOS-KO mice, chronic β-AR stimulation-induced elevation of CRP remains, indicating that chronic β-AR stimulation increases CRP production in an iNOS-independent fashion (i.e., iNOS is not the upstream molecule for CRP production).

**How Does β-AR Stimulation Induce iNOS Overexpression?** The mechanisms for β-AR stimulation-induced iNOS expression and NO production remain to be illustrated. A recent study from our laboratory has shown that β-AR stimulation significantly increases NO production in the ischemic aging rat heart, which is related to aging-induced phenotypic up-regulation of iNOS and ischemic stress in the heart (Li et al., 2006). The present study found that chronic β-AR stimulation markedly increases CRP release in mice. CRP is a proinflammatory mediator and an inflammatory marker (Yeh, 2004). It was found that CRP directly enhanced NO production in interleukin (IL)-1β-stimulated cardiac myocytes through a nuclear factor κB-independent mechanism (Ikeda et al., 2002). CRP may also increase NO production by acting as a proinflammatory stimulus to phagocytic cells by binding to the FcyRII receptor (Bharadwaj et al., 1999) or stimulating monocyte release of inflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor-α (Ballou and Lozanski, 1992). β-AR stimulation may also enhance iNOS expression and NO production through other mechanisms, including enhancing the IL-1β effect by stabilizing the iNOS message (Gustafsson and Brunton, 2000), increasing macrophage arginase activity (Bernard et al., 2000), enhancing l-arginine transport in activated macrophages (Lin et al., 2005), and producing a synergic effect with lipopolysaccharide or cytokines (Kurosaki et al., 2000; Chi et al., 2003). Further studies are needed to illustrate potential interactions between proinflammatory mediators (CRP, cytokines) and iNOS in reinforcing the inflammatory response triggered by chronic β-AR stimulation.

**Mechanisms of β-AR Stimulation-Induced Cardiac Injury.** Reactive oxygen species have been proposed to be responsible for β-AR-induced cardiac myocyte apoptosis. For example, Remondino et al. (2003) have shown that the superoxide dismutase/catalase mimetics markedly decreased β-AR-stimulated apoptosis in cardiac myocytes, thus implicating reactive oxygen species in this process (Singh et al., 2001). However, it remains unknown about whether iNOS-mediated NO production is involved in β-AR-induced cardiac toxicity. In the present study, a series of pathological reactions, such as ISO → iNOS expression → nitrative stress → myocardial apoptosis, is blocked by selectively inhibiting iNOS pharmacologically with 1400W or genetically with iNOS-KO, which provides direct evidence that iNOS-mediated nitrative stress plays a pivotal role in chronic β-AR stimulation-induced myocardial apoptosis.

NO is an important regulator of apoptosis within the mammalian system, capable of both inducing and preventing
apoptosis, depending on the level of NO production and environmental milieu (Nathan, 1997; Kim et al., 1999; Ferdinandy and Schulz, 2003). This bifunctional capacity is well illustrated in the heart. It seems that high levels of NO produced by iNOS promote apoptosis, whereas basal levels of NO production from eNOS protect cardiomyocytes from apoptosis (Kim et al., 1999; Razavi et al., 2005). Accumulating evidence has shown the role of iNOS in mediation of cell apoptosis as follows: 1) tumor necrosis factor-α induces apoptosis via iNOS expression and NO production in neonatal mouse cardiomyocytes (Song et al., 2000); 2) cytokine-induced apoptosis in rat ventricular myocytes is mediated by iNOS induction and ONOO− formation and is associated with an increase in Bax levels (Arstall et al., 1999); 3) IL-1β-induced iNOS expression can trigger NO-dependent apoptosis in adult cardiac fibroblasts, which seems to result from DNA damage and may be mediated by a p53-dependent apoptotic pathway (Tian et al., 2002); and 4) our previous studies showed that iNOS-mediated NO production and ONOO− formation increased markedly myocardial caspase-3 activation, which was attenuated significantly by the pre-treatment of a selective iNOS inhibitor, 1400W (Li et al., 2006).

NO is short-lived and a relatively unreactive radical, but NO can combine with superoxide to form the potent oxidant ONOO−, which has been shown to play a significant role in iNOS-mediated posts ischemic cell damage (Arstall et al., 1999; Walker et al., 2000). Peroxynitrite is the reaction product between superoxide and NO. Under normal physiological conditions, NO concentration is not high enough to compete with superoxide dismutase for superoxide; thus peroxynitrite production is very low. However, under pathological conditions in which iNOS expression and NO production are increased, a significant amount of peroxynitrite is formed. Substantial evidence exists that peroxynitrite is a highly cytotoxic molecule that contributes to cell death and tissue injury under a variety of cardiovascular diseases, including MI/R (Vinten-Johansen 2000; Ferdinandy and Schulz, 2003). As shown in this study, chronic β-AR stimulation up-regulated iNOS expression, increased NO production, and markedly enhanced myocardial nitrotyrosine formation. Based on these results, it is conceivable to conclude that chronic β-AR stimulation likely results in cardiomyocyte injury by increasing peroxynitrite formation.

Catecholamine, Cytokines, and RNS. The neurohumoral and immune systems are two major systems that maintain homeostasis in vivo. The present study evidence that chronic β-AR stimulation markedly breaks down such homeostasis and results in paradoxical responses from the immune system as manifested by iNOS up-regulation, CRP release, and nitrative stress, and that iNOS-mediated nitrative stress functions as a main interface linking chronic β-AR activation and myocardial cell apoptosis. More studies are needed to illustrate the intricate relationships among catecholamine, CRP, cytokines, and RNS. Nevertheless, our studies provide a unique insight into the mechanism for chronic β-AR stimulation-induced cardiac damage, that is, prolonged β-AR stimulation induces expression of iNOS and increases production of RNS and toxic peroxynitrite associated with CRP release, indicating an activated proinflammatory process. These changes accelerate cardiac cell apoptosis and ultimately create a biochemical milieu that predisposes the heart to potential pathological changes, including MI.

References


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