Hyperoxia elicits myocardial protection through a nuclear factor κ B–dependent mechanism in the rat heart

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Objective: Hyperoxia has been previously shown to protect the heart from ischemia-reperfusion injury. In the present study we investigated whether the cardioprotective effects of hyperoxia were dependent on the redox-sensitive transcription factor nuclear factor κ B.

Methods: Rats were kept in a hyperoxic ($\geq 95\%$ O₂) environment for 60 minutes. Their hearts were isolated immediately afterward, buffer perfused in a Langendorff apparatus, and subjected to 25 minutes of global ischemia and 60 minutes of reperfusion. Cardiac pressures and coronary flow were measured, and infarct size was determined by means of triphenyl tetrazolium chloride staining. Activation of nuclear factor κ B was assessed by means of the electrophoretic mobility shift assay, whereas the inhibitor I κ B α was evaluated by means of immunoblotting. Pharmacologic inhibition of nuclear factor κ B was achieved with 2 different agents, SN50 and pyrrolidine dithiocarbamate.

Results: Preischemic exposure to hyperoxia improved postischemic recovery of myocardial contractile function and coronary flow and reduced infarct size. Hyperoxia activated pulmonary and myocardial nuclear factor κ B. Pretreatment with SN50 (400 μ g/kg administered intraperitoneally) or pyrrolidine dithiocarbamate (100 mg/kg administered intraperitoneally) before hyperoxia abolished the functional and infarct-limiting protection. Hyperoxia reduced nuclear factor κ B activation in the heart during sustained ischemia and reperfusion and increased the cytoplasmic inhibitory factor I κ B α . Administration of pyrrolidine dithiocarbamate or SN50 during ischemia and reperfusion to isolated hearts from normoxic control animals improved postischemic contractile function and coronary flow and reduced infarct size.

Conclusions: Hyperoxia protects the rat heart against ischemia-reperfusion injury. The cardioprotection depends on myocardial activation of the transcription factor nuclear factor κ B. Our results support evidence for a dual role of nuclear factor κ B in the heart.

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Despite the surgical and technical improvements of cardiac surgery over the last decades, there are still patients who require improved myocardial protection before surgical intervention. Recent reports suggest the benefit of supplemental intraoperative oxygen in patients undergoing major surgical procedures.^{1,2} However, hyperoxia might lead to excessive generation of reactive oxygen species and cause tissue injury.³

Reactive oxygen species are involved in the regulation of a number of cellular processes.⁴ Transcription factors, such as nuclear factor κ B (NF κ B) and activator protein 1 (AP-1), are, to a great extent, controlled by oxidative stress.⁵ NF κ B and AP-1 are involved in the early activation of genes in response to various types of cell

stress alone or in cooperation with each other. Under normal physiologic conditions, NF κ B is held in the cytoplasm in an inactive form by the inhibitory protein I κ B. During cellular stress, I κ B is phosphorylated and released from the NF κ B homodimer or heterodimer.⁵ NF κ B translocates to the cell nucleus, binds to promoter-enhancer regions of the genes it regulates, and induces their transcription. NF κ B regulates a battery of genes associated with proinflammatory effects, such as leukocyte adhesion molecules, cytokines, and chemokines.^{5,6} NF κ B also regulates genes that might be associated with tissue repair and protection, such as the genes encoding inducible nitric oxide synthase and inducible cyclooxygenase and the antioxidant manganese superoxide dismutase.^{6,7}

Recent studies have suggested that NF κ B plays an essential role in ischemic preconditioning, an endogenous phenomenon by which short periods of ischemia and reperfusion can protect the heart against a subsequent sustained ischemic insult.^{8,9} Inhibition of NF κ B activation during adaptation to ischemia abolished the functional protection and infarct-limiting effect of ischemic preconditioning.^{8,10} However, activation of NF κ B in ischemia-reperfusion injury without prior adaptation appears to be detrimental because inhibition of NF κ B activation reduced infarct size and improved heart function.^{11,12} Thus the role of NF κ B in myocardial ischemia and reperfusion might be dual, with both a cardioprotective role in ischemic preconditioning and a detrimental role during sustained ischemia and reperfusion.

