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in vivo Cardiac Gene Transfer of Dominant Negative IKK-β Reduces Myocardial Inflammation, Apoptosis, and Infarction after Ischemia-Reperfusion Injury

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ABSTRACT

Background and Objectives : NF-κB transcription factors drive the expressions of many genes involved in inflammation and cell survival, which are both important in ischemia-reperfusion (IR) injury. IKK-β can mediate NF-κB activation through the phosphorylation of IκB; however, alternative pathways of activation exist. **Materials and Methods** : To test the role of IKK-β in cardiac IR injury, cardiac gene transfer of dominant negative IKK-β (dnIKK-β) was performed in rats 48 hr prior to IR. **Results** : Adenoviral gene transfer was found to result in regional transgene expression encompassing ~60% of the ischemic area. Ad.dnIKK-β reduced the IR-induced NF-κB translocation and IκB-α degradation, and blocked induction of the NF-κB-dependent inflammatory chemokine, MCP-1, in the ischemic area compared to the Ad.EGFP.β-gal treated rats ($p<0.05$). Neutrophil infiltration in the ischemic area (as indicated by myeloperoxidase activity) was decreased by 33% in the Ad.dnIKK-β treated rats compared to the Ad.EGFP.β-gal treated rats ($p<0.05$). Ad.dnIKK-β also reduced IR-induced apoptosis, as reflected by the attenuated DNA laddering compared to rats injected with either buffer or Ad.EGFP.β-gal. The ischemic area was not affected by dnIKK-β expression. However, Ad.dnIKK-β reduced infarction(%MI) by 57% compared to the Ad.EGFP.β-gal treated rats ($p<0.01$). **Conclusion** : Thus, *in vivo* gene transfer of dnIKK-β prevents the IR-induced activation of NF-κB. In this setting, abrogation of pro-inflammatory signals appears more important than loss of NF-κB dependent survival factors, resulting in an overall reduction in apoptosis and infarct size. These data suggest that IKK-β may represent a valuable target for therapeutic intervention in IR injury. **(Korean Circulation J 2005; 35:206-214)**

KEY WORDS : Reperfusion injury ; Gene transfer ; Signal transduction ; Adenovirus.

Introduction

While the mainstay of current therapy in acute myocardial infarction (MI) is reperfusion, with many studies having demonstrated the clinical benefit of this approach, reperfusion is itself associated with myocardial damage; termed ischemia-reperfusion (IR) injury. In animal models, cardiac IR injury is associated with early endothelial dysfunction,[1] increased adhesion molecule and cytokine expression,[2)3)] activation of the alternate complement pathway,[4] infiltration of circulating neutrophils,[5] release of oxygen free radicals, as well as other potentially toxic products,[4)6)7)] and programmed cell death or apoptosis.[8)9)] Understanding the basis of, and learning to minimize, the injury due to IR could maximize the benefits of reperfusion therapy in acute infarction.

The family of transcription factors, nuclear factor kappa B (NF-κB), is activated by diverse stimuli, including oxidative stress and inflammatory cytokines, which drive the expressions of many genes involved in inflammation and cell survival, both critical elements of IR injury.[1)10)] In IR, NF-κB is activated early[11] and drives the expressions of cytokines and adhesion molecules,[2)3)12)] which enhance local recruitment of inflammatory leukocytes that contribute to IR injury. However, NF-κB also drives the expression of survival factors that appear particularly important in cardiomyocytes.[13] Thus, while NF-κB inhibition might limit inflammation in IR, it could also potentiate cardiomyocyte apoptosis. Clinical manipulation of NF-κB is further complicated by the difficulty of targeting transcription factors through traditional pharmaceutical approaches. Conversely, a successful precedent exists for the clinical

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development of highly specific and effective kinase inhibitors,[14] and recent advances in our understanding of the mechanisms of NF-κB activation could lay the foundation for effective clinical manipulation of this pathway if appropriate molecular targets can be identified.

NF-κB generally exists as a dimer in the cytosol bound to one of three inhibitory, IκB, subunits.[15] A major mechanism of NF-κB activation is the serine phosphorylation and degradation of IκB, followed rapidly by translocation of NF-κB to the nucleus, where it activates transcription of specific promoter targets. In addition, some members of the NF-κB family, such as p65, can be regulated through direct phosphorylation of the transactivation domain (TAD), further enhancing gene transcription.[16] Two known kinases, IKK-α and IKK-β, can each phosphorylate IκB.[17] IKK-β is activated rapidly in the heart after IR.[10] Mice lacking IKK-β die as embryos, but their embryonic fibroblasts have defective NF-κB activation in response to cytokine stimulation.[18] IKK-α appears important for activation of one of the NF-κB family members (NF-κB2) and a subset of NF-κB dependent genes, particularly in B cells.[19] IKK-α is also important in skin development, but this function is independent of its kinase activity.[19] In addition, NF-κB activation can also be regulated by *tyrosine* phosphorylation of IκB, which leads to dissociation from p65 without IκB degradation.[20] The kinase responsible for this event has not been identified, but appears to be particularly important in NF-κB activation after hypoxia-reoxygenation.[20] Finally, recent data suggests reversible acetylation of p65 also modulates its association with IκB and transcriptional activity.[21] Thus, multiple mechanisms of NF-κB regulation exist, but their relative contribution to myocardial injury after IR is currently unknown.

To test the role of IKK-β in cardiac IR injury, the adenoviral gene transfer of a highly specific and effective dominant negative IKK-β mutant (K44A) was used and the effects on NF-κB activation, inflammation, apoptosis and cardiac injury examined. Our results suggest that targeting IKK-β may be an effective strategy to maximize the benefits derived from reperfusion therapy.

