Cardioprotection Afforded by Inducible Nitric Oxide Synthase Gene Therapy Is Mediated by Cyclooxygenase-2 via a Nuclear Factor-κB–Dependent Pathway

Qianhong Li, MD, PhD; Yiru Guo, MD; Wei Tan, MD; Qinghui Ou, MD; Wen-Jian Wu, MSc; Diana Sturza, MD; Buddhadeb Dawn, MD; Greg Hunt, BS; Chuanjue Cui, MD; Roberto Bolli, MD

Background—Gene therapy with inducible nitric oxide synthase (iNOS) markedly reduces myocardial infarct size; this effect is associated with cyclooxygenase-2 (COX-2) upregulation and is ablated by COX-2 inhibitors. However, pharmacological inhibitors are limited by relative lack of specificity; furthermore, the mechanism whereby *iNOS* gene therapy upregulates COX-2 remains unknown. Accordingly, we used genetically engineered mice to test the hypothesis that the cardioprotection afforded by *iNOS* gene transfer is mediated by COX-2 upregulation via a nuclear factor (NF)-κB–dependent pathway.

Methods and Results—Mice received an intramyocardial injection of Av3/LacZ (LacZ group) or Av3/iNOS (iNOS group); 3 days later, myocardial infarction was produced by a 30-minute coronary occlusion followed by 4 hours of reperfusion. Among Av3/LacZ-treated mice, infarct size was similar in *COX-2*$^{-/-}$ and wild-type groups. *iNOS* gene transfer (confirmed by iNOS immunoblotting and activity assays) markedly reduced infarct size in wild-type mice but failed to do so in *COX-2*$^{-/-}$ mice. In transgenic mice with cardiac-specific expression of a dominant-negative mutant of IκBα (IκBα^{S32A,S36A}), the upregulation of phosphorylated IκBα, activation of NF-κB, and cardiac COX-2 protein expression 3 days after *iNOS* gene therapy were abrogated, which was associated with the abolishment of the cardioprotective effects afforded by *iNOS* gene therapy.

Conclusions—These data provide strong genetic evidence that COX-2 is an obligatory downstream effector of iNOS-dependent cardioprotection and that NF-κB is a critical link between iNOS and COX-2. Thus, iNOS imparts its protective effects, at least in part, by recruiting NF-κB, leading to COX-2 upregulation. However, COX-2 does not play an important cardioprotective role under basal conditions (when iNOS is not upregulated). (***Circulation*. 2007;116: 1577-1584.**)

Key Words: nitric oxide synthase ■ gene therapy ■ myocardial infarction ■ NF-kappa B ■ cyclooxygenase 2

The late phase of ischemic preconditioning (PC) is a delayed adaptation that requires the synthesis of new proteins and enhances the tolerance of the heart to a subsequent ischemic stress.[1–4] The study of late PC has led to the identification of genes that are mobilized endogenously by the heart in response to stress and can be exploited for gene therapy. Among these, considerable evidence points to a pivotal role of inducible nitric oxide synthase (iNOS), and the concept that the cardioprotection afforded by late PC is mediated by upregulation of iNOS is now widely accepted.[1–3] These findings have motivated the use of *iNOS* gene transfer to emulate the beneficial effects of late PC. Previous studies have demonstrated that *iNOS* gene therapy enhances the resistance of the heart to ischemia/reperfusion injury for at least 2 months.[5,6] Nevertheless, the mechanism whereby *iNOS* gene transfer confers protection against myocardial ischemia/reperfusion injury remains poorly understood.

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Pharmacological evidence implicates cyclooxygenase-2 (COX-2) as a key effector of the salubrious actions of iNOS.[4,5,7,8] In the context of ischemia-induced late PC, studies using COX-2 inhibitors have shown that both iNOS and COX-2 are necessary for the protection to occur[8,9] and that the activity of COX-2 is driven by iNOS activity (ie, COX-2 is downstream of iNOS).[4,7] In the context of *iNOS* gene therapy, it has been shown that the cardiac protection afforded by *iNOS* gene transfer is associated with COX-2 upregulation and is ablated by COX-2 inhibitors.[5] However, all of the evidence supporting a critical role of COX-2 as a mediator of the salutary effects of iNOS[5,7,8] is predicated on

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From the Institute of Molecular Cardiology, University of Louisville, Louisville, Ky.

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Correspondence to Roberto Bolli, MD, Division of Cardiology, University of Louisville, Louisville, KY 40292. E-mail rbolli@louisville.edu

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the use of pharmacological inhibitors of COX-2 and thus relies on the specificity of these drugs in vivo. Molecular genetic evidence based on deletion of COX-2 is lacking. In addition, the mechanism whereby iNOS activity upregulates COX-2 remains unknown.

The promoter region of the *COX-2* gene contains several consensus sequences for known DNA-binding proteins, including nuclear factor (NF)-κB.[10,11] NF-κB, an oxidant-sensitive transcription factor, plays a critical role in late PC[12] and in the activation of a multitude of genes in response to various stimuli and therefore serves as a general mediator of cellular responses to stress. These facts, coupled with the observation that NO activates NF-κB in noncardiac myocytes,[13,14] support the hypothesis that NF-κB may participate in the upregulation of COX-2 induced by *iNOS* gene therapy. However, virtually nothing is known about the role of NF-κB in modulating COX-2 expression in response to iNOS-derived NO in the myocardium.