We have previously shown that breathing a hyperoxic gas ($\geq 95\%$ O₂) induces systemic low-grade oxidative stress in rats, which is evident as increased systemic conjugated dienes, and improves heart function and reduces myocardial necrosis after ischemia-reperfusion injury.¹³ The purpose of this study was to investigate whether this preconditioning-like response was dependent on NF κ B.

Materials and Methods

The investigation conforms with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health and was approved by the Ethics Committee for Animal Research at the Karolinska Institutet.

Rat Heart Perfusion

Details of heart isolation and perfusion are provided elsewhere.¹³ Briefly, male Sprague-Dawley rats (250–300 g) were anesthetized with midazolam (Dormicum; 1.5 mg/kg administered intramuscularly) and fentanyl-fluanisone (Hypnorm; 0.1 mg/kg fentanyl and 3 mg/kg fluanisone administered intramuscularly). Heparin (200 IU) was injected into the femoral vein, and thereafter, the hearts were excised and the aorta was cannulated. The hearts were retrogradely Langendorff perfused with gassed (5% CO₂, 95% O₂, pH 7.35–7.45) Krebs-Henseleit buffer at 37°C at a constant pressure of 70 mm Hg. Isovolumetric recordings of left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP)

were obtained by using a balloon inserted into the left ventricle through the left atrium. LVEDP was set to 5 mm Hg at the end of the stabilization period. Coronary flow (CF) was measured by means of timed collections of the coronary effluent. Left ventricular developed pressure (LVDP) was calculated as follows:

$$\text{LVDP} = \text{LVSP} - \text{LVEDP}.$$

Heart rate (HR) was counted from the pressure curves. Global ischemia was achieved by clamping the inflow tubing.

The experimental protocol is shown in Figure 1. Before heart isolation and Langendorff perfusion, the rats were kept for 60 minutes in a cage, where a normoxic (atmospheric air) or hyperoxic ($\geq 95\%$ O₂, monitoring with a gas analyzer) environment was established. The length of hyperoxia was selected on the basis of previous studies.¹³ Immediately after exposure to hyperoxia or normoxia, the hearts were excised and buffer perfused by using the Langendorff technique with 25 minutes of stabilization, 25 minutes of global ischemia, and 60 minutes of reperfusion. Only hearts with an LVSP of 60 to 160 mm Hg, a CF of 8 to 16 mL/min, and an HR of 240 to 360 beats/min at the end of stabilization were included. The NF κ B inhibitors SN50 (400 μ g/kg; Calbiochem-Novabiochem Corp) or pyrrolidine dithiocarbamate (PDTC; 100 mg/kg; Sigma Chemical Co) were injected intraperitoneally 20 minutes before 60 minutes of hyperoxia or normoxia, followed by Langendorff perfusion (groups 3–6 in Figure 1, respectively). Studies investigating the effects of SN50 or PDTC on hyperoxia-induced myocardial protection were performed at a different calendar time and are accompanied by separate normoxic control and hyperoxic groups. Selection of drug concentrations and routes of administration were based on pilot studies on functional effects (results not shown).

Groups 7 and 8 were included to investigate whether NF κ B inhibition during reperfusion influences ischemia-reperfusion injury in isolated hearts. Hearts were harvested from normoxic control animals, isolated, and buffer perfused. Starting from 3 minutes before global ischemia and throughout reperfusion, SN50 (18 μ mol/L final concentration)⁸ or PDTC (1 mmol/L)¹⁴ was added to the perfusion buffer. During the preischemic period, the drugs had no effect on baseline functional variables (LVEDP, LVDP, HR, and CF).

Determination of Infarct Size

At the end of reperfusion, the hearts were perfused with 1% triphenyl tetrazolium chloride (TTC; Sigma Chemical Co) at a total volume of 3.0 mL delivered at 70 mm Hg. TTC stains viable tissue red, whereas necrotic tissue remains unstained. The hearts were fixed in 4% formaldehyde for 24 hours and thereafter preserved in 10% sucrose in phosphate-buffered saline. The hearts were cut manually into 1-mm transverse slices. The sections were visualized with a computer imaging system (LEICA Qwin; Leica Imaging Systems), and infarct size was calculated by using Adobe Photoshop 5.0. The infarcted area was calculated as a percentage of total myocardial area. From each slice, an image was obtained from both sides, and all calculations from one heart were averaged into one value for statistics.