Materials and Methods

Recombinant adenoviral vectors

Two recombinant type 5 adenoviruses (Ad.EGFP. β-gal and Ad.dnIKK-β) were used in these studies. Ad.EGFP. β-gal contains cytomegalovirus-driven expression cassettes for β-galactosidase, with an enhanced green fluorescent protein (EGFP) substituted for E1 through homologous recombination. Ad.dnIKK-β was constructed by subcloning the cDNA for a kinase-inactive mutant (K44A) of IKK-β with a carboxy-terminal Flag epitope (a generous gift of Dr. David Goeddel, Tularik) into the shuttle plasmid, pAdTrack-CMV, which also encodes a separate expression cassette for CMV-driven EGFP expression. Full length adenoviral DNA clones incorporating this shuttle vector were obtained through homologous recombination with pAdEasy-1 in E. coli (BJ5183), which were prepared as high titer stocks, as previously described.[23] Adenoviral vectors were amplified in 293 cells, with the particle count estimated from the OD_{260} and the titer determined using the plaque assay. The stock titers were $>10^9$ pfu/ml for each vector, with a particle/pfu ratio of 20-50. Vector doses are expressed as multiplicity of infection (MOI), defined as plaque-forming units per cell. Wild-type adenovirus contamination was excluded by the absence of PCR-detectable E1 sequences.

Neonatal cardiomyocytes

Cardiomyocytes (CM) were prepared from 1-to 2-day-old rats, using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corp), plated in 60 mm dishes at 1×10^5 or 5×10^5 cells/dish, respectively, and cultured in RPMI 1640/5% FCS/10% horse serum (HS) for 72 hours before infection. Cells were then incubated for 2 hours in RPMI 1640 alone, and then with adenoviral vectors of Ad.EGFP. β-gal (MOI 20) or Ad.dnIKK-β (MOI 20) for 24 hours prior to activation by recombinant rat tumor necrosis factor (TNF)-α (50 ng/mL). An equal volume of RPMI 1640/5% FCS/10% HS was then added to each well, and the cells cultured for an additional 48 hours. Nuclear and cytoplasmic extracts were prepared with NE-PER™ nuclear and cytoplasmic extraction reagents (Pierce), according to the manufacturer's protocol.

Animal studies

All animal experiments were performed according to the guideline of the Chonnam National University Experimental Animal Care and Use Committee. Male 250-to 300-g Sprague-Dawley rats were anesthetized (pentobarbital), intubated and ventilated (SAR-830, CWE Inc), and then 200 μL of buffer containing ≈ 1.0×10^{12} particles/mL Ad.EGFP. β-gal

or Ad.dnIKK-β were injected via a left thoracotomy into the anteroapical myocardium. Forty-eight hours later, a left thoracotomy was performed again, and the left anterior descending coronary artery (LAD) ligated with a 6−0 silk suture, ≈4 mm from its origin, with a slipknot. Ischemia was confirmed by myocardial blanching and ECG evidence of injury. Five minutes into ischemia, 300 μL of fluorescent microspheres (10-μm FluoSpheres, Molecular Probes) were injected into the left ventricular (LV) cavity. After 30 minutes, the LAD ligature was released and reperfusion visually confirmed. As a sham ischemia-reperfusion injury (IR), a thoracotomy was performed without LAD ligation. The overall survival at 24 hours was ≈80−90%. To evaluate early changes in IκB, animals were evaluated after 30 minutes of ischemia, with only 30 minutes of reperfusion, where indicated. Rats were sacrificed 24 hours after ischemia and the infarct area (%MI), as a proportion of the area-at-risk (AAR), determined as previously described.[22]

DNA laddering

Fresh tissues were microdissected, under UV light, into ischemic and non-ischemic regions. All tissue from each region was lysed (100 mmol/L Tris [pH 8.5], 5 mmol/L EDTA, 0.2% SDS, 200 mmol/L NaCl, 100 μg/mL proteinase K) at 37℃ for 18 to 20 hours. DNA was prepared, labeled with [α-^{32}P] dCTP, subjected to electrophoresis and autoradiography, as described previously.[22]

Immunohistochemistry and H & E

Hearts were fixed in 4% paraformaldehyde and 5 μm sections treated with 0.1% SDS and incubated with primary antibody to NF-κB p65 (Santa Cruz) (90 minutes, 37℃). Sections were rinsed in PBS and incubated with anti-mouse IgG conjugated to tetramethyl rhodamine (Sigma) (60 minutes, 37℃). Confocal images were obtained using a laser confocal system (Biorad 1024). Hematoxylin and eosin (H & E) staining was performed for histomorphological evaluation of neutrophil infiltration.

Western blotting

Proteins were separated by SDS-PAGE, performed under reducing conditions, on 7.5, 10 and 12% separation gels with a 4% stacking gel. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by semi-dry blotting, and incubated overnight with primary antibodies to IκB-α, phospho-IκB-α (Ser 32, Cell signaling), NF-κB p65 and IKK-β (Santa Cruz) at 4℃. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary Ab and the immunoreactive bands detected by enhanced chemiluminescence (Santa Cruz).