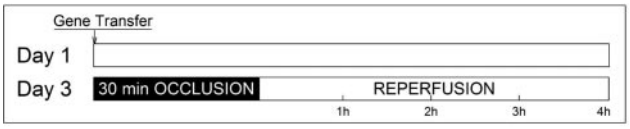
The overall goal of the present study was to use a molecular genetic approach to test the hypothesis that the cardioprotection afforded by *iNOS* gene therapy is mediated by COX-2 upregulation via NF-κB activation. Four fundamental questions were addressed: Does COX-2 play an obligatory role in the infarct-sparing effects of *iNOS* gene therapy? Does *iNOS* gene therapy activate NF-κB in myocardium? If so, does NF-κB activation play an essential role in the upregulation of COX-2 induced by *iNOS* gene therapy? Finally, does NF-κB activation play an essential role in the infarct-sparing effects of *iNOS* gene therapy? To address these issues, molecular analyses were combined with physiological studies in a well-characterized murine model of infarction. Genetically engineered mice were used in lieu of pharmacological agents. Mice with targeted disruption of the *COX-2* gene (*COX-2*$^{-/-}$) were studied to conclusively establish whether COX-2 plays an obligatory role in the cardioprotection afforded by *iNOS* gene transfer. Transgenic mice with cardiac-specific abrogation of NF-κB activation were used to investigate whether upregulation of COX-2 after *iNOS* gene transfer is mediated by NF-κB.

Methods

This study was performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (DHHS Publications No. 85–23, revised 1996) and with the guidelines of the Animal Care and Use Committee of the University of Louisville, School of Medicine (Louisville, Ky).

Genetically Engineered Mice

Two different mouse lines were used. *COX-2*$^{-/-}$ and wild-type (WT) (B6/129) were a kind gift from Dr Robert Langenbach.[15] Transgenic (Tg) mice that express a phosphorylation-resistant mutant of IκBα (IκBα^{S32A,S36A}) under the direction of a cardiac-specific promoter have been previously described[16]; in these mice, expression of the dominant-negative mutant IκBα results in cardiac-specific inhibition of NF-κB activation.[16] Tg mice were identified by polymerase chain reaction (PCR)–based DNA screening.[16] All analyses of IκBα^{S32A,S36A} Tg mice were performed using nontransgenic (NTg) littermates as controls. All mice were maintained in microisolator cages under specific pathogen-free conditions in a room with a temperature of 24°C, 55% to 65% relative humidity, and a 12-hour light-dark cycle.



Mouse Strains	Gene Transfer on Day 1	
	Av3/LacZ	Av3/iNOS
WT	+	+
COX-2$^{-/-}$	+	+
NTg	+	+
IκBα$^{S32A, S36A}$ Tg	+	+

Figure 1. Experimental protocol. On day 1, mice were subjected to intramyocardial injections of Av3/LacZ (LacZ group) or Av3/iNOS (iNOS group). On day 3, both groups underwent a 30-minute coronary occlusion followed by 4 hours of reperfusion to determine infarct size.

Adenoviral Vectors

Recombinant adenoviral vectors deleted in the E1, E2a, and E3 regions and carrying either a nuclear-targeted β-galactosidase reporter gene (Av3/LacZ) or the human *iNOS* gene (Av3/iNOS) were constructed essentially as previously described by homologous recombination between pAvS6/LacZ or pAvS6/iNOS and the large *Cla*I fragment constituting the right side of a novel Ad5 mutant that contains deletions in the E2a and E3 regions.[5,6,17] Plaque-isolated viral clones were propagated at a high titer in an A549-derived cell line, AE1-2a, which contains the Ad5 E1 and E2a region genes; they were then purified over 2 CsCl gradients and titered by plaque assay.[5,6]

In Vivo Gene Transfer

Mice (11 to 12 weeks old; body weight, 26.0±0.8 g) were anesthetized with sodium pentobarbital (50 mg/kg IP) and intubated. After the chest was opened through a midline sternotomy, mice received an intramyocardial injection in the anterior left ventricular wall of Av3/LacZ (1×10^7 plaque-forming units; Av3/LacZ group) or Av3/iNOS (1×10^7 plaque-forming units; Av3/iNOS group). Three days later, mice underwent the infarction protocol described below (Figure 1). The intramyocardial injection was 10 μL in volume and was performed with a 50-μL syringe with a 30-gauge needle; each mouse received 1 injection in the soon-to-be-ischemic region of the left ventricle.[5,6]

Coronary Occlusion/Reperfusion Protocol

The murine model of myocardial ischemia and reperfusion has been described in detail.[18,19] Briefly, mice were anesthetized with sodium pentobarbital (50 mg/kg IP) and ventilated using carefully selected parameters. After administration of antibiotics, the chest was opened through a midline sternotomy, and a nontraumatic balloon occluder was implanted around the mid left anterior descending coronary artery with an 8-0 nylon suture. To prevent hypotension, blood from a donor mouse was given at serial times during surgery.[18,19] Rectal temperature was monitored carefully and maintained between 36.7°C and 37.3°C throughout the experiment. In all groups, myocardial infarction was produced by a 30-minute coronary occlusion followed by 4 hours of reperfusion (Figure 1). The LacZ and iNOS groups received intramyocardial injections of Av3/LacZ or Av3/iNOS, respectively, as described above, 3 days before the 30-minute occlusion. In all groups, successful performance of coronary occlusion and reperfusion was verified by visual inspection (ie, by noting the development of a pale color in the distal myocardium after inflation of the balloon and the return of a bright red color as a result of hyperemia after deflation) and by observing ST-segment elevation and widening of the QRS on the ECG during ischemia and their

resolution after reperfusion. After the coronary occlusion/reperfusion protocol was completed, the chest was closed in layers, and a small catheter was left in the thorax for 10 to 20 minutes to evacuate air and fluids. The mice were removed from the ventilator, kept warm with heat lamps, given fluids (1.0 to 1.5 mL of 5% dextrose in water IP), and allowed 100% oxygen via nasal cone.

