Tissue-Specific Effects of the Nuclear Factor κ B Subunit p50 on Myocardial Ischemia-Reperfusion Injury

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Nuclear factor κ B (NF- κ B) is a ubiquitous transcription factor activated by various stimuli implicated in ischemia-reperfusion injury. However, the role of NF- κ B in cardiac ischemia-reperfusion injury has not yet been well defined. Therefore, we investigated reperfusion damage in mice with targeted deletion of the NF- κ B subunit p50. Electrophoretic mobility shift assays validated NF- κ B activation in wild-type (WT) but not p50 knockout (KO) mice. KO and WT animals underwent 30 minutes of coronary artery ligation and 24 hours of reperfusion *in vivo*. Ischemia-reperfusion damage was significantly reduced in the p50 KO when compared with matching WT mice. Although adhesion molecules such as intercellular adhesion molecule were up-regulated in left ventricles of p50 KO animals, fewer neutrophils infiltrated the infarct area, suggesting leukocytes as a potential mediator of the protection observed in the p50 KO. This was confirmed in adoptive transfer experiments: whereas transplantation of KO bone marrow in KO animals sustained the protective effect on ischemia-reperfusion injury, transplantation of WT bone marrow in KO animals abolished it. Thus, deletion of the NF- κ B subunit p50 reduces ischemia-reperfusion injury *in vivo*, associated with less neutrophil infiltration. Bone marrow transplantation experiments indicate that impaired NF- κ B activation in p50 KO leukocytes attenuates cardiac damage. (*Am J Pathol* 2007, 171:507–512; DOI: 10.2353/ajpath.2007.061042)

The activation of various cytokines and Toll-like receptors has been described as part of an activation of the innate immune response in ischemia-reperfusion (IR) injury.¹ All these innate immune proteins share the downstream activation of nuclear factor κ B (NF- κ B). NF- κ B is a transcription factor regulating the expression of immediate-early and stress response genes following a variety of stimuli mainly implicated in acute inflammatory responses.² After IR injury, NF- κ B activation is biphasic, with peaks after 15 minutes and 3 hours attributed to the release of reactive oxygen species and the production of inflammatory cytokines, respectively.^{3,4} Inhibition of NF- κ B might therefore be an interesting new therapeutic strategy to reduce the activation of the innate immune response to ischemic injury, with potential protective effects.

Different pharmacological approaches are in use to inhibit NF- κ B activation mainly in experimental settings, such as pyrrolidine dithiocarbamate or 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂. However, all of these drugs lack specificity for NF- κ B and have effects that are not related to the inhibition of NF- κ B. For example, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ is also a potent agonist of the nuclear receptor peroxisome proliferator-activated receptor- γ .⁵

Thus, the only possibility to investigate specifically NF- κ B in experimental settings is evidently a molecular approach. Functional NF- κ B in mammals consists of heterodimers comprising altogether five members of the NF- κ B family in the heart with a p50 and a p65 subunit.⁶ Whereas the p65 knockout (KO) is embryonically lethal because of liver apoptosis,⁷ the p50 KO mouse displays a rather mild phenotype; p50 KO mice have normal development. However, the phenotype in various disease models is heterogeneous: p50 KO mice have multifocal defects in the immune response⁸ and in secondary lymphoid organ development.⁹ p50^{-/-} mice develop severe

Supported by grants from the Deutsche Forschungsgemeinschaft (Fr. 1377/4-3, Sonderforschungsbereich 688 TPA10).

Accepted for publication May 2, 2007.

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colitis after *Helicobacter hepaticus* infection.¹⁰ On the other hand, p50 KO mice are protected from streptozotocin-induced diabetes,¹¹ have reduced atherosclerotic lesions,¹² and less damage in response to focal cerebral ischemia.¹³ Because of the overall mild phenotype, p50 might be an ideal target to suppress the innate immune response and to explore the role of NF- κ B in ischemia-reperfusion injury. Therefore, in the present study, we evaluated the role of NF- κ B on ischemia-reperfusion injury molecularly using p50 knockout and matching WT mice.