Tissue myeloperoxidase (MPO) activity

The myocardial MPO activity was determined as an index of neutrophil infiltration. Frozen normal, ischemic and non-ischemic heart samples (20 mg) were homogenized in 50 mmol/L potassium phosphate buffer (PPB). After centrifugation (12,500×g, 20 minutes, 4℃), the pellets were resuspended in PPB containing 0.5% hexadecyltrimethyl ammonium bromide (HTAB) (Sigma). Samples were sonicated on ice, freeze-thawed (×4) and centrifuged (12,500×g, 20 minutes, 4℃). Supernatants were collected and incubated (37℃, 20 min) with reaction buffer (0.167 mg/mL of *o*-dianisidine dihydrochloride (Sigma) and 0.0005% H_2O_2 (Sigma) in 50 mmol/L PPB). The absorbance was measured spectrophotometrically at a wavelength of 470 nm and the MPO activity expressed as $OD_{(sample-blank)}$/mg protein/minute.

MCP-1 ELISA

Myocardial homogenates were suspended in PBS solution containing protease inhibitors (PMSF 1 mmol/L, leupeptin 1 μg/mL, aprotinin 1 μg/mL) and 1% Triton-×100. After incubation (1 hour, 4℃), the extracts were centrifuged (20,000×g, 20 minutes, 4℃) to remove the cellular debris. The protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories). Expression of rat monocyte chemotactic protein (MCP)-1 was quantified by ELISA, according to the manufacturer's instructions (Biosource International).

Quantitative RT-PCR

Neonatal CM were incubated with TNF-α (50 ng/mL, 3 hrs) and then harvested in Trizol reagent (Gibco). Samples were centrifuged (12,000×g, 10 minutes, 4℃), and the supernatants removed and vortexed (20 seconds) with an equal volume of isopropanol. Total RNA was precipitated by centrifugation (12,000×g, 10 minutes, 4℃) and the RNA further purified using the RNeasy kit (Qiagen). The RNA concentration was determined using Ribogreen (Molecular Probes), with any DNA carryover was removed by DNase (Gibco) treatment (10 minutes, room temperature). The expressions of the vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1 and MCP-1 in the samples

was determined using quantitative RT-PCR analysis (M×4000, Stratagene) and sequence-specific primers. RNA (100 ng/reaction) was reverse transcribed (30 minutes, 48℃), and the cDNA subsequently amplified (0.5 minute, 95℃; 1 minute, 60℃; 40 cycles) using the Brilliant Single-Step QRT-PCR kit (Stratagene). The amplified product was detected by SYBR 1 incorporation, and the specificity of the product confirmed by inclusion of no-template and no-RT controls as well as by analysis of the post-PCR melt curve. The fold

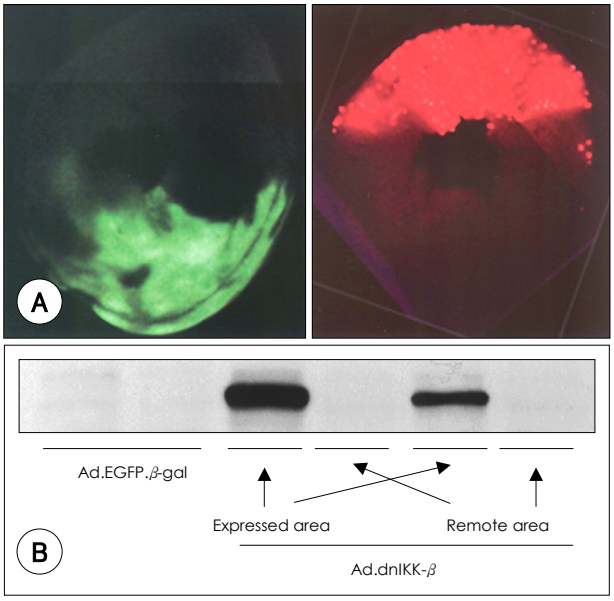

Fig. 1. A: direct injection of adenoviral vectors resulted in regional transgene expression. The green color in the left column indicates GFP expression, and the red color in the right column the perfused area. B: expression of the appropriate size protein was detected by immunoblotting with IKK-β specific antibodies only in the Ad.dnIKK-β injected myocardium. GFP: green fluorescent protein, IKK-β: inhibitor of kappa B kinase-β.

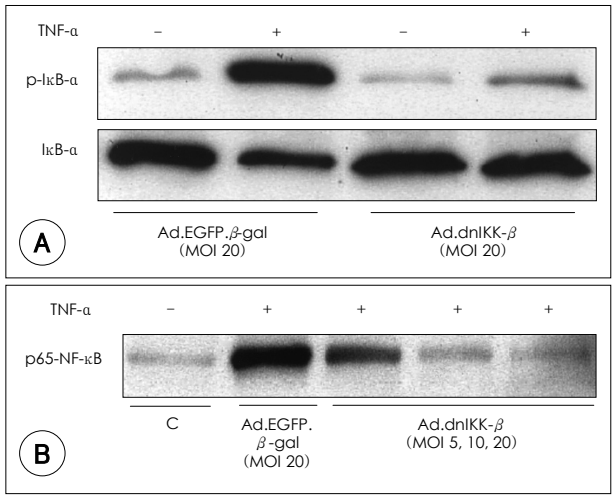

Fig. 2. dnIKK-β blocks NF-κB activation in cardiomyocytes *in vitro*. A: immunoblotting for total IκB-α and phospho-IκB-α in cardiomyocytes. IκB-α degradation and phosphorylation after rat TNF-α stimulation (50 ng/mL, 10 min) were inhibited in the Ad.dnIKK-β treated cells compared with the Ad.EGFP.β-gal infected cardiomyocytes. Data shown are representative of three independent experiments. B: immunoreactive nuclear p65-NF-κB in cardiomyocytes. Nuclear p65 nuclear translocation increased after rat TNF-α stimulation (50 ng/mL, 30 min) in the Ad.EGFP.β-gal infected cells. This increase was inhibited in the Ad.dnIKK-β transduced cardiomyocytes, in a dose-dependent manner. Data shown are representative of three independent experiments. TNF-α: tumor necrosis factor-α, MOI: multiplicity of infection.

