Tumor Necrosis Factor-α Contributes to Ischemia- and Reperfusion-Induced Endothelial Activation in Isolated Hearts

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Abstract—During myocardial reperfusion, polymorphonuclear neutrophil (PMN) adhesion involving the intercellular adhesion molecule-1 (ICAM-1) may lead to aggravation and prolongation of reperfusion injury. We studied the role of early tumor necrosis factor-α (TNF-α) cleavage and nuclear factor-κB (NF-κB) activation on ICAM-1 expression and venular adhesion of PMN in isolated hearts after ischemia (15 minutes) and reperfusion (30 to 480 minutes). NF-κB activation (electromobility shift assay) was found after 30 minutes of reperfusion and up to 240 minutes. ICAM-1 mRNA, assessed by Northern blot, increased during the same interval. Functional effect of newly synthesized adhesion molecules was found by quantification (in situ fluorescence microscopy) of PMN, given as bolus after ischemia, which became adherent to small coronary venules (10 to 50 μm in diameter). After 480 minutes of reperfusion, ICAM-1–dependent PMN adhesion increased 2.5-fold compared with PMN adhesion obtained during acute reperfusion. To study the influence of NF-κB on PMN adhesion, we inhibited NF-κB activation by transfection of NF-κB decoy oligonucleotides into isolated hearts using HJV-liposomes. Decoy NF-κB but not control oligonucleotides blocked ICAM-1 upregulation and inhibited the subacute increase in PMN adhesion. Similar effects were obtained using BB 1101 (10 μg), an inhibitor of TNF-α cleavage enzyme. These data suggest that ischemia and reperfusion in isolated hearts cause liberation of TNF-α, activation of NF-κB, and upregulation of ICAM-1, an adhesion molecule involved in inflammatory response after ischemia and reperfusion. (*Circ Res*. 1999;84:392-400.)

Key Words: ischemia ■ reperfusion ■ cytokine ■ polymorphonuclear neutrophil

Although reperfusion of an occluded coronary artery is standard therapy to prevent myocardial necrosis, tissue damage may be induced by reperfusion itself. Numerous observations have confirmed a role of leukocytes, specifically polymorphonuclear neutrophils (PMNs), in mediating functional damage to endothelial and myocardial cells during acute and late reperfusion.[1–3] To exert this detrimental role, PMNs must interact with the vascular wall, eg, by adherence to the postcapillary coronary venules. In a well-established 2-step model of PMN adhesion, PMNs are first decelerated by interaction of selectins with their ligands, whereas leukocyte β_2 integrins and endothelial intercellular adhesion molecules (ICAMs-1 to -3) provide firm adhesion.[4,5]

Leukocyte adhesion may be caused by acute endothelial activation, which takes place within seconds through translocation of stored P-selectin[6] and release of proadhesive mediators like platelet-activating factor.[7,8] Later, with these acute trigger mechanisms fading, leukocyte recruitment is still observed. Subacute leukocyte adhesion, requiring hours for its occurrence, is thought to rely on distinct processes, most prominently altered protein expression. More generally, reperfusion induces a functional change from a usually antiadhesive and anticoagulant endothelium toward a proadhesive[9] and procoagulatory[10] cell lining, a process that fits the concept of subacute endothelial activation.[11]

De novo synthesis of adhesion molecules like ICAM-1[12,13] provides functionally active protein within hours after the onset of reperfusion. Therapeutic strategies aimed at interrupting leukocyte binding to these receptors appear to protect from subacute, leukocyte-dependent tissue damage, if covering the appropriate time window. In contrast, therapeutic interventions limited to the acute reperfusion phase and thereby neglecting subacute endothelial activation suffer loss of cardioprotection over time.[14,15] These findings indicate that

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subacute endothelial activation is an independent risk factor for reperfusion injury of the heart.

The driving force of subacute endothelial activation seems to be enhanced formation of proinflammatory and procoagulatory proteins, mediated by activation of transcription factors, eg, nuclear factor-κB (NF-κB). A well-characterized activation cascade of NF-κB includes proteolysis of its cytosolic inhibitor IκBα by the IκB kinase IKK.[16] Thereafter, nuclear localization of NF-κB and transactivation of promoters containing the appropriate binding sequence, eg, ICAM-1[17,18] come into effect. NF-κB activation can occur after ischemia and reperfusion of the heart, as shown in a study by Chandrasekar and Freeman.[19]

In the context of myocardial reperfusion injury, 3 questions prompted us to characterize the postischemic subacute endothelial activation more fully. First, does NF-κB activation induce changes in transcription of ICAM-1, a gene with an NF-κB binding promoter? For this purpose, hearts were subjected to increasing intervals of reperfusion after ischemia, with tissue NF-κB activation as well as ICAM-1 expression being analyzed. Because recent findings suggest that transfection of short oligonucleotides containing NF-κB DNA binding sequence (decoy oligonucleotides) inhibits NF-κB–dependent transcription (for review, see Reference [20]), we used transfection of NF-κB decoy oligonucleotides to demonstrate a causal relation of both processes. Second, does enhanced transcription (and translation) of ICAM-1 lead to enhanced PMN adhesion in coronary venules? Fluorescence microscopy of rhodamine-labeled PMN was performed acutely as well as subacutely, in the absence or presence of NF-κB blocking interventions. Third, may rapid release of TNF-α, a cytokine found in reperfused myocardium,[21] contribute to the transcriptional activation of ICAM-1? To address this question, BB 1101, an inhibitor of a TNF-α cleavage enzyme (TACE),[22] was applied.

Materials and Methods

p50, p65, and IκBα antibodies were purchased from Santa Cruz. dIdC was purchased from Pharmacia. Polyacrylamide was obtained from Biozym. Hybond N and Hybond enhanced chemiluminescence (ECL) membranes (Amersham) were used for Northern blotting and Western blotting, respectively. All chemicals were purchased from Sigma, if not indicated otherwise. Oligonucleotides were obtained from MWG. BB 1101 was kindly provided by British Biotech (Oxford, UK). ICAM-1 full-length cDNA was provided by Dr Horiuchi (Daiichi Pharmaceuticals, Tokyo, Japan).