Postmortem Tissue Analysis

At the conclusion of the study, the occluded/reperfused vascular bed and the infarct were identified by postmortem perfusion of the heart with phthalo blue dye (Heucotech, Fairless Hill, Pa) and triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, Mo).[18,19] The corresponding areas were measured by computerized videoplanimetry (Adobe Photoshop version 7.0, Adobe Systems, San Jose, Calif); from these measurements, infarct size was calculated as a percentage of the region at risk.[18,19]

Reverse-Transcription PCR Study

Total RNA was isolated from the left ventricle with the TRI reagent (Sigma-Aldrich, St Louis, Mo). After total RNA extraction, all samples were tested for RNA integrity by electrophoresis. For reverse-transcription PCR detection of COX-1 and GAPDH transcripts, 100 ng total RNA was used for first-strand cDNA synthesis and PCR amplification with the One-Step Platinum Taq reverse-transcription -PCR kit (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions.[20] A 352-bp fragment for COX-1 or a 494-bp fragment for GAPDH was amplified for 30 cycles, respectively, with the following mouse COX-1–specific primers: forward, 5′-AGGAGATGGCTGCTGAGTTGG-3′, and reverse, 5′-CTCAGAGCTCAGTGGAGCGTC-3′ (Genbank accession No. NM008969), or with the following mouse GAPDH-specific primers: forward, 5′-GGCGCCTGGTCACCAGGGCTG-3′, and reverse, 5′-ATGGACTGTGGTCATGAGCCC-3′ (Genbank accession No. NG005915). PCR products were then visualized on a 1.8% agarose gel. Each sample was assayed in triplicate.

Quantification of NF-κB Activation

Nuclear protein fractions were prepared from freshly isolated mouse hearts using the Nuclear Extract Kit (Active Motif, Carlsbad, Calif) according to the manufacturer's instructions.[21,22] The activity of NF-κB subunit p50 or p65 was determined with the Trans-AM NFκB ELISA kit (Active Motif). Briefly, nuclear proteins (10 μg) were incubated with an oligonucleotide containing the NF-κB consensus binding site (5′-GGGACTTTCC-3′) bound to a 96-well microtiter plate. After extensive washes, the NF-κB complexes bound to the oligonucleotides were further incubated with a specific anti–NF-κB p50 or p65 antibody (1:1000 dilution). After the incubation and extensive washings, the plates were further incubated with a secondary antibody (goat anti-rabbit horseradish peroxidase-IgG, 1:1000 dilution), and tetramethyl benzidine (substrate) was added for color development, which was quantified by spectrophotometry (reading absorbance at 450 nm). Lysis buffer of nuclear extracts was used as a negative control.[23,24] In all groups, duplicate assays were performed for each sample, and the activity of NF-κB p50 or p65 was expressed as micrograms per milligram of nuclear protein.

Western Immunoblotting Analysis

Samples were homogenized in buffer A (25 mmol/L Tris · HCl [pH 7.5], 0.5 mmol/L EDTA [pH 7.5], 0.5 mmol/L EGTA [pH 7.5], 1 mmol/L phenylmethylsulfonyl fluoride, 2 μmol/L leupeptin, 1 μmol/L pepstatin, 1 μmol/L aprotinin, 10 mmol/L NaF, and 100 μmol/L dephostatin) and centrifuged at 14 000*g* for 15 minutes at 4°C. The resulting supernatants were collected as cytosolic fractions. The pellets were incubated in a lysis buffer (buffer A+10% glycerol+20 mmol/L CHAPS) for 4 hours at 4°C and centrifuged at 10 000*g* for 15 minutes at 4°C. The resulting supernatants were used as membranous fractions. The protein content in the cytosolic and membranous fractions was determined by the Bradford technique (Bio-Rad Laboratories, Hercules, Calif). The expression of IκBα, IκBβ, and IκBα phosphorylated at serine residues 32 and 36, COX-2, COX-1, and GAPDH was assessed by standard SDS/PAGE Western immunoblotting techniques.[5,19] Briefly, 80 μg protein was separated on an SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. Gel transfer efficiency was recorded carefully by making photocopies of membranes dyed with reversible Ponceau staining; gel retention was determined by Coomassie blue staining.[5,6,19] Proteins were probed with specific anti-IκBα, anti-IκBβ antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif), antiphosphorylated IκBα (Ser32, Ser36) antibody (Imgenex), anti–COX-1 and anti–COX-2 antibodies (Cayman Chemical), and anti-GAPDH antibody (Cell Signaling Technology, Danvers, Mass). Immunoreactive bands were visualized with horseradish peroxidase–conjugated anti-rabbit IgG using an enhanced chemiluminescence detection kit (NEN), quantified by densitometry, and normalized to the Ponceau stain density.

Statistical Analysis

Data are reported as mean±SEM and analyzed with 1-way ANOVA followed by Student's *t* tests. Because of the small sample sizes, data also were analyzed with nonparametric tests (Kruskal-Wallace test and Mann-Whitney test). The results were similar to those obtained with ANOVA and Student *t* tests; therefore, for the sake of simplicity and clarity, the latter (parametric) results are reported here. A value of $P<0.05$ was considered statistically significant. All statistical analyses were performed with the SigmaStat software system.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Exclusions

A total of 161 mice were used for this study: 72 mice for studies of infarct size, 43 mice for studies of NF-κB activation, and 46 mice for studies of COX protein expression. Nineteen mice died during or shortly after the surgical procedure, and 4 were excluded because of technical problems. Thus, a total of 138 mice were included in the final analyses.