Tissue Sampling for Molecular Biology

Additional hearts and lungs were sampled for tissue analysis. The hearts were freeze clamped at the end of 60 minutes of hyperoxia

Figure 1. Flow chart of the experimental groups included for evaluation of hemodynamic parameters and infarct size. Rats were kept in a cage breathing 95% or greater O_2 for 60 minutes (*hyperoxia*), or control animals were kept in the same environment for a corresponding time period breathing atmospheric air (*normoxia*). Thereafter, hearts were excised and Langendorff perfused, allowing 25 minutes of stabilization before 25 minutes of global ischemia and 60 minutes of reperfusion. In other groups the NF κ B inhibitors SN50 (400 μ g/kg) or PDTC (100 mg/kg) were administered intraperitoneally 20 minutes before either hyperoxia or normoxia (group 3, 4, 5, and 6, respectively) or infused (SN50, 18 μ mol/L; PDTC, 1 mmol/L) during ischemia and reperfusion to hearts isolated from normoxic control animals (groups 7 and 8).

($n = 7$) or normoxia ($n = 7$) for nuclear protein extraction and electrophoretic mobility shift assay (EMSA) to evaluate whether hyperoxia resulted in myocardial NF κ B activation. Hearts of 3 animals injected with SN50 before hyperoxia were analyzed to assess the effect of SN50 on myocardial NF κ B activation. These organs were freeze clamped after exposure to 0, 2, 5, 10, 20, or 60 minutes of hyperoxia ($n = 3$ of each tissue for each time point) to evaluate the time course of NF κ B activation in lungs and hearts. Finally, the hearts were sampled for immunoblotting serially during

stabilization, ischemia, and reperfusion ($n = 3$ from each group at each time point) to investigate whether hyperoxia influenced NF κ B activation and content of the NF κ B inhibitor I κ B α .

Preparation of Nuclear Protein Extracts

Nuclear proteins were extracted from frozen heart samples, as previously described in detail.¹⁵ Briefly, hearts were homogenized in a microdismembrator, and lysis buffer was added. After incu-

Figure 2. A, LVDP, LVEDP, and CF in Langendorff-perfused rat hearts subjected to 25 minutes of global ischemia and 60 minutes of reperfusion. Rats were kept in a normoxic (*control*) or hyperoxic ($\geq 95\%$ O_2 , *hyperoxia*) environment for 60 minutes before heart isolation or pretreated with the NF κ B inhibitor SN50 (400 μ g/kg administered intraperitoneally) before exposure to hyperoxia (*SN50+hyperoxia*) or before normoxia (*SN50+normoxia*), followed by Langendorff perfusion. B, Rats were subjected to either normoxia (*control*) or hyperoxia, and the NF κ B inhibitor PDTC (100 mg/kg administered intraperitoneally) was administered before exposure to either normoxia (*PDTC+normoxia*) or hyperoxia (*PDTC+hyperoxia*), followed by Langendorff perfusion, as for panel A. Data are given as means $\pm$ SEM. BI, Before global ischemia. * $P < .05$ versus control; # $P < .05$ versus hyperoxia.

bation on ice, nuclei were collected by means of centrifugation for 1 minute at 8000g. The pellet was washed with 20 mmol/L KCl buffer, centrifuged, and resuspended in 20 mmol/L KCl buffer, and 0.6 mol/L KCl was added and kept at +4°C for 30 minutes. After

centrifugation for 15 minutes at 8000g, the supernatant containing nuclear proteins was obtained. The protein concentration was determined by using the bicinchonic acid reagent (Pierce, Rockford, Ill) with bovine serum albumin as a standard.

Figure 3. A, LVDP, LVEDP, and CF in Langendorff-perfused rat hearts subjected to 25 minutes of global ischemia and 60 minutes of reperfusion. Rats were either normoxic control animals (*control*) or perfused with the NF κ B inhibitor SN50 (18 μ mol/L) during ischemia and reperfusion (*SN50*). B, Isolated hearts were normoxic in the absence (*control*) or presence (*PDTC*) of PDTC (1 mmol/L) during ischemia and reperfusion. Data are given as means $\pm$ SEM. * $P < .05$ versus control.