Heart Preparation

The care of the animals and all experimental procedures conform with the *Guide for the Care and Use of Laboratory Animals*. Hearts were isolated and perfused as previously described. Briefly, animals (male, 250 to 350 g) were anesthetized (saturated ether) and decapitated. The aorta was quickly cannulated, and hearts were retrogradely perfused with a modified Krebs-Henseleit buffer gassed with 94.5% O_2/5.5% CO_2 (37°C, pH 7.40±0.05).

The working heart preparation was established as previously described.[8] The caval and azygos veins were ligated. Pulmonary venous entry was used for a cannula that, in the working mode of the heart, provided orthograde access for the perfusate. In the perfusion apparatus, it was possible to switch between nonworking (Langendorff) mode and working heart mode. External heart work was calculated as the sum of pressure-volume work (developed aortic pressure×cardiac output) and acceleration work.

Experimental Protocols

In experiments assessing external heart work, rat hearts performed pressure volume work (preload 12 cm H_2O, afterload 80 mm Hg) for 20 minutes. Hearts displaying a cardiac output under 35 mL/min were excluded from the study. Thereafter, global ischemia (15 minutes, 37°C) was imposed. After ischemia, hearts were initially reperfused in the nonworking Langendorff mode (5 mL/min, 15 minutes) before a second period of external heart work at the given conditions was performed to assess acute reperfusion injury (myocardial stunning). Application of the TACE inhibitor BB 1101 was performed for 240 minutes of reperfusion or until the end of the shorter experiments. Oligonucleotide transfection using HVJ-liposomes was performed 10 minutes before ischemia and lasted 5 minutes. Immediately at the end of the experiment hearts, were snap-frozen in liquid nitrogen and stored at -80°C. Three hearts per group were analyzed, if not indicated otherwise.

In experiments assessing PMN adhesion by in situ fluorescence microscopy, hearts and PMNs of guinea pigs were used, as previously established.[23] After Langendorff preparation, hearts were subjected to ischemia, various intervals of reperfusion, and a second trigger of ischemia (15 minutes). Then, a PMN bolus (10^7 homologous cells, prestained with rhodamine 6G, diluted in 1 mL of Tyrode's solution) was infused 1 minute after the onset of reperfusion, bringing the final concentration to 1666 PMN/μL. In one experimental group, a monoclonal CD18 antibody (10 μg/mL MCA 503, Serotec) was added to the PMN for 10 minutes before injection.

After washout of nonadherent PMN (3 minutes), hearts were arrested by cold cardioplegia (potassium 28 mmol/L) and placed on a microscopic stage, the surface of the left ventricle being exposed to a microscope (Ploemopak, Leitz) with a ×10 objective (L10, 0.22 aperture, Leitz). Images were generated by a charge-coupled device camera (COHU 4400, Prospective Measurements). To analyze vessel distribution, fluorescein isothiocyanate dextran (FITC 0.1%, M_r 150 000) was infused (15 mL/h) and excited by epi-illumination with an H130 mercury light source and filtered with an $I_{2,3}$ filter block (Leitz). The distribution of rhodamine 6G prestained PMNs was studied under a rhodamine 6G selective N_2 filter block (Leitz).

This method allowed for localization of PMNs with respect to the vascular lumen. Postcapillary venular adhesion was observed in venules of 10 to 50 μm in diameter (small venules; n=18 to 25 per heart).

ELISA Measurements (TNF-α and Interleukin-1)

Coronary effluent and transudate (interstitial/lymphatic fluid appearing on the epicardial surface) of hearts undergoing ischemia and reperfusion were collected at preischemic and postischemic time points. Fluid samples were frozen on liquid nitrogen and stored at -80°C for <8 weeks. Two different ELISA kits were used to detect TNF-α (Endogen and Laboserv). Interleukin-1 (IL-1) was also assessed with ELISA (Endogen). Because cytokine levels in the coronary effluent were consistently below the detection limit (≈5 pg/mL; data not shown), we only report coronary transudate levels.

Electrophoretic Mobility Shift Assay (EMSA)

Left ventricular tissue (200 mg) was lysed in a reducing Triton lysis buffer (Tris 1 mmol/L (pH 7.4), NaCl 50 mmol/L, Triton 1×, Na vanadate 5 mmol/L, Na fluoride 50 mmol/L, Na pyrophosphate 30 mmol/L, DTT 1 mmol/L, PMSF 1 mmol/L, leupeptin 10 μmol/L, and pepstatin 10 μmol/L). Separation of cytosolic and nuclear protein lysates was achieved by 5-second centrifugation (10 000g), according to the protocol of Schreck and Baeuerle.[24] The membrane fraction was separated by 15 minutes of centrifugation at 10 000g (4°C) in both cases. Protein concentration of the lysates was measured with a detergent-compatible assay (BCA, Pierce). Ten micrograms of protein from lysates was incubated with dIdC (1 μg) in binding buffer (10 mmol/L Tris [pH 7.5], 50 mmol/L NaCl, 0.5 mmol/L EDTA, 1 mmol/L DTT, 0.05 mmol/L PMSF, 2%/volume glycerol, and 2% Ficoll 400) for 20 minutes. Thereafter, lysates were exposed for 15 minutes to double-stranded oligonucleotides encoding an NF-κB consensus binding site (5'-AGT TGA

GGG GAC TTT CCC AGG C-3′ and 5′-GCC TGG GAA AGT CCC CTC AAC T-3′) or a nonbinding oligonucleotide (5′-TTG CCG TAC CTG ACT TAG CC-3′ and 5′-GGC TAA GTC AGG TAC GGC AA-3′) labeled with [^{32}P] ATP using polynucleotide kinase (Boehringer-Mannheim). Two microliters of p50 antibody (Santa Cruz) or 7 μL of p65 antibody (Santa Cruz) were used for supershift or deletion of specific bands. Protein-oligonucleotide mixtures were separated on a 6% polyacrylamide gel. Finally, gels were dried and exposed to x-ray film.