Materials and Methods

Animals and Surgery

p50 KO mice were generated and characterized by Sha et al.⁸ p50 mice have been backcrossed for 10 generations in the C57BL/6 background by S.G. Mice 8 to 12 weeks old, with a body weight of 20 to 28 g, underwent coronary artery ligation for 30 minutes and reperfusion for 24 hours as recently described.¹⁴ The Standing Committee on Animal Research at our institution has approved the animal study protocol. Area at risk and infarct size were assessed by standard staining with 5% Evans blue and 1.5% triphenyltetrazolium chloride as recently described.¹⁴

Histochemistry

Polymorphonuclear neutrophil infiltration in the area at risk (AAR) was determined in 5- μ m sections, cut from the paraffin-embedded median section of the heart, with a specific stain (naphthol AS-D chloroacetate esterase; Sigma) according to the manufacturer's protocol. Polymorphonuclear neutrophils were counted in the AAR at a magnification of $\times 400$ as previously described.¹⁵

Bone Marrow Transplantation

Animals were treated with acidified water (pH 2) ad libitum containing 100 mg/L neomycin and 10 mg/ml polymyxin B sulfate (Sigma) for 10 days before and until 2 weeks after lethal irradiation. Mice were irradiated (10 Gy) with a cesium γ source, followed by bone marrow transplantation 4 hours later. Bone marrow cells were prepared from the tibia and femur bones by flushing the bones with RPMI 1640 medium (Gibco, Grand Island, NY) containing 2% fetal bovine serum and 5 U/ml heparin. Cells were filtered through a spleen mesh, counted, and resuspended in medium 199 containing 1% HEPES buffer, 400 U/ml DNase, and 200 μ g/ml gentamicin. Recipient animals received 5×10^6 bone marrow cells in 0.5 ml of bone marrow transplantation medium (formula) by tail vein injection.

Real-Time Polymerase Chain Reaction

Myocardial RNA isolation and real-time polymerase chain reaction measurements were performed as previously

described¹⁶ with commercially available TaqMan probes for 18S rRNA, tumor necrosis factor (TNF), intercellular adhesion molecule-1 (ICAM-1), P-selectin, and lipopolysaccharide-inducible CXC chemokine (LIX) (Applied Biosystems, Foster City, CA). cDNA samples were normalized to 18S rRNA.

Preparation of Nuclear and Cytosolic Extracts

Nuclear and cytosolic proteins were extracted from lymphocytes of the spleen after stimulation with lipopolysaccharide.^{17,18} After Dounce homogenization cells were lysed for 10 minutes on ice in a solution containing 10 mmol/L HEPES, pH 7.6, 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.5% Nonidet P-40, 1 mmol/L dithiothreitol, and 0.5 mmol/L phenylmethylsulfonyl fluoride. Nuclei were precipitated by centrifugation at $800 \times g$ for 30 seconds, supernatants were saved as cytosolic extracts, and the nuclei resuspended in a solution of 20 mmol/L HEPES, 1.5 mmol/L MgCl₂, 420 mmol/L KCl, 0.2 mmol/L ethylenediamine tetraacetic acid, 1 mmol/L dithiothreitol, and 0.5 mmol/L phenylmethylsulfonyl fluoride. The nuclei mixture was incubated on ice for 30 minutes, the supernatant collected after centrifugation for 15 minutes at $13,000 \times g$, and an equal amount of glycerol buffer added (20 mmol/L HEPES, 100 mmol/L KCl, 0.2 mmol/L ethylenediamine tetraacetic acid, and 20% glycerol).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays were performed as described.¹⁸ DNA-binding reactions were performed with 10 μ g of nuclear protein. Control reaction mixtures contained a 100-fold excess of unlabeled oligonucleotide and were incubated with nuclear extracts as indicated. DNA complexes were separated on a 5% nondenaturing polyacrylamide gel in Tris-borate ethylenediamine tetraacetic acid buffer. NF- κ B oligonucleotides were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