Electrophoretic Mobility Shift Assay

Nuclear extracts (16 μ g of protein per lane) were preincubated for 10 minutes in binding buffer (20 mmol/L Hepes [pH 7.9], 5% glycerol, 5 mmol/L MgCl₂, 0.5 mmol/L ethylenediamine tetraacetic acid, and 1 mmol/L dithiothreitol), followed by 30 minutes' incubation at room temperature with 50,000 cpm of phosphorous 32-labeled probe containing the NF κ B binding site 5 AGT TGA

GGG GAC TTT CCC AGG C or the AP-1 binding site 5 CGC TTG ATG AGT CAG CCC GGA A (both from Promega Biosciences, Inc). DNA-protein complexes were electrophoresed on a 4% polyacrylamide gel. For supershift analysis, a rabbit polyclonal anti-p50 antibody or a rabbit polyclonal anti-c-jun antibody (both Santa Cruz Biotechnology) were incubated with the binding buffer for 15 minutes before adding the radiolabeled probe. For competition analysis, unlabeled

beled probe in 100-fold excess was added before radiolabeled probe. Quantification of band densities was performed with the software Tina 2.0. Only bands on the same gel were used for calculation and comparison.

Immunoblotting

Cytoplasmatic cardiac proteins were extracted by homogenizing frozen tissue at a ratio of 40 mg of tissue per milliliter of lysis buffer (1% sodium dodecylsulfate, 1 mmol/L Na vanadate, and 1 mmol/L of the protease inhibitor phenylmethylsulphonyl fluoride), and insoluble material was removed by means of centrifugation. Protein content was determined by using the bicinchonic acid reagent (Pierce), with bovine serum albumin as a standard. The lysates were mixed with 5× Laemmli buffer at a ratio of 5:1 and boiled, and proteins were electrophoresed under reducing conditions (16 µg per lane), followed by transfer to presoaked nitrocellulose membranes (Hybond-C pure; Amersham Life Science). The membranes were blocked in phosphate-buffered saline/Dulbecco medium (Gibco BRL, Life Technology) with 0.1% Tween and 5% nonfat dry milk, followed by incubation with rabbit polyclonal IκBα (Santa Cruz sc-371) diluted 1:1000. A goat anti-rabbit (StressGen Biotechnologies) IgG-alkaline phosphatase 1:1000 dilution and an alkaline phosphatase conjugate substrate kit (BioRad Laboratories) were used for visualization.

Statistical Analysis

Functional data are given as means ± SEM. Differences in functional recovery were tested by using 2-way analysis of variance (ANOVA) with repeated measures on one factor, taking treatment as an independent factor and time as a dependent factor. The treatment-time interaction in ANOVA refers to the statistical test of whether mean profiles for one group are the same as for the other groups. In the case of significant interaction, simple effects (ie, effects of one factor holding another factor fixed) were examined. Planned comparisons between the groups across factor time were then performed. The *P* values were thereafter corrected according to the Bonferroni procedure. If the sphericity assumption was not met, the degrees of freedom of the *F* tests associated with the time factor were reduced by multiplying each degree of freedom by the Greenhouse-Geisser ϵ value. Normal probability plots were performed to confirm that the underlying model assumptions were met by the data.

Infarct size is presented as box plots with median and quartiles and with whisker plots as minimum and maximum values. Optical densities of EMSA bands are presented either as box plots or as scatterplots. The infarct sizes were approximately normally distributed, and thus comparisons of optical densities and infarct sizes were performed by means of 1-way ANOVA. When significant *P* values were calculated, intergroup comparisons were performed with the Duncan post hoc test.

Results

Postischemic Contractile Function and Coronary Flow

Left ventricular developed pressure. With SN50, LVDP decreased during reperfusion of normoxic control hearts (group 1), and this was attenuated by hyperoxia (group 2; *P* = .001; Figure 2, A). Pretreatment with SN50 (group 3)