Fundamental Physiological Parameters

Heart rate and body temperature, fundamental physiological parameters that may affect infarct size, were similar in all 8 groups of mice used in studies of coronary occlusion (Figure 1). By experimental design,[18,19] rectal temperature remained within a narrow, physiological range (36.8°C to 37.3°C) in all groups. Five minutes before the 30-minute coronary occlusion, the average heart rate in the 8 groups ranged from 530 to 595 bpm (P=NS). Heart rate did not differ significantly among the 8 groups at any time during the 30-minute occlusion or the ensuing reperfusion (the Table). The size of the region at risk, expressed as a percentage of left ventricular weight, did not differ among the 8 groups: WT+Av3/LacZ, 43±3%; WT+Av3/iNOS, 42±3%; COX-$2^{-/-}$+Av3/LacZ, 36±2%; COX-$2^{-/-}$+Av3/iNOS, 41±4%; NTg+Av3/LacZ, 41±4%; NTg+Av3/iNOS, 42±4%; IκB$\alpha^{S32A,S36A}$ Tg+Av3/LacZ, 39±1%; and IκB$\alpha^{S32A,S36A}$ Tg+Av3/iNOS, 43±2%.

Infarct Size in *COX-2*$^{-/-}$ Mice After *iNOS* Gene Therapy

Western immunoblotting confirmed that COX-2 protein expression was absent in the myocardium of *COX-2*$^{-/-}$ mice; no changes occurred in COX-1 protein levels and mRNA expression (Figure 2), consistent with previous reports.[15,25] As

Heart Rate on the Day of the 30-Minute Coronary Occlusion

		Occlusion		Reperfusion	
Groups	Preocclusion	5 minutes	30 minutes	5 minutes	15 minutes
WT+Av3/LacZ	546±15	567±15	572±11	558±14	579 ±8
WT+Av3/iNOS	556±25	584±27	600±23	590±21	593±18
COX-$2^{-/-}$ + Av3/LacZ	552±28	578±26	567±27	583±31	581±32
COX-$2^{-/-}$ + Av3/iNOS	595±22	605±27	633±28	637±20	640±22
NTg+Av3/LacZ	537±18	577±21	547±17	574±27	550±16
NTg+Av3/iNOS	530±13	553±16	578±18	563±16	569±13
IκB$\alpha^{S32A,S36A}$ Tg+Av3/LacZ	535±11	561±16	558±24	552±23	552±21
IκB$\alpha^{S32A,S36A}$ Tg+Av3/iNOS	534±12	580±21	585±35	566±29	573±22

Data are mean±SEM.

previously reported,[5,6] *iNOS* gene transfer resulted in upregulation of cardiac iNOS (data not shown). Three days after gene transfer in WT mice, infarct size was reduced by an average of 42% in Av3/iNOS-treated mice versus Av3/LacZ-treated WT mice, indicating an infarct-sparing effect of *iNOS* gene therapy (Figure 3). In *COX-2*$^{-/-}$ mice that received *LacZ* gene transfer, the infarct size was similar to that seen in WT mice that received *LacZ* gene transfer (Figure 3), implying that COX-2 does not modulate ischemia/reperfusion injury under basal conditions. However, in *COX-2*$^{-/-}$ mice given *iNOS* gene therapy, infarct size (43.1±3.7% of the risk region; n=6) did not differ significantly from that observed in mice that received Av3/LacZ (48.9±4.0% of the risk region; n=6) (Figure 3), demonstrating that COX-2 plays an obligatory role in the cardioprotection afforded by *iNOS* gene therapy.

Myocardial IκB Protein Content

Quantitative analysis of Western immunoblots demonstrated that the total amount of myocardial IκBα protein was increased 2.8-fold in IκB$\alpha^{S32A,S36A}$ Tg mice compared with NTg mice (Figure 4). No change occurred in the levels of myocardial IκBβ protein between IκB$\alpha^{S32A,S36A}$ Tg and NTg mice (Figure 4). *iNOS* gene transfer resulted in increased myocardial content of phosphorylated IκBα (at serine residues 32 and 36) in NTg mice (208±28% versus LacZ group, n=4; $P<0.05$) but not in IκB$\alpha^{S32A,S36A}$ Tg mice (Figure 5), indicating that Tg expression of the mutant IκB$\alpha^{S32A,S36A}$ effectively blocks phosphorylation of IκBα. *iNOS* gene transfer did not change the protein expression of GAPDH, an internal control, in either IκB$\alpha^{S32A,S36A}$ Tg mice or NTg mice (Figure 5).

Myocardial NF-κB Activation

Nuclear proteins extracted from the transduced myocardium of NTg or IκB$\alpha^{S32A,S36A}$ Tg mice were assayed for the presence of the active p50 or p65 subunit of NF-κB with an ELISA-based assay. As shown in Figure 6, in NTg mice, a robust activation of NF-κB (manifested as binding of NF-κB proteins to nuclear DNA) was observed 3 days after *iNOS* gene transfer in both the p50 subunit (303±31%, n=4, versus the LacZ group, n=4; $P<0.05$) and the p65 subunit (219±28%, n=4, versus the LacZ group, n=4; $P<0.05$).

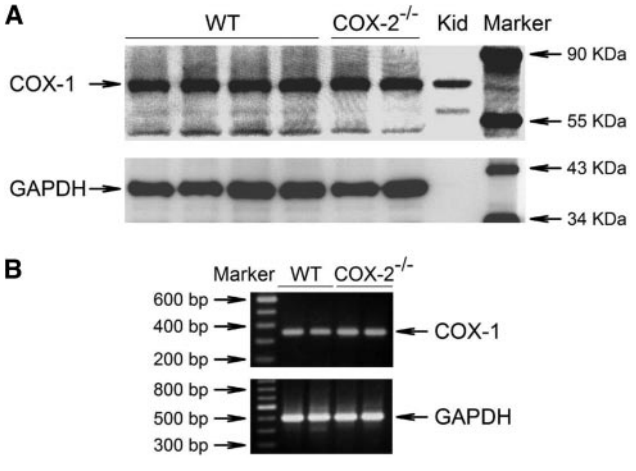


Figure 2. COX-1 protein and mRNA content in myocardium of *COX-2*$^{-/-}$ mice. No differences existed in myocardial COX-1 protein levels (A) or COX-1 mRNA levels (B) between *COX-2*$^{-/-}$ mice (n=2) and WT mice (n=4). The kidney served as a positive control for COX-1 protein expression, and GAPDH served as an internal control for both COX-1 protein and mRNA detection. Assays were performed in triplicate.