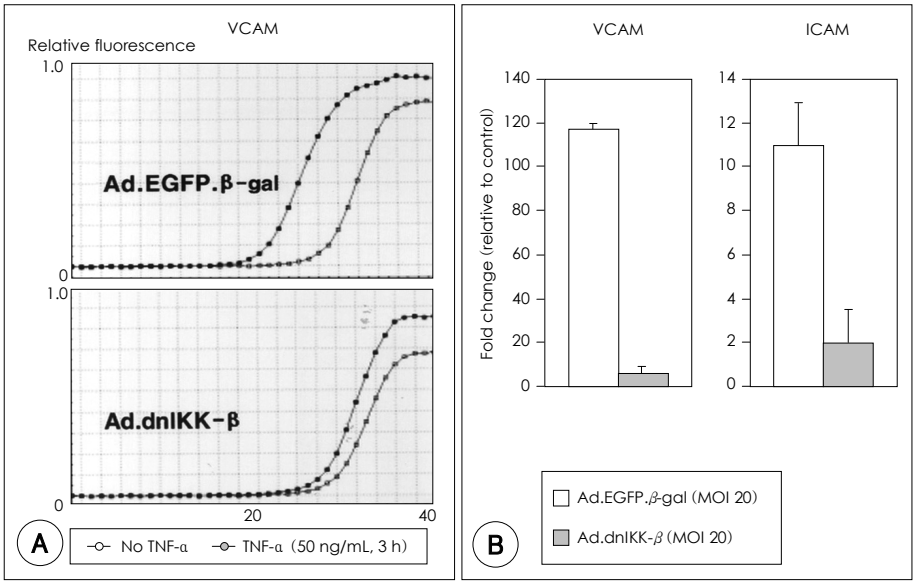

Fig. 3. QRT-PCR. A: representative amplification plots are shown for VCAM-1 quantitative RT-PCR in cells infected with Ad.dnIKK-β or a control virus, and stimulated with TNF-α (50 ng/mL). After TNF-α treatment, there was a leftward shift of the amplification curve, indicating significant induction of VCAM-1 mRNA in the Ad.EGFP.β-gal infected CM (upper panel). This leftward shift was substantially inhibited in cells expressing dnIKK-β (lower panel). Similar plots were obtained for ICAM-1. B: in the cumulative data from three independent experiments, the inductions of VCAM-1 and ICAM-1 were significantly inhibited by dnIKK-β expression ($p<0.05$, for both); by 90±11 and 80±13%, respectively. VCAM: vascular cell adhesion molecule, ICAM: intercellular adhesion molecule, QRT-PCR: quantitative reverse transcription-polymerrase chain reaction, RT-PCR: reverse transcription-polymerase chain reaction, CM: cardiomyocytes. MOI: multiplicity of infection.

induction was calculated according to previously validated mathematical models utilizing the cycle threshold for each reaction, which were performed in triplicate.[25)26)]

Statistical analysis

All data were evaluated with a 2-tailed, unpaired Student's *t* test and expressed as the mean±SEM. p less than 0.05 were considered significant. All data shown represent at least three independent experiments.

Results

Adenoviral gene transfer *in vivo*

Direct injection of adenoviral vectors resulted in regional transgene expression in an area encompassing ~60% of the area subjected to ischemia (Fig. 1A). Expression of the IKK-β protein was detected by immunoblotting only in Ad.dn-IKK-β injected myocardium (Fig. 1B).

dnIKK-β blocks IκB-α phosphorylation and NF-κB activation in cardiomyocytes

We examined whether dnIKK-β expression would block IκB phosphorylation and NF-κB activation in CM *in vitro*. Cultured CM, transduced with Ad.EGFP.β-gal or Ad.dnIKK-β for 24 hours, were treated with recombinant rat TNF-α (50 ng/mL) for 10 to 30 minutes. After 10 minutes, TNF-α stimulation induced a significant increase in immunoreactive phospho-IκB-α and a decrease in total IκB-α in the Ad.EGFP.β-gal infected cells (Fig. 2A). In contrast, the IκB-α and phospho-IκB-α levels were only minimally affected by TNF-α treatment in the Ad.dnIKK-β infected cells (Fig. 2A). By 30 minutes, the rat TNF-α induced a significant increase in nuclear p65-NF-κB in the Ad.EGFP.β-gal infected cells (Fig. 2B). This increase was significantly blocked by dnIKK-β expression, in a dose-dependent manner (Fig. 2B). To determine whether transcription of NF-κB dependent genes was effectively blocked, we performed quantitative RT-PCR (QRT-PCR) on the RNA (100 ng) from the CM infected with Ad.dnIKK-β or Ad.EGFP.β-gal and stimulated with TNF-α (50 ng/mL, 3 hours). The amplified product was detected using SYBR1 fluorescence (Fig. 3A). Importantly, a post-PCR melt curve analysis confirmed a single peak of amplified product of the appropriate T_m, but there was no amplification in the absence of the template (data not shown). After TNF-α treatment, a leftward shift was observed in the amplification curves, indicating a significant increase in the mRNA levels for each of the examined genes in the Ad.EGFP.β-gal infected CM. This leftward shift was substantially inhibited in cells expressing dnIKK-β (shown for VCAM-1 in Fig. 3A). Overall, dnIKK-β inhibited the induction of mRNA for VCAM-1 and ICAM-1 ($p<0.05$, $n=4$ in each group) by 90±11 and 80±13%, respectively (Fig. 3B).