Northern Blot Analysis

To analyze mRNA induction, 200 mg of heart tissue were lysed with guanidine-isothiocyanate/phenol. Phase separation was achieved with 20% chloroform and centrifugation (12 000g, 15 minutes, 4°C). Total RNA was precipitated by addition of 2-propanol (20 minutes, room temperature), and the precipitate was centrifuged at 12 000g. After removal of 2-propanol, the pellet was washed with 1 mL of 75% ethanol, centrifuged at 7000g, air-dried for 15 minutes, and quantified in Tris-EDTA at 260 nm.

Aliquots (10 μg) of total RNA were separated on a 1% agarose gel containing 5% formaldehyde and 1× MOPS. Overnight transfer was performed using Hybond N filters, which thereafter were cross-linked by UV light, prehybridized in salmon sperm DNA (50 μg), and hybridized in 5× SSC, 0.5% SDS, and 1× Denhardt's solution for 12 hours using cDNA specific for the indicated gene, which was ^{32}P labeled using random primers (Prime It II, Stratagene). After washing in 4× SSC, 0.5% SDS, 1× Denhardt's for 30 minutes, and 2× SSC, 0.5% SDS for 15 minutes 2 times, filters were exposed to x-ray film.

Hybridization probes were used as full-length cDNA for ICAM-1 or obtained from rat mRNA that was reversely transcribed to cDNA. The following primers were used to isolate the corresponding cDNA: TNF-α, 5′-CTC TTC TGT CTA CTG AAC TTC GG-3′ and 5′-AGA TAG CAA ATC GGC TGA CGG-3′; IL-1, 5′-CAC AGC AGC ACA TCA ACA AGA GC-3′ and 5′-GTC CTG CTT GTG AGG TGC TGA TG-3′.

Western Blot Analysis

Protein lysates (40 μg) were separated on a 12% polyacrylamide gel, transferred to a nitrocellulose membrane (Hybond ECL, Amersham), blocked with buffer containing 5% nonfat dry milk, incubated with 2 μg/mL primary antibody (IκBα and p65, Santa Cruz), washed 4× with buffer containing 1% nonfat dry milk, exposed to 0.2 μg/mL secondary antibody (Santa Cruz) bound to horseradish peroxidase, incubated with ECL enhancer kit (Pierce) for 1 minute, and exposed to x-ray film.

Preparation of HVJ-Liposomes

HVJ-liposomes were prepared according to Kaneda[25] with minor modifications. Briefly, 10 mg of lipids (phosphatidylcholine, cholesterol, phosphatidylserine 4.8:2:1) were dissolved in 5 mL diethyl-ether. Two hundred microliters of a 10 μmol/L solution of oligonucleotides dissolved in BSS (137 mmol/L NaCl, 5.4 mmol/L KCl, and 200 μL Tris HCl [pH 7.0]) were injected, vortexed thoroughly, and sonicated for 10 seconds in a bath-type sonicator. (The sequence of the NF-κB oligonucleotide and the nonsense oligonucleotide were identical to the sequences used in (EMSA), as described above.) Thereafter, ether was removed using a rotary evaporator. From the gel that formed under these conditions, liposomes were prepared by vortexing. Liposomes were then fused with 30 000 hemagglutinating units of UV-inactivated Sendai virus. All additional steps were performed according to Kaneda.[25] After dilution, 10 mL of HVJ-liposomes carrying 5 μmol/L oligonucleotides were injected 10 minutes before ischemia and reperfusion.

Statistical Methods

The results are given as mean±SEM. Statistical analysis was performed with 1-way ANOVA. Whenever a significant effect was obtained with ANOVA, we performed multiple-comparison tests between the groups using the Dunn test (Sigmastat statistical

Influence of Ischemia and Reperfusion on Myocardial Function

Group	Recovery, %	CF1, mL/min	CF2, mL/min	n
Control	95±3	10±1	10±1	3
Ischemia and reperfusion	68±6*	16±2	14±2	6

Recovery is defined as postischemic/preischemic external heart work×100%. CF1 indicates preischemic coronary flow; CF2, postischemic coronary flow.

*$P<0.05$ vs control.

program). Differences between groups were considered significant for $P<0.05$.

Results

Hemodynamic and Contractile Effects of Ischemia and Reperfusion

We first tested if our model of ischemia (15 minutes) and reperfusion induces reperfusion injury, ie, myocardial and endothelial stunning in isolated rat hearts. External heart work and coronary flow were monitored in a preischemic working phase (phase 1) and postischemically (phase 2). In nonischemic hearts, external heart work in phase 2 (without foregoing ischemia) was 95% of the external heart work in phase 1 (Table). Coronary flow under fixed preload and afterload (12 cm H$_2$O and 80 mm Hg, respectively) was identical in both periods. In contrast, hearts subjected to 15 minutes of ischemia and subsequent reperfusion displayed a distinct loss of the capacity to perform external heart work, recovery being 68±6% of the preischemic value (myocardial stunning). At the same time, coronary flow tended to decrease during postischemic reperfusion.

Activation of Transcription Factors During Early Reperfusion

Because we have shown previously that NF-κB is a crucial transcription factor for posthypoxic ICAM-1 induction in a cell culture model,[26] we now investigated the activation pattern of NF-κB in an isolated heart model displaying "nonlethal" infringement of function during early reperfusion. The activation was assessed by EMSA, relying on the ability of activated NF-κB, which has dissociated from IκBα, to bind to a specific double-stranded DNA sequence. As shown in Figure 1A, NF-κB activation can be induced in isolated hearts by TNF-α (100 pg/mL). NF-κB band retardation is inhibited by 100-fold excess competition with the specific NF-κB oligonucleotide, but not with a nonspecific (scrambled) oligonucleotide. (Both the specific and the scrambled oligonucleotide are also used for HVJ-liposome transfection; see Figure 4B.) No activation, however, is found after ischemia (45 minutes). In contrast, the activated form of NF-κB is present in the cytosol and translocated to the nucleus as early as 30 minutes after ischemia (15 minutes) and is detected up to 2 hours of reperfusion in the isolated rat heart model (Figure 1B).