Bone Marrow Cell Adhesion Assay

Total mononuclear bone marrow cells were isolated as described.¹⁹ A total of 1×10^6 cells were seeded on fibronectin-coated 12-well plates for 3 hours. After careful washing with phosphate-buffered saline, the number of adherent cells/microscopic field was determined. At least three different microscopic fields per well were counted of $n = 5$ individual donors per group.

Statistical Analyses

All replicate data are expressed as mean and SE of mean. Absolute differences between groups were compared using an analysis of variance adjusted by Fisher's rule. Statistical significance was achieved when two-

Figure 1. Targeted deletion of the NF- κ B subunit p50 in blood cells reduces IR damage. The top panel presents representative sections of mice hearts after 30 minutes of coronary ischemia and 24 hours of reperfusion. There were no significant differences in the area at risk. Infarct/AAR was significantly smaller in p50 KO mice compared with WT. After transplantation of WT bone marrow in KO animals, infarct sizes were similar to those in WT animals. * $P < 0.05$.

tailed $P < 0.05$. Statistical analyses were performed using the StatView statistics program (Abacus Concepts, Inc., Berkeley, CA).

Results

Myocardial Area at Risk and Infarct Size

After 30 minutes of ligation of the left anterior descending artery and 24 hours of reperfusion, the left ventricular area affected by coronary ligation, referred to as the AAR, was similar between the groups [WT ($n = 11$) 38.2 ± 2.5 versus KO ($n = 9$), $43.3 \pm 2.6\%$, $P = \text{N.S.}$]. However, p50 KO mice had significantly smaller myocardial infarctions compared with WT mice (infarct/AAR, WT versus KO, 48.7 ± 7.1 versus $21.4 \pm 4.8\%$, $P = 0.004$; Figure 1). Thus, mice with targeted deletion of the NF- κ B subunit p50 displayed a significantly reduced IR injury.

Inflammatory Markers

Because NF- κ B is known to induce transcription of proinflammatory cytokines and proinflammatory cytokines are known to mediate ischemic injury, myocardial TNF was measured by real-time polymerase chain reaction. Surprisingly, significantly more TNF was expressed in hearts with targeted disruption of the NF- κ B subunit p50 after IR (TNF/18S, WT versus KO, 0.3 ± 0.1 versus 1.0 ± 0.1 arbitrary units, $P = 0.001$, data not shown) despite a lack of NF- κ B activation in the p50 KO mice as measured by

Figure 2. NF- κ B is not activated in the p50 KO. Electrophoretic mobility shift assays were performed on nuclear extracts of spleen lymphocytes for NF- κ B-mediated signaling after stimulation with lipopolysaccharide. The data shown are representative of three experiments. There was no activation of NF- κ B in the p50 KO.

electrophoretic mobility shift assay (Figure 2). Furthermore, by polymerase chain reaction the expression of ICAM, a protein regulated by NF- κ B and responsible for neutrophil infiltration, as well as of LIX, a leukocyte attractant chemokine, was significantly up-regulated in p50 KO hearts (Figure 3). The adhesion protein P-selectin was not influenced by the genotype (P-selectin/18S, WT versus KO, 0.29 ± 0.10 versus 0.53 ± 0.18 arbitrary units, $P = \text{N.S.}$). In contrast to the molecular data, however, neutrophil accumulation in the area at risk was diminished in p50 KO hearts (WT versus KO, 24.7 ± 10.1 versus 6.6 ± 1.4 cells/mm², $P = 0.05$; Figure 3).