dnIKK-β inhibits NF-κB activation *in vivo*

To investigate whether dnIKK-β expression could block NF-κB activation after IR *in vivo*, the myocardial nuclear and cytoplasmic proteins isolated from the ischemic areas of the hearts subjected to 30 minutes of ischemia and 30 minutes of reperfusion for IκB-α, as well as 30 minutes of ischemia and 24 hours of reperfusion for nuclear p65-NF-κB. The level of immunoreactive IκB-α decreased in the Ad.EGFP.β-gal treated hearts after IR. This decrease was blocked by dnIKK-β

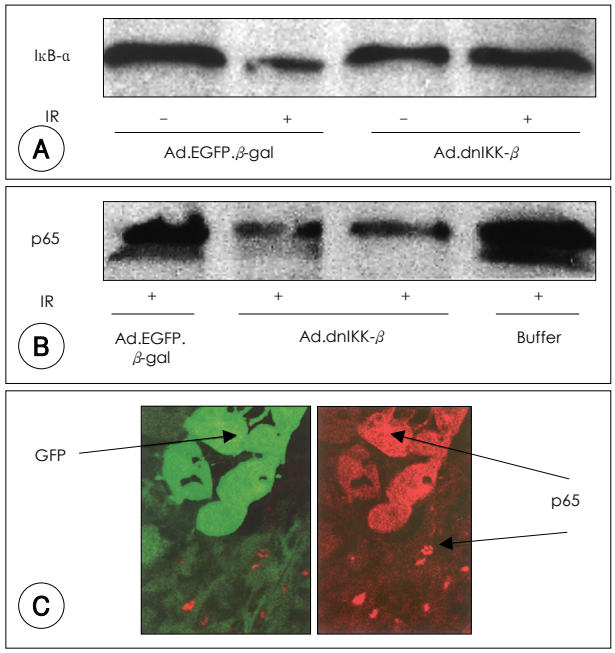

Fig. 4. dnIKK-β inhibits NF-κB activation *in vivo*. A: immunoblotting for IκB-α. IκB-α degradation was evident after 30 min of ischemia followed by 30 min of reperfusion in the Ad.EGFP.β-gal infected hearts compared with the non-ischemic hearts. In contrast, Ad.dnIKK-β treatment reduced IκB-α degradation after 30 min ischemia and 30 min reperfusion. B: immunoblotting for nuclear p65 in myocardium. Nuclear p65 increased after IR (30 min ischemia, 24 hr reperfusion) in the Ad.EGFP.β-gal and buffer treated myocardia. This increase was substantially inhibited in the Ad.dnIKK-β treated myocardium despite IR. All blots are representative of three independent experiments. C: confocal microscopy on Ad.dnIKK-β treated myocardium after IR. Confocal microscopy for GFP, which is co-expressed by Ad.dnIKK-β (left panel) or immunoreactive p65 (right panel) after IR (30 min ischemia, 24 hr reperfusion), revealed that p65 remained predominantly in the cytoplasm in Ad.dnIKK-β transduced (GFP-expressing) cells, but moved to the nucleus in cells not expressing the transgene (seen in bottom half of right panel). Data shown are representative of three independent experiments. GFP: green fluorescent protein, IR: ischemia-reperfusion.

expression (Fig. 4A). Similarly, a dramatic increase in nuclear p65 was evident in both the Ad.EGFP.β-gal and buffer treated rats by the immunoblotting after IR. In contrast, p65 nuclear translocation was substantially blocked in Ad.dnIKK-β treated rats (Fig. 4B). In Ad.dnIKK-β injected myocardium, inhibition of p65 nuclear translocation was also evident on immunohistochemical examination. Confocal microscopy for GFP, which is co-expressed by Ad.dnIKK-β (left panel) or immunoreactive p65 (right panel, arrows), revealed that p65 remained predominantly in the cytoplasm of Ad.dnIKK β-transduced (GFP-expressing) cells, but moved to the nucleus in cells not expressing the transgene (seen in right panel of bottom half) (Fig. 4C).

We also examined the effect of dnIKK-β expression on the *in vivo* induction of the NF-κB-dependent inflammatory chemokine, MCP-1, since a quantitative ELISA assay specific for *rat* MCP-1 is commercially available. The tissue MCP-1 expression increased after IR in the ischemic myocardium of Ad.EGFP.β-gal treated rats compared with normal hearts (87.3±7.3 vs. 192.6±85.1 pg/mL, $p<0.05$, n=4 in each group) (Fig. 5). This increase was completely blocked in Ad.dnIKK-β treated animals (95.2±41.8 vs. 192.6±85.1 pg/mL, $p<0.05$), reducing the chemokine concentration to the levels seen in non-ischemic or normal myocardium (Fig. 5).