Antibody supershift experiments reveal that the NF-κB–specific complexes detected comprise dimers of p65 and p50 (Figure 1C). A p50 antibody shifts 2 bands, the lower of which is completely transposed. The upper of the shifted
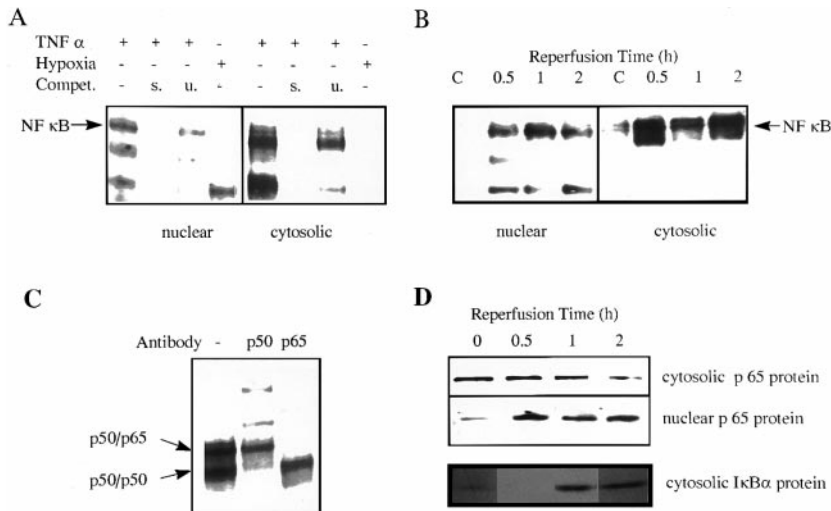
Figure 1. A, EMSA of nuclear and cytosolic extracts of isolated rat hearts demonstrates NF-κB activation by TNF-α (100 pg/mL), whereas ischemia (45 minutes) does not activate NF-κB. Specific competition with 100-fold excess of unlabeled oligonucleotide encoding NF-κB binding site (s.) but not unspecific competition (u.) with scrambled oligonucleotide blocked NF-κB band retardation in the nuclear and cytosolic compartment. B, EMSA time course of NF-κB activation after ischemia (15 minutes) and various periods of reperfusion. NF-κB activation and translocation to the nucleus is present from 30 minutes to 2 hours after ischemia. C, Supershift EMSA experiment using p50 and p65 antibodies (Santa Cruz) to identify NF-κB–containing complexes. p50 antibody shifts 2 of the demonstrated bands, whereas the upper band disappears in the presence of the deletional p65 antibody. Most likely, the lower band represents p50 homodimers, whereas the upper band contains p50/p65 dimers. D, Immunoblot of p65. Cytosolic (upper panel) and nuclear extracts (lower panel) of the same hearts were used, demonstrating p65 translocation to the nucleus as soon as 30 minutes of reperfusion. Time course of cytosolic IκBα by Western blot. Results are representative for at least 3 separate experiments for each condition.

bands is entirely deleted by a p65 antibody. We conclude that both bands contain p50, the lower band as a homodimer, the upper band as a p65/p50 heterodimer.

Parallel to the time course of NF-κB binding activity in the nucleus, Western blot analysis detected p65 protein in nuclear extracts as early as 30 minutes and up to 2 hours of reperfusion (Figure 1D). Accordingly, nuclear translocation of p65 coincides with a decrease in IκBα levels. The reappearance of IκBα after 60 minutes of reperfusion has been described by others and is itself an NF-κB–dependent process.[27] These experiments demonstrate that NF-κB is activated and translocated to the nucleus not earlier than 30 minutes of reperfusion and remains activated for at least 2 hours.

Reperfusion Induces mRNA Specific for Inflammatory Proteins

Because activation of NF-κB is involved in enhanced transcription of several proinflammatory proteins such as ICAM-1, we next investigated mRNA levels of ICAM-1 during postischemic reperfusion in our model. As shown in Figure 2A, ICAM-1 mRNA is practically absent in normoxic hearts (time-matched controls) but occurs in increasing amounts from 60 minutes of reperfusion to a maximum of 2 hours postischemically, thereafter decreasing again. Inhibition of protein synthesis by addition of cycloheximide (CHX) revealed that enhanced ICAM-1 mRNA expression is independent of protein synthesis, suggesting that nuclear translocation of transcription factors, which do not require protein synthesis, is involved.

A similar pattern of postischemic gene induction was found using probes for other proinflammatory proteins, such as TNF-α and IL-1 (Figure 2C). Therefore, induction of cytokines does not precede that of ICAM-1 in isolated postischemic hearts, indicating that additional factors contribute to the NF-κB activation and ICAM-1 mRNA increase after ischemia (see Figure 5).

ICAM-1 Induction Affects Postischemic PMN Adhesion in Reperfused Hearts

Postischemic induction of ICAM-1 mRNA is accompanied by increased translation of the protein (Figure 2B). To investigate if enhanced ICAM-1 mRNA and protein levels are correlated with a functional effect, we performed microscopic evaluation of PMN adhesion in superficial coronary vessels in a guinea pig model, which displayed an NF-κB activation pattern similar to the rat heart (Figure 3A). The protocol of the adhesion assay is displayed in Figure 3B.
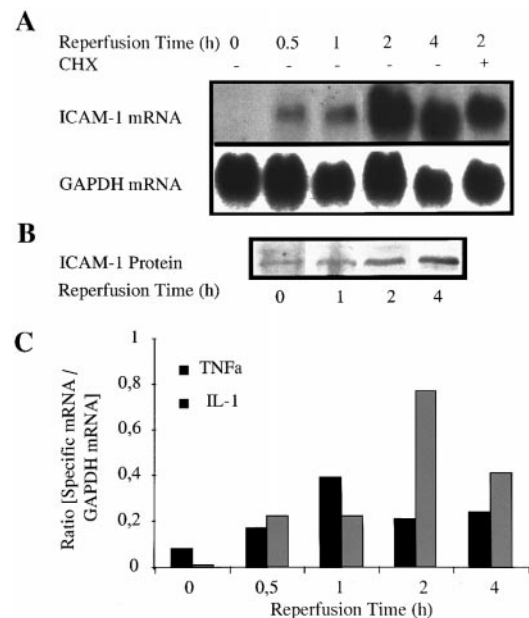