Figure 4. A, Infarct size at the end of 25 minutes of global ischemia and 60 minutes of reperfusion in hearts isolated immediately after 60 minutes of normoxia (*control*, *n* = 18) or 60 minutes of hyperoxia (*hyperoxia*, *n* = 17) or from animals pretreated with the NFκB inhibitor SN50 (400 µg/kg administered intraperitoneally) before either hyperoxia (*SN50+hyperoxia*, *n* = 6) or normoxia (*SN50+normoxia*, *n* = 6) before Langendorff perfusion or SN50 infused during ischemia and reperfusion (18 µmol/L, *SN50 buffer*, *n* = 7). B, Infarct size in control (*control*, *n* = 18) and hyperoxic (*hyperoxia*, *n* = 17) hearts or from animals pretreated with the NFκB inhibitor PDTC (100 mg/kg intraperitoneally) before hyperoxic (*PDTC+hyperoxia*, *n* = 13) or normoxic (*PDTC+normoxia*, *n* = 10) conditions or PDTC (1 mmol/L) infused during ischemia and reperfusion (*PDTC buffer*, *n* = 7). Data are presented as medians and quartiles, with *whiskers* as minimum and maximum values. **P* < .05 versus control; #*P* < .05 versus hyperoxia.

abolished the protective effect of hyperoxia (*P* = .003). The differences between normoxic control hearts (group 1) and hearts from normoxic animals pretreated with SN50 (group 4) or between normoxic control hearts (group 1) and hearts from hyperoxic animals pretreated with SN50 (group 3) could be due to chance (*P* > .2). When SN50 was added to the perfusate during Langendorff perfusion, functional recovery after global ischemia was not influenced (group 7; Figure 3, A).

Figure 5. A, EMSA of nuclear protein extracts of 6 rat hearts after 60 minutes of exposure to hyperoxia ($\geq 95\% \text{ O}_2$, *hyperoxia*) incubated with a radiolabeled NFκB probe. In the middle of the blot, an antibody to the p50 subunit of NFκB (+p50) was added to 2 hyperoxic heart extracts, which restricted the mobility of the probe. No bands were apparent in the extracts of 6 normoxic control hearts (*control*). B, EMSA of nuclear extracts of 6 rat hearts after 60 minutes of exposure to hyperoxia (*hyperoxia*) and 6 normoxic control hearts (*control*) incubated with a radiolabeled AP-1 probe. C, Optical density of the NFκB bands of 6 hyperoxic and 6 control hearts. D, Optical density of the AP-1 bands of 6 hyperoxic and 6 control hearts. E, EMSA of nuclear protein extracts of 3 hearts from rats pretreated with SN50 (400 $\mu\text{g/kg}$ administered intraperitoneally) before 60 minutes of exposure to hyperoxia ($\geq 95\% \text{ O}_2$ + SN50) and incubated with a radiolabeled NFκB probe. In the next lanes competition with cold probe (+CP) or anti-p50 antibody (+p50) have been used on nuclear extracts from hyperoxic hearts, and finally, nuclear extracts from 3 hyperoxic (*hyper*) and 3 normoxic control hearts (*con*) are blotted. F, EMSA of nuclear extracts of the same hearts as shown in panel E but incubated with a radiolabeled AP-1 probe. In the next lanes competition with cold probe (+CP) or anti-junD antibody (+junD) have been used on nuclear extracts from hyperoxic hearts, and finally, extracts from 3 hyperoxic (*hyper*) and 3 normoxic control hearts (*con*) are blotted. G, Optical density of the NFκB bands shown in panel E. H, Optical density of the AP-1 bands shown in panel F.

Pretreatment with PDTC abolished the protective effect of hyperoxia (group 5; $P < .001$; Figure 2, B). The differences between normoxic control hearts and hearts from hyperoxic animals pretreated with PDTC (group 3) could be due to chance ($P > .2$). However, pretreatment

of normoxic control animals with PDTC improved postischemic recovery of LVDP ($P = .03$; Figure 2, B). When PDTC was added to the perfusate (group 8), postischemic left ventricular function was not influenced (Figure 3, B).

Figure 6. A, NFκB activation serially in the lungs (*upper panel*) and hearts after 0, 2, 5, 10, 20, and 60 minutes of hyperoxic exposure. Band identities were verified by means of supershift analysis with an anti-p50 antibody (+p50) added to nuclear protein extract after 10 (lungs) and 60 (hearts) minutes of hyperoxia and by means of cold-probe competition (+CP) in nuclear extracts from hearts after 60 minutes of hyperoxia. B, NFκB activation during ischemia and reperfusion in the hearts from normoxic (C) and hyperoxic (H) rats. Nuclear proteins were extracted after 25 minutes of stabilization, 12.5 and 25 minutes of global ischemia, and 5 minutes of reperfusion before incubation with a radiolabeled NFκB probe. Competition analysis was performed by means of addition of cold probe before adding the labeled probe (+CP), and supershift analysis was performed by adding an anti-p65 antibody that caused restriction of the probe (+p65). C, A representative immunoblot of cytoplasmic proteins after incubation with a rabbit polyclonal anti-IκB-α antibody from the same heart samples as shown in the *upper panel*.