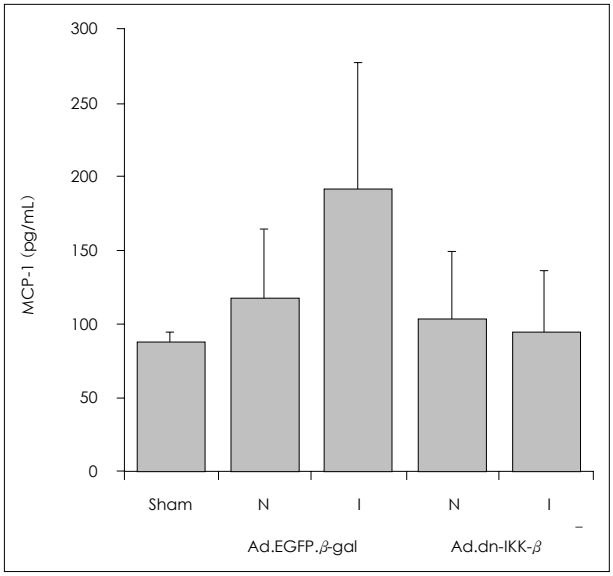

Fig. 5. MCP-1 induction blocked by dnIKK-β after IR *in vivo*. Tissue MCP-1 was measured by ELISA from the normal myocardium (NL) and from hearts infected with either Ad.EGFP.β-gal or Ad.dnIKK-β and subjected to IR (30 min ischemia, 24 hr reperfusion). The MCP-1 concentration increased significantly in the ischemic regions (I) of the hearts treated with Ad.EGFP.β-gal compared to the non-ischemia regions (N) ($p<0.05$). In contrast, the MCP-1 concentration was significantly lower in the ischemic regions of the Ad.dnIKK-β treated hearts compared with the ischemic regions of the Ad.EGFP.β-gal infected hearts ($p<0.05$), but was not significantly different from the non-ischemic or normal myocardia ($p=NS$). Data shown are cumulative with four animals in each group. MCP-1: monocyte chemotactic protein-1.

Ad.dnIKK-β reduces neutrophil infiltration after IR

NF-κB activation of inflammatory pathways is thought to

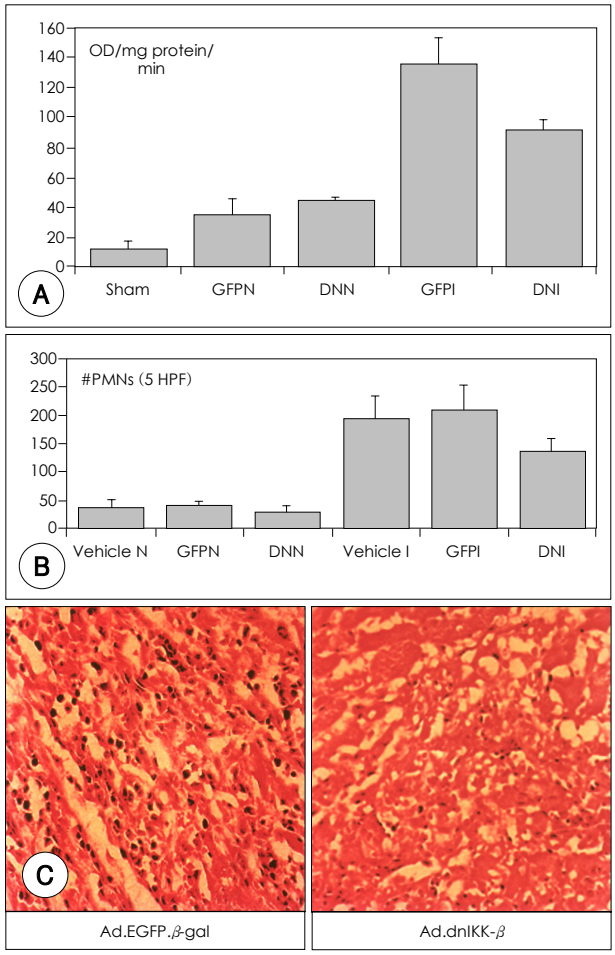

Fig. 6. Effect of dnIKK-β on neutrophil infiltration after IR. A: myocardial MPO activity. As an index of neutrophil infiltration, the MPO activity was measured in ischemic and non-ischemic regions after IR (30 min ischemia, 24 hr reperfusion). The level of MPO increased significantly in the ischemic regions (I) from the Ad.EGFP.β-gal treated animals compared to the non-ischemic (N) or normal myocardia ($p<0.01$). In the Ad.dnIKK-β treated rats, this increase was significantly reduced compared with that in the Ad.EGFP.β-gal treated animals ($p<0.05$), but remained above the levels seen in the non-ischemic regions or control myocardia ($p<0.05$). B: the neutrophil counts were consistently and significantly lower in the dnIKK-β treated ischemic myocardium compared with the vehicle or control virus treated myocardia ($p<0.01$, n=4 in each group). The data shown is cumulative, with four animals in each group. C: H & E staining of tissue from Ad.EGFP.β-gal and Ad.dnIKK-β treated myocardium after IR. Many neutrophils are evident infiltrating the ischemic tissue from the Ad.EGFP.β-gal treated myocardium (left panel), while fewer neutrophils are seen in the ischemic tissue from the Ad.dnIKK-β treated animals (right panel). Data shown are representative of four independent experiments. MPO: myeloperoxidase, GFPN: non-ischemic regions from the Ad.EGFP.β-gal treated rats, DNN: non-ischemic regions from the Ad.dnIKK-β treated rats, GFPI: ischemic regions from the Ad.EGFP.β-gal treated rats, DNI: ischemic regions from the Ad.dnIKK-β treated rats, IR: ischemia-reperfusion, PMNs: polymorphonuclear neutrophils.