Figure 2. A, Time course of postischemic rat ICAM-1 mRNA expression (upper panel) in comparison to GAPDH mRNA expression (lower panel) by Northern blot. CHX 1 μmol/L was added to exclude posttranscriptional modification at 2 hours of reperfusion. B, Time course of postischemic rat ICAM-1 translation, detected by Western blot of whole cell extracts (300 μg/lane). C, Time course of cytokine induction, given as a ratio of specific mRNA to GAPDH mRNA.
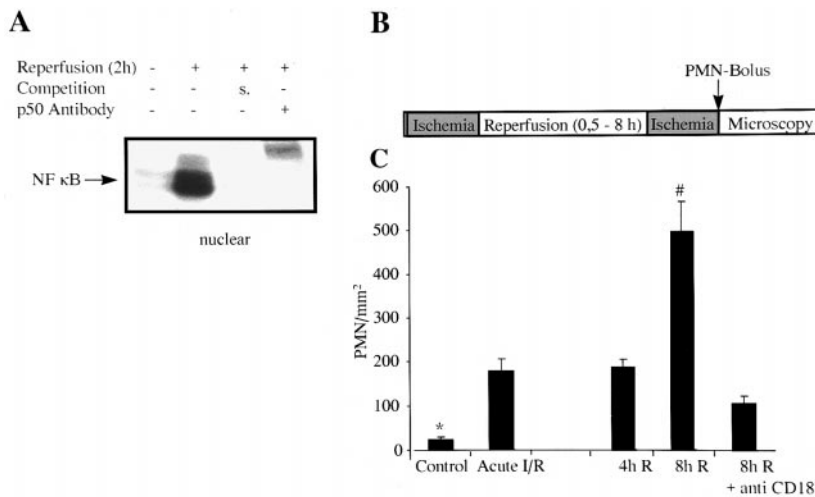
Figure 3. A, EMSA demonstrating NF-κB activation in the nuclear compartment of isolated guinea pig hearts after 2 hours of reperfusion. Specific competition abolished the retarded band, whereas p50 antibody incubation (2 μL) supershifted it. B, Protocol of PMN adhesion assay during acute and subacute reperfusion. A bolus 10^7 homologous PMN was injected into the coronary system. After 3 minutes of washout, cardioplegic arrest and in situ microscopy were performed. PMNs in small venules (10 to 50 μm in diameter) of isolated hearts are given as cell number/mm² of vessel surface. C, Acute adhesion was assessed directly after ischemia, whereas subacute adhesion (4h R, 8h R, and 8h R+anti-CD18) was assessed after 4 to 8 hours of reperfusion. *$P<0.05$ vs all other groups; #$P<0.05$ vs Acute I/R and 8h R+anti-CD18. Venules of 3 hearts were investigated per time point; values are mean±SEM.

Double-fluorescence technique provided images of the vascular bed (detected by FITC-dextran) as well as localization of adherent PMN (prestained with rhodamine 6G).[23] As shown in Figure 3C for small coronary venules (10 to 50 μm in diameter), PMN adhesion to an untreated, intact endothelium is minimal (Control). However, injected at the onset of reperfusion after ischemia, PMNs acutely adhere to an 8-fold greater extent (Figure 3C, Acute I/R). Physiologically, PMN adhesion declines progressively with duration of reperfusion (data not shown). To ensure comparability of acute PMN adhesion and PMN adhesion after 4 or 8 hours of reperfusion (termed subacute PMN adhesion), PMNs were always injected after a standard ischemia (15 minutes, 37°C). The ischemic event was therefore the second one in the subacute adhesion groups.
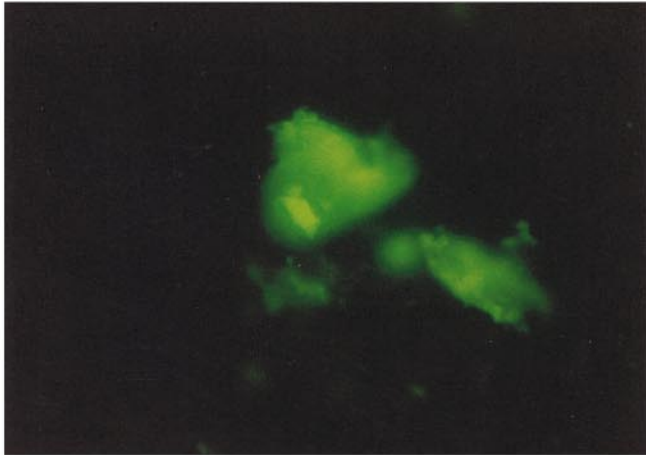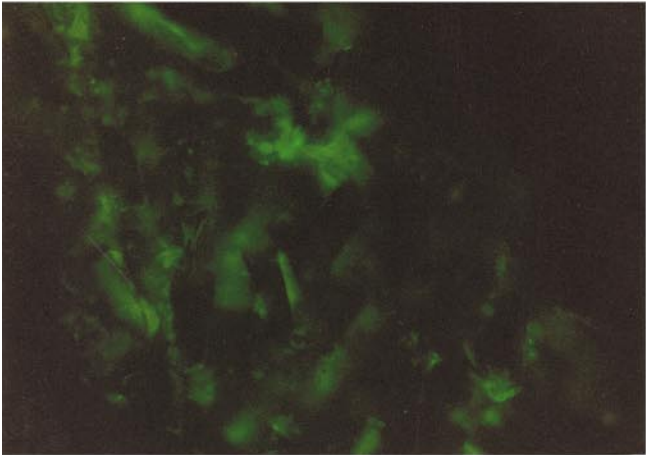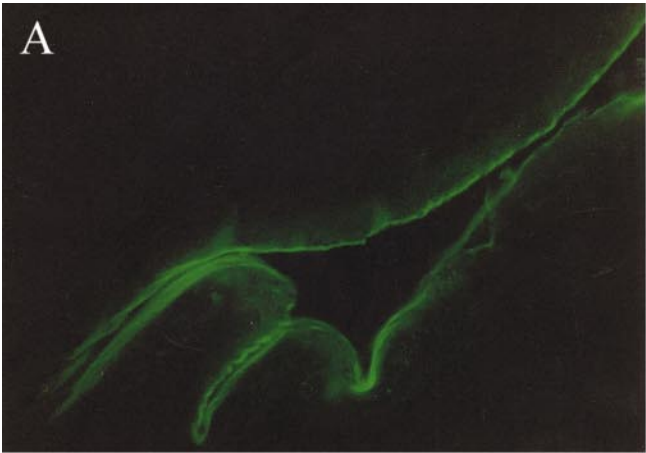
Interestingly, no further increase in adhesion could be seen after 4 hours of reperfusion (4h R), compared with either early acute postischemic PMN adhesion (0h R) or time-matched controls (data not shown). In contrast, a significant increase of postischemic PMN adhesion was found subacutely after 8 hours of reperfusion (8h R) but not in time-matched controls (data not shown).