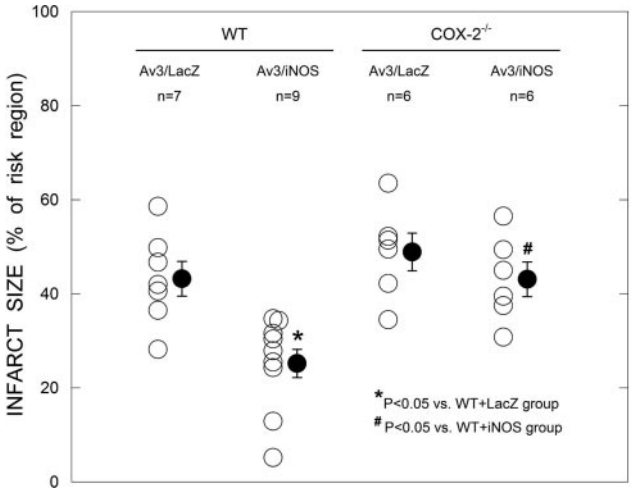


Figure 3. Effect of ablation of the *COX-2* gene on the infarct-sparing effect of *iNOS* gene therapy. Myocardial infarct size is expressed as a percentage of the region at risk. ○ Indicates individual mice; ●, mean±SEM.

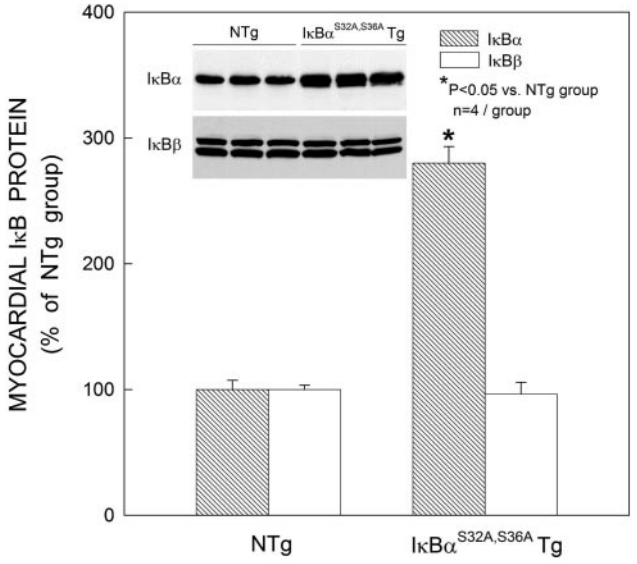


Figure 4. Representative Western blots of total myocardial IκBα and IκBβ protein content. Total myocardial IκBα protein levels increased 2.8-fold with no changes in IκBβ protein content in $I\kappa B\alpha^{S32A,S36A}$ Tg vs NTg. Data are mean±SEM of experiments performed in duplicate.

Activation of myocardial NF-κB in response to *iNOS* gene transfer was completely abrogated in $I\kappa B\alpha^{S32A,S36A}$ Tg mice with respect to both the p50 subunit (n=4) and the p65 subunit (n=4) (Figure 6), demonstrating that Tg expression of the mutant $I\kappa B\alpha^{S32A,S36A}$ effectively blocks iNOS-induced translocation of NF-κB to the nucleus and binding to DNA.

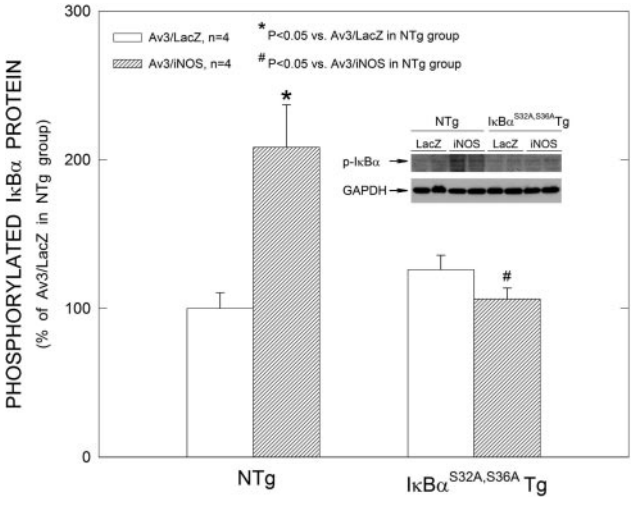


Figure 5. Phosphorylation of myocardial IκBα at serine residues 32 and 36 after *iNOS* gene transfer. Both NTg and $I\kappa B\alpha^{S32A,S36A}$ Tg mice received Av3/LacZ or Av3/iNOS gene transfer 3 days before immunoblotting analyses. GADPH served as an internal control for protein expression. NTg mice subjected to *iNOS* gene transfer showed an increased phosphorylation of IκBα (p-IκBα) in the transduced myocardium. Conversely, *iNOS* gene transfer failed to do so in the transduced myocardium of $I\kappa B\alpha^{S32A,S36A}$ Tg mice, indicating that cardiac-specific Tg expression of the mutant $I\kappa B\alpha^{S32A,S36A}$ completely abrogated the phosphorylation of IκBα at serine residues 32 and 36 in response to *iNOS* gene transfer. Data are mean±SEM of experiments performed in triplicate.