Left ventricular end-diastolic pressure. With SN50, LVEDP increased during reperfusion of normoxic control hearts (group 1). Sixty minutes of hyperoxia immediately before perfusion (group 2) did not significantly influence postischemic LVEDP (Figure 2, A). The differences between normoxic control hearts (group 1) and hearts from normoxic animals pretreated with SN50 (group 4) or between hearts from hyperoxic animals pretreated with SN50 (group 3) and normoxic control hearts could be due to

chance ($P > .2$). Infusion of SN50 during ischemia and reperfusion (group 7) did not influence postischemic LVEDP (Figure 3, A).

With PDTCT, 60 minutes of hyperoxia immediately before perfusion attenuated an increase of LVEDP. Pretreatment with PDTCT before exposure to hyperoxia (group 5) did not significantly influence postischemic LVEDP compared with that seen in hyperoxic hearts (group 2; Figure 2, B). The differences between hearts from normoxic animals

pretreated with PDTC (group 6) and normoxic control hearts or between hearts from hyperoxic animals pretreated with PDTC (group 5) and normoxic control hearts could be due to chance ($P > .2$). PDTC added to the perfusate during ischemia and reperfusion (group 8) reduced the increase of LVEDP during reperfusion ($P = .004$; Figure 3, *B*).

Left ventricular systolic pressure and heart rate. The differences in LVSP within or between the groups could be due to chance ($P > .2$, data not shown). In the SN50 study the mean HR immediately before global ischemia was 281 ± 5 beats/min for all groups, whereas in the PDTC study the value was 310 ± 5 beats/min. Both preischemic and postischemic differences within or between the groups could be due to chance ($P > .2$).

Coronary flow. With SN50, CF was reduced during reperfusion of normoxic control hearts (group 1). Pretreatment with hyperoxia (group 2) inhibited this reduction ($P < .001$; Figure 2, *A*). SN50 given before exposure to hyperoxia (group 3) abolished the beneficial effect of hyperoxia ($P < .001$). The differences between normoxic control hearts and hearts from normoxic animals pretreated with SN50 (group 4) and between normoxic control hearts and hearts from hyperoxic animals pretreated with SN50 (group 3) could be due to chance ($P > .2$; Figure 2, *A*). When SN50 was administered to the isolated heart during ischemia and reperfusion (group 7), CF was not influenced (Figure 3, *A*).

PDTC pretreatment (group 5) abolished the beneficial effect of hyperoxia on CF ($P < .001$; Figure 2, *B*). The differences between normoxic control hearts and hearts from hyperoxic animals pretreated with PDTC or between normoxic control hearts and hearts from normoxic control animals pretreated with PDTC could be due to chance ($P > .2$). Infusion of PDTC to the isolated heart during ischemia and reperfusion (group 8) improved postischemic CF ($P = .05$; Figure 2, *B*).

Infarct size

In hearts from normoxic control animals (group 1), $33\% \pm 3\%$ of total myocardial tissue was calculated as unstained by TTC after 60 minutes of reperfusion. Hyperoxia (Group 2) reduced this area to $19\% \pm 3\%$ ($P = .04$; Figure 4, *A*). Pretreatment with SN50 before exposure to hyperoxia (group 3) inhibited the effect of hyperoxia ($35\% \pm 7\%$, $P = .003$). The differences between normoxic control hearts and hearts from hyperoxic animals pretreated with SN50 (group 3) or hearts from normoxic control animals pretreated with SN50 (group 4) could be due to chance ($P > .2$). Infarct size was profoundly reduced when SN50 was infused during ischemia and reperfusion (group 7, $13\% \pm 2\%$, $P = .004$).