To verify that ICAM-1 contributes to the subacute postischemic increase of PMN adhesion, we incubated PMN with an antibody against CD18, part of the leukocyte β_2 integrin ligands of ICAM-1 (guinea pig model, 10 minutes of incubation before bolus injection). Indeed, the reperfusion-induced increase in PMN adhesion after 8 hours was specifically reduced by this intervention (8h R+anti-CD18).
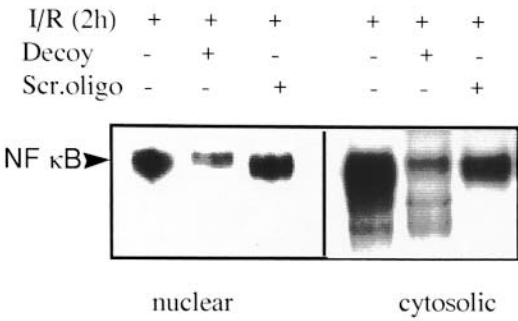
Role of NF-κB for Postischemic Induction of Adhesion Molecules

Because the time courses of NF-κB activation, increase in ICAM-1 mRNA, and enhanced ICAM-1–mediated PMN adhesion suggested a causal relation of the 3 events, we attempted to prevent the latter phenomena by inhibiting the former. To investigate the role of NF-κB for postischemic ICAM-1 induction during postischemic reperfusion, we used transfection with decoy oligonucleotides containing a consensus NF-κB binding site. As previously reported, decoy oligonucleotides occupy the DNA binding domain of a transcription factor, preventing binding of the corresponding DNA sequence of promoters. Using HVJ-liposomes as vehicles to incorporate fluorescence-labeled decoy NF-κB oligonucleotides, we detected preferentially transfection of vessel walls, whereas the parenchyma showed a sprinkled pattern of partial transfection (Figure 4A). Moreover, cytosolic and nuclear NF-κB binding activities were decreased more than by scrambled oligonucleotides not binding NF-κB, even though not entirely blocked (Figure 4B). Accordingly, postischemic ICAM-1 mRNA transcription was reduced in the presence of the NF-κB decoy oligonucleotide (Figure 4B). To investigate the functional significance of the NF-κB activation for the subacute increment of postischemic PMN adhesion, decoy transfection was also used in experiments assessing subacute PMN adhesion. In those experiments, PMN adhesion was reduced to the range of acute PMN adhesion, in effect subtracting the subacute increase of postischemic PMN adhesion (Figure 4C). In contrast, transfection with scrambled oligonucleotide had only a slight, insignificant effect on
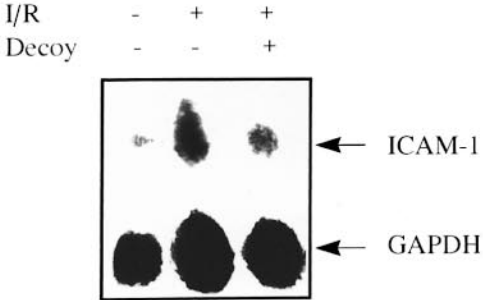
Figure 4. A, Three examples of hearts transfected with a carboxyfluorescein-labeled NF-κB decoy oligonucleotide (5 μmol/L) using HVJ-liposomes. Preferentially, vessels are transfected (top). Parenchymal transfection is achieved to a certain degree, reflected by the sprinkled fluorescence pattern (middle). Intracellularly, cytosol and nuclei are stained (right). No difference was found between normoxic and postischemically reperfused hearts. B, EMSA of rat nuclear extracts of hearts exposed to ischemia and 2 hours of reperfusion (I/R) without or with transfection of HVJ-liposomes containing decoy NF-κB oligonucleotide (Decoy, 5 μmol/L) or scrambled oligonucleotides (Scr.oligo, 5 μmol/L). C, Northern blot of rat ICAM-1 mRNA demonstrating reduction of ICAM-1 mRNA level after transfection with decoy NF-κB oligonucleotide. D, Guinea pig PMN adhesion immediately after ischemia (Acute I/R) in comparison to PMN adhesion after 8 hours of reperfusion without (8h R) and with transfection with decoy NF-κB oligonucleotide (8h R+Decoy), which was imposed at the beginning of the experiment. Transfection with scrambled oligonucleotides or mock transfections with unloaded liposomes served as control (8h R+scrambled oligo and 8h R+Mock). *$P<0.05$ vs Acute I/R and 8h R+decoy. Figures demonstrate representative results of 3 independent experiments.
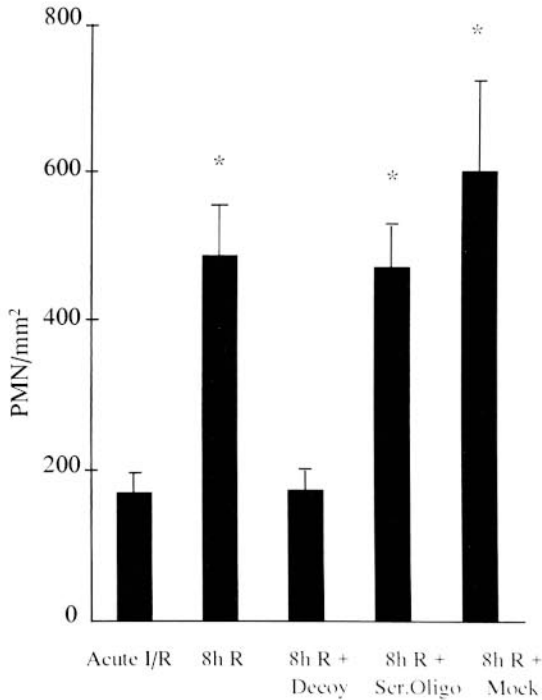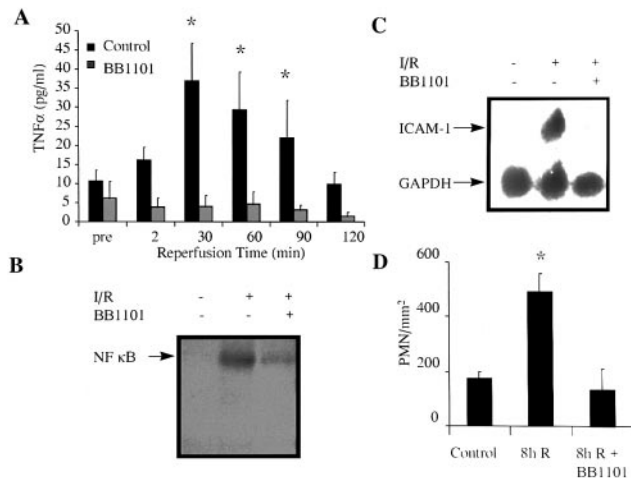
Figure 5. A, Rat TNF-α concentrations of transudate after various reperfusion intervals measured by ELISA. Five hearts were followed up without treatment (black bars), whereas 3 hearts (gray bars) received BB 1101, a TACE inhibitor. B, EMSA of nuclear extracts of rat hearts without or with BB 1101 treatment. C, Northern blot of ICAM-1 mRNA of rat hearts without or with BB 1101 treatment. D, PMN adhesion assay (guinea pig), showing that reduction of ischemia triggered PMN adhesion after BB 1101 treatment. Results of 3 independent experiments are given.
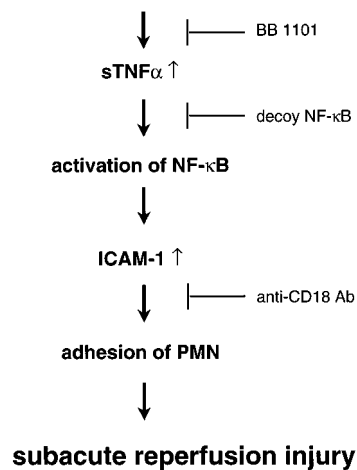