contribute to IR injury through recruitment of neutrophils, which mediate, at least in part, myocardial injury.[1)3)] Leukocyte infiltration into damaged myocardium following IR was assessed by measurement of the MPO activity, a specific marker for neutrophils.[27)] The MPO activity was increased in ischemic regions following 30 minutes of ischemia and 24 hours of reperfusion in both Ad.EGFP.β-gal and Ad.dnIKK-β treated rats compared with normal hearts. However, the MPO activity in the ischemic area was decreased by 33% in the Ad.dnIKK-β treated compared with the Ad.EGFP.β-gal treated rats (90.7±7.8 vs. 134.9±17.9 OD/minute/mg protein, $p<0.05$, n=4 in each group) (Fig. 6A). These data were consistent with the results of the histological evaluation of H & E-stained myocardium (Fig. 6C). Neutrophils were rarely seen in the non-ischemic myocardium of either group, but were readily detected in ischemic myocardium. The neutrophil counts in the non-ischemic and ischemic myocardia were analyzed. For each heart, a midventricle cross section was taken, and 5 high power fields (HPFs, ×400) randomly chosen for counting. Polymorphonuclear neutrophils (PMNs) were identified on the basis of their unique appearance in hematoxylin and eosin-stained tissues. Neutrophil counts were consistently and significantly lower in the dnIKK-β treated ischemic myocardium compared with the vehicle or control virus treated myocardia ($p<0.01$, n=4 in each group) (Fig. 6B). The number of neutrophils in the control virus treated myocardium was similar to that in the vehicle controls.

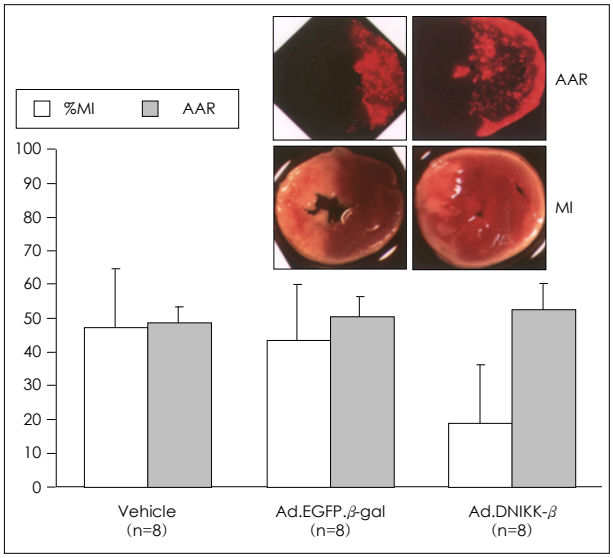

Fig. 7. Ad.dnIKK-β reduces infarction after IR. Representative micrograph (right panel) revealing the fluorescent microsphere distribution (top) and TTC staining (bottom) in rats subjected to IR after gene transfer with either Ad.EGFP.β-gal (Left) or Ad.dnIKK-β (Right). The bar graph shows cumulative data for AAR and %MI from Ad.EGFP.β-gal and Ad.dnIKK-β treated animals (n=8 in each group) after IR. There was no significant difference in the ischemia areas (AAR) between the groups. In contrast, infarction (%MI) was reduced by 57% in the Ad.dnIKK-β treated animals ($p<0.01$). MI: myocardial infarction, AAR: area-at-risk.

Infarct size and area at risk

To determine the impact of the observed reduction in inflammation on clinically relevant endpoints, the cumulative ischemic and infarcted areas were examined in hearts from animals treated with either Ad.dnIKK-β or Ad.EGFP.β-gal. The ischemic area induced by LAD ligation (AAR) did not differ between the animals infected with the Ad.dnIKK-β or Ad.EGFP.β-gal. However, the Ad.dnIKK-β treated rats demonstrated a dramatic 78% reduction in infarct size (%MI) compared with the Ad.EGFP.β-gal treated rats (18.8±16.1% vs. 43.5±14.5%, $p<0.01$, n=8 in each group) (Fig. 7).

Apoptosis

Since apoptosis can contribute to myocardial injury in IR,[22)] and CM require NF-κB-dependent survival factors in some settings,[13)] the effect of dnIKK-β expression was examined on the DNA laddering, a biochemical hallmark of apoptosis. Left ventricular (LV) samples were divided into ischemic and non-ischemic areas, as delineated by the fluorescent microsphere distribution. DNA were extracted from LV samples, and subjected to agarose gel electrophoresis. DNA laddering was not observed in the non-ischemic myocardium of animals treated with buffer alone, Ad.EGFP.β-gal or Ad.dnIKK-β (n=4 in

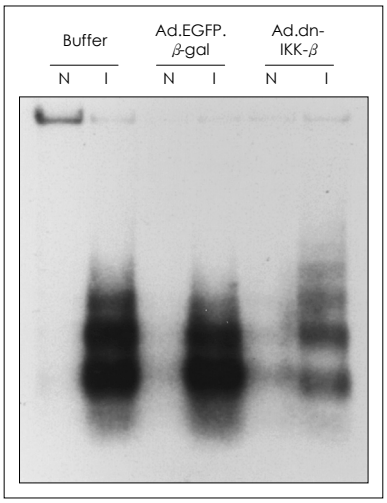

Fig. 8. Reduced DNA laddering in dnIKK-β expressing myocardium. DNA isolated from the ischemic (I) and non-ischemic (N) regions of hearts after IR (30 min ischemia, 24 hr reperfusion) were subjected to gel electrophoresis. No DNA laddering was evident in the non-ischemic regions from any of the groups. A significant increase in DNA laddering was evident in the ischemic regions of animals treated with buffer alone or Ad.EGFP.β-gal. Laddering was attenuated in the ischemic region of animals treated with Ad.dnIKK-β. Data shown are representative of four independent experiments.

each group). However, prominent DNA laddering was evident in the ischemic myocardium from the buffer and Ad.EGFP.β-gal treated rats. This laddering was markedly attenuated by dnIKK-β expression (Fig. 8).