Cell-Specific Rescue of NF- κ B by Bone Marrow Transplantation

Because neutrophil infiltration was decreased despite increased expression of ICAM and LIX in p50 KO mice, we tested the hypothesis that compromised NF- κ B function in p50 KO leukocytes itself may be responsible for attenuation of IR injury in p50 KO. Indeed, in an *in vitro* model p50 KO bone marrow cells adhered significantly less on fibronectin-coated plates than WT cells (see Figure 4). To confirm the hypothesis *in vivo*, we generated chimeric mice by transplanting WT bone marrow cells in lethally irradiated KO animals. Six weeks later, these mice were subjected to 30 minutes of ischemia and 24 hours of reperfusion *in vivo*. Attenuation of IR injury observed in the

Figure 3. Inflammatory markers are elevated, but infiltrating neutrophils are reduced in the p50 KO after reperfusion. RNA expression of ICAM (**A**), a protein responsible for leukocyte invasion, as well as of LIX (**B**), a leukocyte attractant chemokine, are increased in p50 KO mice (* $P < 0.05$, arbitrary units, normalized to 18S rRNA). However, the number of infiltrating neutrophils (**C**) is reduced in the p50 KO (red naphthol esterase staining for neutrophils).

p50 KO was reversed in the chimeric animals (infarct/AAR, $n = 4$, WT versus KO with WT bone marrow, 48.7 ± 7.1 versus $50.9 \pm 9.5\%$, $P = \text{N.S.}$; Figure 1), whereas the protective effects were unaltered in KO mice with KO bone marrow (infarct/AAR, $n = 5$, KO versus KO with KO bone marrow, $21.4 \pm 4.8\%$ versus $29.0 \pm 3.7\%$, $P = \text{N.S.}$). Thus, inhibition of p50 in bone marrow-derived inflammatory cells is responsible for reduced IR injury in p50 KO mice.

Figure 4. Reduced adherence of p50 KO bone marrow-derived cells. Significantly less p50 KO bone marrow-derived cells adhered on fibronectin-coated plates when compared with WT cells. ** $P < 0.001$.

Discussion

The present study demonstrates for the first time reduced cardiac damage in response to IR injury in mice with targeted deletion of the NF- κ B subunit p50. Cardioprotection was absent after transplantation of WT bone marrow cells in KO animals, indicating a decisive role of NF- κ B activation in bone marrow-derived cells for cardiac ischemia-reperfusion injury *in vivo*.

NF- κ B is a ubiquitous transcription factor regulating the expression of immediate-early and stress response genes following a variety of stimuli. Over 150 stimuli are known to activate NF- κ B, and these have traditionally been mainly implicated in acute inflammatory responses.² Recently, an activation of NF- κ B in ischemia-reperfusion injury and in patients with unstable angina²⁰ has also been described. Inhibition of NF- κ B might therefore be a potential novel approach to reduce the activation of the immune response to ischemic injury.²¹

Few studies have thus far tested the functional consequences of NF- κ B inhibition on cardiac ischemia-reperfusion injury using molecular (inhibition of p65 by double-stranded oligonucleotides²²) as well as pharmacological methods (I κ B kinase complex inhibition²³). Approaches that inhibited NF- κ B globally by pharmacological or molecular intervention resulted in reduced IR injury. However, it is difficult to imagine that the inhibitors used can be easily transferred into clinical applications because

they are potentially toxic: mice with deletion of p65,⁷ the molecular approach used previously, as well as mice with deletion of I κ B kinase complex,²⁴ the pharmacological approach used before, have an embryonic lethal phenotype. On the contrary, the mice used by us with targeted deletion of the NF- κ B subunit p50 have a mild phenotype with only minor immune defects and normal embryonic development.⁸ Infarct size after 30 minutes of ischemia and 24 hours of reperfusion *in vivo* was reduced to less than 50% of that of WT mice. These data suggest that approaches directed against p50 may be a valuable tool to reduce ischemia-reperfusion injury.