Figure 6. Model of subacute endothelial activation. Postischemic TNF-α cleavage causes TNF-α release and subsequent TNF-α–dependent activation of NF-κB. Thereafter, transcription of ICAM-1 is enhanced, leading to formation of functional adhesion molecules, as suggested by the PMN adhesion assay. Because the process of ICAM-1 formation requires several hours, it accounts for subacute reperfusion injury.

subacute PMN adhesion, and mock transfection did not influence PMN adhesion at all. These experiments demonstrate a specific inhibition of NF-κB activation and downstream events of subacute endothelial activation by NF-κB decoy transfection in postischemically reperfused hearts.

Influence of TNF-α on Induction of ICAM-1

Postischemic induction of genes might include the ones encoding cytokines, subsequently leading to release of a class of proteins that are well-known activators of the NF-κB transcription factors. Therefore, we studied the time course of postischemic cytokine induction during reperfusion of rat hearts. Interestingly, TNF-α and IL-1 were induced by a kinetic comparable to that of ICAM-1, although peaking already after 1 hour of reperfusion (Figure 2B). Because NF-κB activation already occurred as early as 30 minutes after ischemia (Figure 1), cytokine induction may not account for the initial postischemic NF-κB activation.

On the other hand, cleavage of cytokines from stored precursor molecules might be a faster mechanism of cytokine release. Figure 5A indicates that, in comparison to preischemic levels, a distinct rise of TNF-α was found in transudates postischemically, reaching significant levels after 30 minutes of reperfusion and disappearing after 2 hours. The time course of IL-1 release was less pronounced and more restricted to early reperfusion (data not shown). Therefore, we investigated the possibility that the TNF-α found during early reperfusion was causally associated with the binding activation of NF-κB. This result was further strengthened, because TNF-α, exogenously added, was able to activate NF-κB when applied for 60 minutes and washed out for 30 minutes (Figure 1A), a regimen similar to the TNF-α release found in postischemic transudates (Figure 5A).

As a second approach, endogenous TNF-α cleavage, most likely the mechanism underlying early TNF-α release, was prevented with BB 1101, an inhibitor of a hydroxamic acid metalloproteinase specific for TNF-α. Figure 5 demonstrates that BB 1101 actually inhibited all 3 events: postischemic increase of TNF-α release of the isolated hearts (Figure 5A) in concurrence with the postischemic activation of NF-κB (Figure 5B) as well as ICAM-1 induction (Figure 5C). Moreover, PMN adhesion after 8 hours of reperfusion and a second ischemic trigger was reduced, almost to time-matched control values (Figure 5D). Therefore, we were able to show that subacute endothelial activation in postischemic hearts can be antagonized by inhibition of TNF-α cleavage during the first 4 hours of reperfusion.