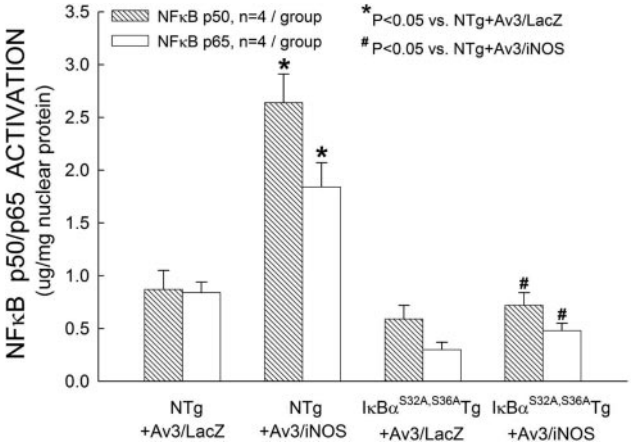


Figure 6. Activity of NF-κB p50 and p65 subunits in the myocardium. Both NTg and $I\kappa B\alpha^{S32A,S36A}$ Tg mice received an intramyocardial injection of Av3/LacZ or Av3/iNOS 3 days before the ELISA-based NF-κB activity analyses. NTg mice subjected to *iNOS* gene transfer showed robust activation of NF-κB p50 and p65 subunits in the transduced myocardium. Conversely, *iNOS* gene transfer failed to induce activation of either the p50 or the p65 NF-κB subunit in the transduced myocardium of $I\kappa B\alpha^{S32A,S36A}$ Tg mice, indicating that cardiac-specific Tg expression of $I\kappa B\alpha^{S32A,S36A}$ completely abrogated activation of NF-κB. The specificity of myocardial NF-κB activity was verified by specific competition with WT and mutated NF-κB consensus oligonucleotides. Data are mean±SEM of experiments performed in duplicate.

Infarct Size in $I\kappa B\alpha^{S32A,S36A}$ Tg Mice After *iNOS* Gene Therapy

When NTg mice were transduced with Av3/LacZ 3 days before the 30-minute coronary occlusion, infarct size averaged 47.3±5.7% of the risk region (Figure 7). As expected,[5] in NTg mice that received Av3/iNOS, the average infarct size was 37% smaller than in the Av3/LacZ-treated NTg group (30.0±3.6% of the risk region versus 47.3±5.7%, respectively; $P<0.05$; Figure 7), indicating that the expression of iNOS was associated with cardioprotection. In $I\kappa B\alpha^{S32A,S36A}$

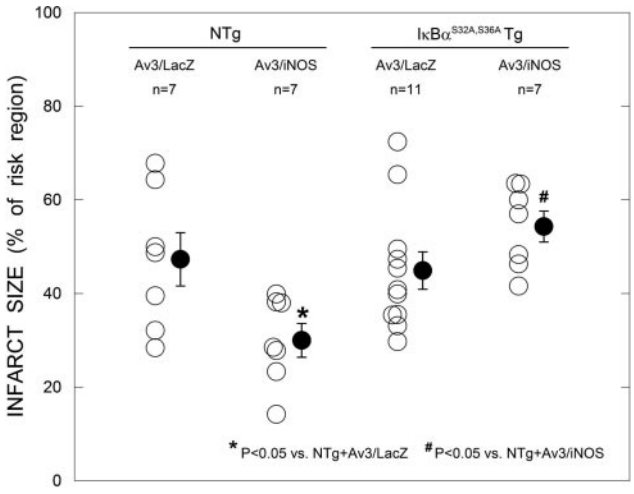


Figure 7. Disruption of NF-κB activation by cardiac-specific expression of a mutant $I\kappa B\alpha^{S32A,S36A}$ abrogates the infarct-sparing effect of *iNOS* gene therapy. Myocardial infarct size is expressed as a percentage of the region at risk. ○ Indicates individual mice; ●, mean±SEM.

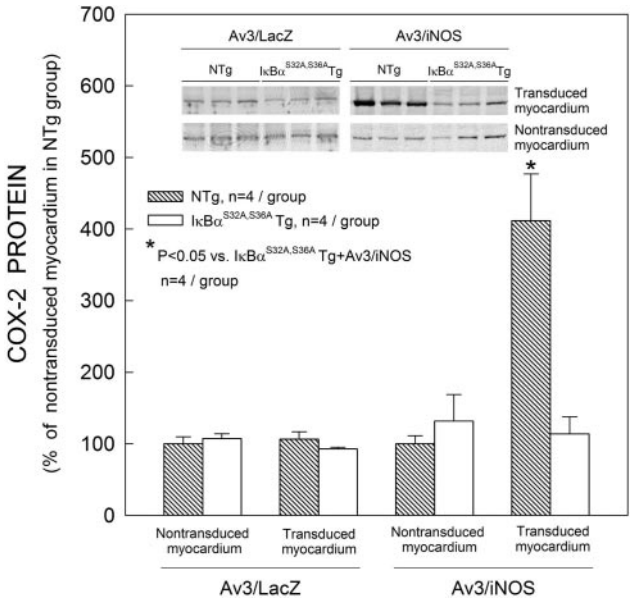


Figure 8. The upregulation of COX-2 elicited by *iNOS* gene transfer is abrogated in IκBα^{S32A,S36A} Tg mice. Both NTg and IκBα^{S32A,S36A} Tg mice received Av3/LacZ or Av3/iNOS gene transfer 3 days before immunoblotting analyses. NTg mice subjected to *iNOS* gene transfer exhibited a 3.6-fold increase in COX-2 protein expression in the transduced myocardium, with no change in nontransduced myocardium. Conversely, *iNOS* gene transfer failed to upregulate COX-2 protein levels in either the transduced or nontransduced myocardium in IκBα^{S32A,S36A} Tg mice. Data are mean±SEM of experiments performed in duplicate.

mice given Av3/LacZ, the infarct size was similar to that observed in NTg mice treated with Av3/LacZ (Figure 7). However, in contrast to NTg mice, in IκBα^{S32A,S36A} Tg mice given Av3/iNOS, the infarct size was not reduced compared with IκBα^{S32A,S36A} Tg mice given Av3/LacZ (Figure 7), indicating that disruption of NF-κB activation by cardiac-specific expression of a mutant IκBα^{S32A,S36A} completely abrogates the infarct-sparing effects of *iNOS* gene therapy.