The transcription factor NF- κ B is involved in the regulation of many genes implicated in IR injury and recruitment of inflammatory cells. Indeed, leukocyte infiltration is an important mediator of cardiac IR injury.²⁵ Various approaches have been used to inhibit neutrophils after IR injury, such as depletion of neutrophils eg, by neutrophil-specific filters and inhibition of adhesion molecules by selective antibodies, mostly reducing reperfusion damage.²⁵ Thus, the protective effects of NF- κ B inhibition could be mediated by a down-regulation of proinflammatory proteins with a consecutive reduction of infiltrating inflammatory cells in the heart after ischemia-reperfusion injury. In fact, a few studies demonstrated a reduction of inflammatory cell infiltration after inhibition of NF- κ B.^{22,23,26} Thus, we postulated that a reduction of proinflammatory proteins and a resultant reduced number of infiltrating inflammatory cells would be responsible for our protective results. Consistent with this hypothesis, we observed a significant reduction of neutrophils in the hearts of p50 KO animals after ischemia-reperfusion. However, all tested proteins involved in the accumulation of leukocytes were up-regulated in the KO when compared with WT animals. This is in accordance with three recent studies, where the expression of proinflammatory proteins was increased in the heart of p50 KO mice after myocardial hypertrophy in response to chronic infusion of angiotensin II, in inflammatory cardiomyopathy or after chronic myocardial infarction.^{21,27,28} This may be related to the different transcriptional activity of p50-p65 heterodimers and p50-p50 homodimers.²⁹ Chronic exposure of proinflammatory cytokines can shift the ratio of transcriptionally active heterodimers to the transcriptionally inactive p50 homodimers, which can act as negative feedback mechanism to prevent excessive production of proinflammatory proteins and may thereby account for the increased expression of proinflammatory proteins in the p50 KO. However, because those factors aggravate the ischemic response in other models,³⁰ they cannot account for cardioprotection in p50 KOs.

Because leukocyte attractant proteins were up-regulated and the number of infiltrating neutrophils was down-regulated after ischemia-reperfusion injury, we hypothesized that compromised NF- κ B activation in inflammatory cells itself would be responsible for reduced IR injury. Several reports demonstrate functional defects of inflammatory cells in the p50 KO mice, which have an impaired T-cell response^{8,31} as well as B cells that do not proliferate in response to bacterial lipopolysaccharide and are defective in basal and specific antibody production.⁸

Indeed, herein we ourselves found cell adherence to be impaired in KO cells *in vitro*. To identify the role of p50 specifically in bone marrow-derived cells *in vivo*, adoptive transfer experiments were performed. Lethally irradiated KO animals were reconstituted with WT bone marrow. The observed loss of cardioprotection in these animals indicates that deletion of p50 in blood cells is responsible for the cardioprotection in p50 KO *in vivo*. To exclude direct effects of the bone marrow transplantation, KO bone marrow was also transplanted in irradiated KO mice, without effects on cardioprotection in the p50 KO.

There are limitations regarding the interpretation of results of the present study. First, although we and others³² did not see direct effects of bone marrow transplantation on cardiac ischemia-reperfusion injury, potential direct effects of bone marrow transplantation per se cannot be totally excluded. Second, although our experiments were well controlled, we did not study bone marrow transplantation in WT mice. Nevertheless, our *in vitro* data as well as our adoptive transfer experiments in KO mice strongly argue for the hypothesis that deletion of p50 in bone marrow-derived cells is responsible for cardioprotection.

In summary, we found a decreased susceptibility to ischemia-reperfusion injury *in vivo* in mice with targeted deletion of the NF- κ B subunit p50 mediated by impaired NF- κ B activation in p50 KO leukocytes. Thus, p50 might be an attractive target for rational drug design in cardiac ischemia-reperfusion injury.

Acknowledgments

We thank B. Bayer, H. Wagner, and G. Ries for their excellent technical support.

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