Discussion

In the present study, we found that NF-κB activation after ischemia leads to postischemically increased ICAM-1 mRNA and protein levels and PMN adhesivity of the endothelial lining, as demonstrated by an ex vivo PMN adhesion assay. Observations of early TNF-α release as well as experiments with a TNF-α cleavage inhibitor (BB 1101) suggest that TNF-α liberation contributes to the postischemic NF-κB activation and subsequent induction of the ICAM-1 gene. Figure 6 displays a model for the underlying signaling pathway. It was possible to block each of the given levels by specific strategies: BB 1101 inhibited postischemic TNF-α liberation and signaling, whereas transfection with decoy oligonucleotides interrupted NF-κB translocation. Finally, antibodies blocking the CD18-containing ligands of, for example, ICAM-1 effectively suppressed subacutely enhanced PMN adhesion after ischemia.

PMN adhesion was performed in a guinea pig model, providing long-term reperfusion of the heart without enhanced vascular permeability as well as highly purified,

modestly preactivated PMNs. Both features are prerequisites for the double-fluorescence microscopy, in which extravasation of FITC-dextran would prevent visualization of the vessel tree, and strong preactivation of PMNs would predispose them to capillary plugging. Although the results of subacute endothelial activation of the rat might not be transferred to the guinea pig model without supporting data, the activation pattern of NF-κB (Figure 3A) and the effects of the CD18 antibody (Figure 3C), NF-κB decoy elements (Figure 4D), and TACE inhibition (Figure 5C) underline the presence of subacute endothelial activation in the given facets in guinea pig hearts as well.

Taken together, these findings suggest that early cleavage of TNF-α triggers a cascade of NF-κB activation and ICAM-1 induction in response to postischemic reperfusion. Similar processes have been observed in the fields of chronic inflammation or transplantation medicine[11] and have been named subacute endothelial activation. The role of NF-κB for ICAM-1 induction and enhanced PMN adhesion after ischemia was confirmed by liposome transfection with an NF-κB decoy oligonucleotide (Figure 4C). The transfected decoy oligonucleotide binds to the activated NF-κB, effectively preventing its assembly at the corresponding sequence of gene promoters that are regulated by this transcription factor,[20] eg, ICAM-1. The experiments might extend understanding of a previous study applying NF-κB decoy transfection to prevent myocardial infarction in vivo in rats.[28] In isolated organs without blood-borne stimuli, we showed that postischemic cardiac NF-κB activation enhances ICAM-1 expression and PMN adhesion, both closely related to infarct size.[1,13] Most interestingly, HVJ-liposomes containing decoy elements easily reached the endothelial vessel lining but did not transfect all parenchymal cells. Whether transfection of the endothelium would suffice to reduce postischemic infarct size in vivo is a question of considerable therapeutic implications that remains to be determined.

Other factors besides enhanced ICAM-1 expression on the endothelial surface may have contributed to enhanced PMN adhesion after prolonged reperfusion, especially induction of P-selectin[29] and E-selectin,[30] adhesion molecules mediating rolling as the first step of PMN adhesion. Given that in our model PMN adhesion was inhibited by the nonselective selectin antagonist Fucoidin, to about the same extent as by the CD18 antibody (data not shown), we cannot rule out this possibility. On the other hand, P-selectin is also acutely upregulated by translocation from Weibel-Palade bodies, becoming abundant on the cell surface rapidly, within minutes of stimulation,[31] eg, after ischemia and reperfusion, as used in our model. Moreover, subacute upregulation of E-selectin during a general inflammatory response is not essential for enhanced PMN rolling to occur,[32] although it modulates rolling velocities[33] and leukocyte recruitment to locally restricted stimuli.[34] Whereas a protective role against ischemia and reperfusion has not been described yet for E-selectin–deficient organisms, protection is present in ICAM-1–deficient mice, at least in kidney and brain.[35,36] Therefore, although P-selectin and E-selectin induction might have contributed to the subacute increase of PMN adhesion

after myocardial ischemia, the findings of the present study and others accentuate the role of ICAM-1.

Which process is responsible for NF-κB activation during early reperfusion? Previous studies have suggested new cytokine synthesis as one possible cause.[37] However, Northern blot analysis (Figure 2C) revealed that de novo synthesis of cytokines cannot account for the NF-κB activation in our experiments, because it occurs at least 30 minutes later. Another process typically occurring during early reperfusion is release of reactive oxygen species, which in cell culture, using hypoxia and reoxygenation, suffices for NF-κB activation independently of receptor stimulation[26] (and data not shown). However, many different antioxidants (including oxypurinol, uric acid, and glutathione) were unable to inhibit NF-κB activation in the isolated heart (data not shown), at least at dosages effectively blunting acute endothelial activation and PMN adhesion.[38]

In contrast, experiments with BB 1101, which affects TNF-α cleavage by TACE,[39,40] displayed inhibition of postischemic NF-κB activation as well as of ICAM-1 induction and acute endothelial activation and acute PMN adhesion (Figure 5C). Although hydroxamic acid compounds like BB 1101 might interfere with other inflammatory proteins,[41] IL-1 release was not affected by BB1101 (data not shown). Moreover, exogenously applied TNF-α (at concentrations as released in the transudate) mimicked the NF-κB activation seen during postischemic reperfusion (Figure 1A). Therefore, TNF-α cleavage by TACE may contribute to NF-κB–dependent ICAM-1 induction during postischemic reperfusion. The process stimulating TACE is not yet identified. However, blockade of the L-type Ca^{2+} channel by nifedipine, even at a dosage severely affecting contractility, did not blunt it (data not shown).

In summary, we describe a model for gene regulatory response of the heart to the challenge of limited ischemia and reperfusion. Enhanced transcription of, among others, ICAM-1 is associated with an enhanced capacity to recruit PMN at postcapillary coronary venules. The NF-κB activation found upstream of these events is, at least in part, triggered by TNF-α stimulation. Further studies will be conducted to elucidate the therapeutic potential of interfering with subacute endothelial activation in the postischemically reperfused heart.

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