Discussion

The role of IKK-β was examined in an *in vivo* model of cardiac ischemia-reperfusion injury through adenoviral gene transfer of a dominant negative mutant. We found that gene transfer achieved local expression of the dnIKK-β in the ischemic region, inhibiting IκB-α degradation and p65 nuclear translocation. The expression of MCP-1, an NF-κB-dependent inflammatory mediator, was reduced, as was the neutrophil infiltration after IR. Overall, apoptosis was reduced in the ischemic regions of hearts injected with the Ad.dnIKK-β. Although the ischemic "area-at-risk" did not differ between animals treated with Ad.EGFP.β-gal or Ad.dnIKK-β, the dnIKK-β expression dramatically protected hearts from injury, reducing the infarct size by 78%. These data establish an important role for IKK-β in the adult heart after IR *in vivo*, and suggest that a significant proportion of the myocardial damage sustained after IR can be prevented by specific inhibition of this kinase.

The IKK complex is composed of two catalytic subunits (IKK-α and IKK-β) and a regulatory subunit (IKK-γ). IKK-α and IKK-β differ in their substrate specificities and have distinct functions. The interaction of IKK-γ with IKK-α and IKK-β is critical for the assembly of the IKK complex leading to NF-κB activation in response to a variety of different signals.[19]

Our results are consistent with prior reports documenting the early activation of NF-κB and IKK-β in cardiac IR,[11][28] with the consequent expression of pro-inflammatory molecules[12] that are thought to mediate neutrophil recruitment. A previous study utilized oligonucleotide decoys to inhibit NF-κB in IR, which demonstrated a similarly dramatic reduction in cardiac injury.[29] However, the current work extends these studies by demonstrating the functional significance of a *specific* pathway of NF-κB activation in this setting. Thus, IKK-β signaling appears necessary for NF-κB activation in cardiac IR, suggesting any contribution by alternative pathways (tyrosine phosphorylation[20]), IKK-α,[19] or acetylation[21] is likely to be minor in this setting. However, from a clinical perspective, these results also suggest that inhibitors of IKK-β might provide an important adjunctive therapy after cardiac reperfusion.

IKK-β may have significant advantages as a pharmacologic target compared with agents directly targeting IκB or NF-κB. Not only is there considerable experience with clinical development of specific kinase inhibitors,[14] but well-characterized compounds (e.g. salicylates) already exist that inhibit IKK-β,[30] which could provide a springboard for the development of future small molecule inhibitors. While it may be tempting to speculate that some of the benefits of aspirin after reperfusion therapy could derive from inhibition of IKK-β, both the pleiotropic effects of aspirin and the relatively low doses administered clinically in this setting suggest other mechanisms may be the primary contributors. Thus, it is possible that specific and effective inhibitors of IKK-β could be developed that might prove to be valuable additions to the current therapy for acute infarctions.

dnIKK-β substantially blocked NF-κB activation and CM expression of pro-inflammatory genes both *in vitro* and *in vivo*. Since MCP-1 is thought to act predominantly on mononuclear cells, it is unlikely to play a major role in acute IR injury, so was used here simply as an index. Interestingly, although NF-κB activation, MCP-1 expression and infarct size were dramatically reduced, the reduction in the MPO activity was less dramatic, but still significant. This apparent discrepancy may reflect a non-linear relationship between these various endpoints or the inadequacy of MPO as a reflection of both the neutrophil numbers and activation. Alternatively, it is possible that neutrophil-independent mediators of tissue injury are favorably modulated by dnIKK-β expression. Thus, it is likely that the inhibition of neutrophil infiltration contributed to the benefits observed, but may not be wholly responsible.

NF-κB has been reported to suppress apoptosis in ventricular myocytes *in vitro*,[13] but we were surprised to observe a reduction in apoptosis *in vivo*. Although previous work has suggested that most of the apoptotic cells after IR are cardiomyocytes,[22] it is possible that alterations in the number of apoptotic neutrophils contributed to this difference. Nevertheless, there was no evidence of increased apoptosis that could have resulted from NF-κB inhibition. Of note, the expression of dnIKK-β dramatically enhanced apoptosis *in vitro* in neonatal cardiomyocytes exposed to TNF-α (50 ng/mL), which has also been reported for IκB-α expression[13] (data not shown). These results suggest that the reduced apoptosis and infarction observed *in vivo* after dnIKK-β expression was secondary to a reduction in NF-κB-dependent inflammatory mediators. In this *in vivo* setting, abrogation of

pro-inflammatory signals appears more important than loss of NF-κB dependent survival factors.

The unpredictable nature of acute coronary syndromes makes gene therapy impractical in this setting. In the current study, gene transfer was utilized as an experimental tool to examine the potential benefits of IKK-β inhibition in IR. Our data suggest that IKK-β may provide a valuable target for therapeutic intervention in IR, but several limitations should be noted. Only the acute effects of dnIKK-β expression after IR were examined. It will be of interest to examine whether transient inhibition of IKK-β signaling during the early phases of reperfusion is sufficient to mediate sustained benefits. Since mice lacking IKK-β are not viable, viral expression of dnIKK-β should provide a valuable tool for examining the role of IKK-β in a variety of cardiac disease models.

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