Pretreatment with PDTC (group 5) abolished the beneficial effect of hyperoxia (group 2) against necrosis ($34\% \pm 1\%$ vs $20\% \pm 2\%$, $P < .001$; Figure 4, *B*). However, pretreatment of either normoxic control or hyperoxic animals with PDTC (group 6) limited myocardial necrosis

compared with that seen in normoxic control hearts ($35\% \pm 2\%$ vs $44\% \pm 3\%$ [$P = .007$] and $34\% \pm 1\%$ vs $44\% \pm 3\%$ [$P = .004$]). Administration of PDTC during ischemia and reperfusion (group 8) also limited the necrotic area ($29\% \pm 2\%$, $P < .001$; Figure 4, *B*).

Activation of Transcription Factors

When hearts were sampled immediately after 60 minutes of hyperoxia (group 2) and compared with normoxic control hearts (group 1), activation of NF κ B and AP-1 was found in nuclear extracts of all hyperoxic hearts. A representative EMSA of nuclear extracts from 6 hyperoxic and 6 normoxic control hearts with radiolabeled NF κ B probe is shown in Figure 5, *A*, where the bands could be supershifted with anti-p50 antibody. The band optical densities were increased by means of hyperoxia (Figure 5, *C*). When the same samples were incubated with a radiolabeled AP-1 probe, activation of AP-1 was found after 60 minutes of hyperoxia (Figure 5, *B* and *D*). In the lungs sampled serially after different durations of hyperoxia, NF κ B was activated already after 2 minutes and thereafter gradually decreased during 60 minutes of exposure (Figure 6, *A*). In the hearts activation of NF κ B could be seen after 2 to 5 minutes of exposure, was thereafter reduced, and increased again at the end of 60 minutes of hyperoxia. The identity of the proteins bound to the probe was verified by means of supershift analysis and cold-probe competition (Figure 6, *A*).

Effect of SN50 on NF κ B and AP-1 Activation

EMSA of nuclear extracts from 3 normoxic control hearts (group 1) and 3 hyperoxic hearts (group 2) after incubation with the radiolabeled NF κ B probe is shown in Figure 5, *E*. Incubation with anti-p50 caused restriction of the probe, and the band could be abolished with cold-probe competition. The NF κ B inhibitor SN50 administered before exposure to hyperoxia (group 3) reduced activation of the transcription factor. The band optical densities are shown in Figure 5, *G*. The NF κ B inhibitor SN50 also reduced the AP-1 band (Figure 5, *F* and *H*). When an anti-junD antibody was included, the probe was restricted and abolished by means of cold-probe competition.

NF κ B During Ischemia and Reperfusion

The myocardial NF κ B translocations during Langendorff perfusion, ischemia, and reperfusion are shown in Figure 6, *B*. In hearts isolated from normoxic control animals (group 1), NF κ B was activated after 25 minutes of Langendorff perfusion and remained activated during ischemia and early reperfusion. In hearts from hyperoxic animals (group 2), in contrast, a consistently reduced activation of NF κ B than seen in hearts from control animals was observed. The band identities were verified by means of supershift and cold-probe competition.

I κ B α Levels During Ischemia and Reperfusion

A representative immunoblot of cytoplasmatic proteins after incubation with a rabbit polyclonal antibody against the inducible inhibitory protein I κ B α is shown in Figure 6, C. Hyperoxia increased the bands for I κ B α during stabilization, ischemia, and reperfusion.

Discussion

In the present study we demonstrate that pretreatment of rats with hyperoxia improved recovery of contractile function and reduced the extent of myocardial necrosis in the isolated hearts subjected to global ischemia and reperfusion. NF κ B and AP-1 were activated in the hearts by means of hyperoxia, and inhibition of NF κ B activation with 2 different pharmacologic agents abolished the beneficial effects. Hearts from hyperoxic animals had less NF κ B activation during ischemia and reperfusion. This might be due to an increase of the inducible NF κ B inhibitory protein I κ B α . Thus we suggest that NF κ B plays a dual role in the heart. It might play a key role in the myocardial adaptation to ischemia presently induced by hyperoxia, but it also has a detrimental role in hearts not adapted to sustained ischemic insult. When the NF κ B inhibitor PDTC was infused into the isolated heart during ischemia and reperfusion, the postischemic recovery of myocardial contractile function was improved, whereas PDTC and SN50 both reduced infarct size.