Myocardial COX-2 Protein Content

As expected,[5] NTg mice transfected with Av3/iNOS exhibited robust expression of COX-2 in the transduced myocardium (Figure 8). In contrast, immunoreactive COX-2 was weakly detectable in the transduced myocardium of IκBα^{S32A,S36A} Tg mice treated under the same conditions. On average, transfection of NTg mice with Av3/iNOS resulted in a 3.6-fold increase in COX-2 protein content compared with the Av3/iNOS-treated IκBα^{S32A,S36A} Tg mice (Figure 8). In nontransduced myocardium, COX-2 protein expression did not change 3 days after *iNOS* gene transfer in either the NTg group or the IκBα^{S32A,S36A} Tg group (Figure 8). Additionally, *iNOS* gene transfer did not change COX-1 protein content in transduced myocardium in either IκBα^{S32A,S36A} Tg mice or NTg mice (Figure I in the online Data Supplement). COX-2 protein levels in other major organs (lung, liver, spleen, and kidney) did not differ between IκBα^{S32A,S36A} Tg and NTg groups after *iNOS* gene transfer (online Figure II), indicating that cardiac-specific abrogation of NF-κB activation by transdominant expression of a mutant IκBα^{S32A,S36A} specifically blocks the upregulation of cardiac COX-2 protein elicited by *iNOS* gene therapy.

Discussion

Although COX-2 has previously been implicated as a mediator of the beneficial effects of iNOS upregulation after ischemic PC[7] or *iNOS* gene therapy,[5] these investigations were predicated on the use of pharmacological COX-2 inhibitors. Furthermore, virtually nothing is known about the mechanism whereby *iNOS* gene therapy upregulates cardiac COX-2 expression. The present study provides new information pertinent to these issues. The salient findings can be summarized as follows. First, targeted disruption of the *COX-2* gene completely abrogates the infarct-sparing effects of *iNOS* gene therapy, demonstrating that COX-2 is necessary for *iNOS* gene transfer–dependent cardioprotection. Second, *iNOS* gene transfer results in increased phosphorylation of IκBα, leading to pronounced activation of cardiac NF-κB. Third, cardiac-specific abrogation of NF-κB activation via expression of a dominant-negative mutant IκBα (IκBα^{S32A,S36A}) abrogates not only the upregulation of cardiac COX-2 protein expression 3 days after *iNOS* gene transfer but also the attendant infarct-sparing effects, demonstrating that both the increased expression of COX-2 and the limitation of infarct size after *iNOS* gene transfer are dependent on an NF-κB–mediated pathway. Finally, after Av3/LacZ gene transfer, the infarct size is similar in *COX-2*$^{-/-}$ and WT mice, indicating that under basal conditions (when iNOS is not upregulated) COX-2 does not play a cardioprotective role. To the best of our knowledge, this is the first demonstration that COX-2 and NF-κB play an obligatory role in the cardioprotection afforded by *iNOS* gene therapy and that iNOS-dependent COX-2 induction is mediated by NF-κB activation.

Role of NF-κB in the Cardioprotection Induced by *iNOS* Gene Transfer

NF-κB is a ubiquitous dimeric transcription factor that exists in the cytosol complexed to an inhibitory κB (IκB) monomer. NF-κB complexes consist most commonly of p50/p65 dimers.[26,27] Various stimuli activate NF-κB, leading to its translocation to the nucleus, where it binds to specific DNA binding sites, called κB, present within the promoter region of various genes including *COX-2*.[27] Activation of NF-κB is controlled by site-specific phosphorylation and ubiquitination of IκB proteins.[26] Among the known IκB proteins, IκBα and IκBβ have been studied most extensively and are thought to be the key regulators of NF-κB nuclear translocation. Phosphorylation of serine residues at positions 32 and 36 is critical for the ubiquitination and degradation of IκBα.[16,28,29]

Pharmacological studies have implicated NF-κB as a necessary transcription factor in the development of late PC.[1,3,12,30] Unequivocal assessment of the functional contribution of NF-κB to this process, however, has been impeded by the lack of tools to achieve selective inhibition of this transcription factor in vivo. Although various pharmacological inhibitors of NF-κB are available, none of them has sufficient specificity to provide conclusive evidence of NF-κB function.[16,31] To overcome this problem, we have

created Tg mice that express a cardiac-specific dominant-negative mutant IκBα protein in which both serines are replaced by alanines ($I\kappa B\alpha^{S32A,S36A}$).[16] We have demonstrated that $I\kappa B\alpha^{S32A,S36A}$ Tg mice exhibit normal cardiac morphology and histology and that the expression of the mutant IκBα completely blocks the NF-κB activation elicited by tumor necrosis factor-α and lipopolysaccharide, two of the most potent stimuli known to activate NF-κB.[16] This Tg mouse overcomes the limitations inherent in pharmacological manipulations of NF-κB and provides an in vivo system to conclusively determine the role of NF-κB in pathophysiological states.