It has previously been demonstrated by us¹⁶ and others¹⁷ that low doses of exogenous reactive oxygen species might protect myocardial function, indicating that moderate oxidative stress is cardioprotective. Normobaric hyperoxia induces oxidative stress in cell cultures.¹⁸ Li and colleagues¹⁹ have demonstrated that NF κ B is activated by means of hyperoxia in cultured human alveolar epithelial cells. In the present study we found that NF κ B becomes activated in a time-dependent manner starting in the lungs and rapidly followed by activation in the heart during hyperoxic exposure. In the lungs NF κ B activation gradually decreased, whereas in the heart it followed a biphasic pattern, with a second peak after 20 to 60 minutes of hyperoxic exposure. At the moment, we do not know how systemic hyperoxia causes activation of cardiac NF κ B. To inhibit cardiac NF κ B activation, we used the synthetic peptide SN50, which is a highly specific NF κ B inhibitor.²⁰ The present study showed that beneficial functional and anti-infarct effects of hyperoxic exposure were completely blocked with SN50 in isolated rat hearts. Results from EMSA analysis suggest that SN50 did not completely inhibit activation of myocardial NF κ B. A higher concentration of SN50 might have abolished the activation. Another NF κ B inhibitor, PDTC, which suppresses NF κ B activation through inhibiting the reversible release of the inhibitory protein I κ B α ,¹⁴ reduced the functional protection and infarct-limiting effects of hyperoxia. PDTC has also been shown to possess antioxidant properties.²¹ Pretreatment of normoxic animals with PDTC,

but not SN50, before heart isolation attenuated the postischemic depression of myocardial function, indicating that antioxidant properties of PDTC might have been of importance.

Our present findings are in accordance with studies investigating the role for NF κ B in ischemic preconditioning. Maulik and coworkers⁸ demonstrated that ischemic preconditioning of isolated rat hearts induced translocation of NF κ B, and SN50 inhibited the achieved cardioprotection. Similarly, Morgan and associates¹⁰ inhibited preconditioning in rabbits by using the NF κ B inhibitor ProDTC. Xuan and associates⁹ showed that even delayed cardioprotection by means of preconditioning of the heart was abolished by means of pharmacologic inhibition of NF κ B.

NF κ B and AP-1 are activated also during prolonged ischemia and reperfusion of the heart.^{22,23} In studies on sustained ischemia-reperfusion injury, NF κ B activation appears to play a detrimental role because its inhibition with nonsense decoy oligos improves functional recovery and reduces infarction.^{11,12} These results are supported by our findings of a moderate reduction of infarction and improvement of postischemic cardiac function when PDTC was administered to control animals before heart isolation. Furthermore, using PDTC or SN50 during perfusion of the isolated heart improved functional recovery and reduced myocardial necrosis after global ischemia. However, the effects of PDTC on postischemic contractile function were more apparent than the effects of SN50 and might have been secondary to its antioxidant effect. Taken together, our data support that activation of NF κ B in ischemia without previous adaptation is detrimental.

The beneficial effects of NF κ B activation by means of hyperoxia or preconditioning could be related to anti-inflammatory effects caused by upregulation of the rapidly inducible I κ B α , thereby reducing inflammation during sustained ischemia by inhibiting NF κ B activation. Hearts from animals subjected to hyperoxia had less NF κ B activation during Langendorff perfusion, induced global ischemia, and reperfusion than hearts from normoxic control animals. Our findings are in accordance with those in isolated, classic, preconditioned rabbit hearts.¹⁰ Furthermore, the NF κ B inhibitor I κ B α , which is transcriptionally induced by means of NF κ B activation, was upregulated in hyperoxic hearts during sustained ischemia. These findings indicate that the benefit of NF κ B activation during adaptation to ischemia is partly an anti-inflammatory effect caused by upregulation of NF κ B activation. However, the beneficial effect of hyperoxia could be also due to transcription of an NF κ B-regulated beneficial gene,²⁴ but the latter is not investigated in the present study.

In conclusion, the present study demonstrates that in vivo exposure to short-term hyperoxia profoundly protects heart function and preserves cell viability through an NF κ B-

dependent mechanism. In this study the heart was the only organ investigated, but it is likely that the protective effects of hyperoxia are systemic. Theoretically, hyperoxia might increase the endogenous defense in all cell types. Such a concept is in agreement with recent findings, in which hyperoxia reduces wound infections¹ and augments antimicrobial defenses.² We speculate that ventilation with a hyperoxic gas might be used in patients to protect any organ or the whole organism, for instance in connection with possible complications accompanying major operations.

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