In the present study, immunoblotting analyses confirmed a selective elevation of total myocardial IκBα protein levels in $I\kappa B\alpha^{S32A,S36A}$ Tg mice with no appreciable change in IκBβ levels (Figure 4). In NTg mice, *iNOS* gene transfer resulted, 3 days later, in increased phosphorylation of IκBα serine residues 32 and 36 (Figure 5) and in robust activation of NF-κB in the nuclear fraction (Figure 6). In contrast, the phosphorylation of IκBα and the activation of NF-κB were completely abrogated in $I\kappa B\alpha^{S32A,S36A}$ Tg mice (Figures 5 and 6), demonstrating that expression of the $I\kappa B\alpha^{S32A,S36A}$ dominant-negative mutant was quite effective in inhibiting the recruitment of this transcription factor. Importantly, repression of NF-κB activation in $I\kappa B\alpha^{S32A,S36A}$ Tg mice was associated with inhibition of cardioprotection after *iNOS* gene therapy (Figure 7), indicating that the nuclear translocation and increased DNA-binding activity of NF-κB observed 3 days after *iNOS* gene therapy are not merely epiphenomena but rather obligatory components of the mechanism underlying the salubrious effect of *iNOS* gene therapy. These results provide the first molecular genetic evidence that NF-κB plays a critical role in cardioprotection.

Role of COX-2 in the Cardioprotection Induced by *iNOS* Gene Transfer

Previous studies have shown that the infarct-sparing effects of *iNOS* gene therapy are associated with COX-2 upregulation and are ablated by COX-2 inhibitors.[5] In addition, iNOS and COX-2 are coinduced during late PC, and the enzymatic activity of COX-2 after PC requires iNOS-derived NO, whereas iNOS activity is independent of COX-2, implying that COX-2 is located downstream of iNOS in the protective pathway of late PC.[7] However, unequivocal determination of the role of COX-2 in pathophysiological processes requires specific inhibition of COX-2 in vivo, which thus far has not been done. As a result, the contribution of COX-2 to the protective effects of iNOS in the setting of *iNOS* gene transfer or late PC has been inferred from the effects of pharmacological inhibitors.[5,7,8]

Unlike previous investigations,[5,7] in the present study, we used a molecular genetic approach by studying mice with targeted disruption of the *COX-2* gene. The similarity in infarct size between WT and $COX\text{-}2^{-/-}$ mice after Av3/LacZ administration (Figure 3) implies that COX-2 does not play a significant cardioprotective role under basal conditions, possibly because of its low level of expression in normal myocardium (Figure 8). However, our finding that the infarct-sparing effects of *iNOS* gene therapy were ablated by *COX-2* gene knockout (Figure 3) provides conclusive evidence that the increased COX-2 protein expression induced by *iNOS* gene transfer is necessary for the acquisition of ischemic tolerance after *iNOS* gene therapy. To test the hypothesis that iNOS-derived NO upregulates COX-2 via activation of NF-κB, we used the $I\kappa B\alpha^{S32A,S36A}$ mice in which, as discussed above, expression of a degradation-resistant IκBα mutant blocks the translocation of NF-κB to the nucleus after *iNOS* gene transfer (Figure 6). The finding that the upregulation of COX-2 by *iNOS* gene transfer was abolished in these mice (Figure 8) demonstrates unequivocally that this phenomenon is mediated by NF-κB activation.

The mechanism whereby COX-2 upregulation limits infarct size likely relates to increased myocardial production of protective prostanoids. Specifically, our previous studies[4,7,9] suggest that the beneficial effects of COX-2 upregulation are mediated by PGE_2 and/or PGI_2, 2 prostanoids shown to exert a number of salutary actions during myocardial ischemia/reperfusion, including antagonism of adenylyl cyclase, activation of ATP-sensitive potassium channels, inhibition of Ca^{2+} influx, and attenuation of neutrophil infiltration.[4]

Conclusions

Using a molecular genetic approach, we have demonstrated for the first time that NF-κB and COX-2 play a necessary role in the cardiac protection afforded by *iNOS* gene therapy and that NF-κB activation is essential for iNOS-dependent upregulation of COX-2. Our findings provide new insights into the mechanisms that regulate COX-2 expression in cardiac tissue and into the biology of the iNOS–COX-2 cardioprotective module.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The concept that the cardioprotection afforded by late phase of ischemic preconditioning is mediated by upregulation of inducible nitric oxide synthase (iNOS) was initially established in 1997 and is now widely accepted. The most promising approach to translate these important findings from the basic science laboratory to the clinical arena is to use *iNOS* gene transfer to emulate the beneficial effects of ischemia-induced late ischemic preconditioning on myocardial infarction. Indeed, prophylactic gene therapy (ie, the transfer of cardioprotective genes to prevent myocardial infarction) is a promising novel approach to limit infarct size. Previous studies indicate that *iNOS* gene therapy enhances the resistance of the heart to ischemia/reperfusion injury for at least 2 months, but the mechanism remains poorly understood. By using a specific gene knockout and a cardiac-specific gene mutation in a mouse model of *iNOS* gene therapy, this study shows that cyclooxygenase-2 is an obligatory downstream effector of iNOS-dependent cardioprotection and that iNOS imparts its protective effects, at least in part, by recruiting nuclear factor-κB, leading to cyclooxygenase-2 upregulation. Additionally, this study shows that cyclooxygenase-2 does not play an important cardioprotective role under basal conditions (ie, when iNOS is not upregulated). These observations demonstrate the feasibility of inducing a late ischemic preconditioning-like cardiac phenotype with *iNOS* gene therapy and advance our understanding of the molecular mechanism that renders this phenotype tolerant to ischemia, thereby providing a strong rationale for further preclinical testing of prophylactic gene therapy.

Cardioprotection Afforded by Inducible Nitric Oxide Synthase Gene Therapy Is Mediated by Cyclooxygenase-2 via a Nuclear Factor- κB–Dependent Pathway

Qianhong Li, Yiru Guo, Wei Tan, Qinghui Ou, Wen-Jian Wu, Diana Sturza, Buddhadeb Dawn, Greg Hunt, Chuanjue Cui and Roberto Bolli

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