Nonanticoagulant heparin inhibits NF-κB activation and attenuates myocardial reperfusion injury

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Departments of 2Internal Medicine and 3Emergency Medicine and the Cannon Research Center, Carolinas Medical Center, Charlotte, North Carolina 28232, 1Division of Cardiothoracic Surgery, Department of Surgery, Emory University School of Medicine and 4Carlyle Fraser Heart Center Cardiothoracic Research Laboratory, Crawford Long Hospital, Atlanta, Georgia 30365; and 5Division of Respiratory, Critical Care and Occupational (Pulmonary) Medicine, University of Utah, Salt Lake City, Utah 84132

Thourani, Vinod H., Sukhdev S. Brar, Thomas P. Kennedy, Lisa R. Thornton, John A. Watts, Russell S. Ronson, Zhi-Qing Zhao, Anne L. Sturrock, John R. Hoidal, and Jakob Vinten-Johansen. Nonanticoagulant heparin inhibits NF-κB activation and attenuates myocardial reperfusion injury. Am J Physiol Heart Circ Physiol 48: H2084–H2093, 2000.—Heparin reduces ischemia-reperfusion injury to myocardium. This effect has been attributed to complement inhibition, but heparin also has other activities that might diminish ischemia-reperfusion. To further probe these mechanisms, we compared heparin or an o-desulfated nonanticoagulant heparin with greatly reduced anticomplement activity. When given at the time of coronary artery reperfusion in a canine model of myocardial infarction, heparin or o-desulfated heparin equally reduced neutrophil adherence to ischemic-reperfused coronary artery endothelium, influx of neutrophils into ischemic-reperfused myocardium, myocardial necrosis, and release of creatine kinase into plasma. Heparin or o-desulfated heparin also prevented dysfunction of endothelial-dependent coronary relaxation following ischemic injury. In addition, heparin and o-desulfated heparin inhibited translocation of the transcription nuclear factor-κB (NF-κB) from the cytoplasm to the nucleus in human endothelial cells and decreased NF-κB DNA binding in human endothelium and ischemic-reperfused rat myocardium. Thus heparin and nonanticoagulant heparin decrease ischemia-reperfusion injury by disrupting multiple levels of the inflammatory cascade, including the novel observation that heparins inhibit activation of the proinflammatory transcription factor NF-κB.

ischemia-reperfusion; myocardial infarction; endothelium dysfunction

DESPITE SUFFERING SIGNIFICANT ISCHEMIA, a large portion of myocytes are still viable immediately after relief of coronary occlusion but undergo progressive necrosis from reperfusion injury (7). Considerable attention has been focused on clot dissolution, therefore, medical and interventional therapy for relief of myocardial ischemia is now quite advanced. Nevertheless, development of therapies for myocardial reperfusion injury has lagged. Currently, there is still no available treatment for reperfusion injury to be used in the clinical arena.

Heparin (HEP) is an almost universal therapy for myocardial infarction to prevent coronary reocclusion and thromboembolic complications. For many years a second pharmacology of HEP, potentially protective against myocardial reperfusion injury, has been recognized at much higher doses than those used currently to produce anticoagulation (27). This effect of HEP is independent of its anticoagulant activity (2, 8, 18, 19) and has been attributed to inhibition of the complement cascade (2, 8). However, N-acetylheparin, a nonanticoagulant HEP derivative, is slightly more potent than unmodified HEP in reducing myocardial ischemia-reperfusion injury but is only about half as active as HEP as an inhibitor of complement-mediated red blood cell lysis (2, 8). Thus other pharmacological actions of HEP may also be important. To further probe the mechanisms by which HEP reduces reperfusion injury, we studied ischemia-reperfusion in a canine infarct model using a novel o-desulfated (ODS) nonanticoagulant HEP with greatly reduced anticomplement activity. Given at reperfusion, this nonanticoagulant HEP decreases neutrophil influx into ischemic-reperfused myocardium and dramatically reduces infarct size, perhaps in part through the novel mechanism of inhibiting activation of the proinflammatory transcription factor nuclear factor-κB (NF-κB).

MATERIALS AND METHODS

Materials

Acetylcholine chloride, the calcium ionophore A-23187, sodium nitroprusside, indomethacin (Sigma, St. Louis, MO), and U-46619 (Upjohn, Kalamazoo, MI) were used in concentrations determined previously (28). Grade I-A heparin sodium salt from porcine intestinal mucosa (Sigma) was resuspended with Krebs-Henseleit (KH) buffer and administered as an intravenous bolus (3 mg/kg to dogs). Partially o-desulfated nonanticoagulant HEP (ODS-HEP) was synthesized by
reduction with sodium borohydride followed by lyophilization under alkaline conditions (9). The resulting HEP derivative was a partially 2-o- and 3-o-desulfated HEP of \( \sim 10,500 \text{kDa} \) with an anticoagulant activity of 7.7 ± 0.9 U/mg in the United States Pharmacopoeia (USP) assay and 4.9 ± 0.8 U/ml anti-factor Xa activity in the amidolytic assay compared with 170 USP/mg anticoagulant activity and 150 U/mg anti-factor Xa activity for the unmodified porcine intestinal HEP from which it was manufactured (9). Whereas 1.0 mg/ml of unmodified HEP inhibited 91% ± 2% of the lysis of human red blood cells by canine plasma, ODS-HEP reduced erythrocyte lysis only by 4% ± 2% at 1.0 mg/ml. ODS-HEP was resuspended in KH buffer and administered as an intravenous bolus (3 mg/kg to dogs; 6 mg/kg to rats, with 100 μg/ml added to KH perfusate for isolated hearts).

In Vivo Studies

Surgical procedure. All animals were handled in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1985). The Institutional Animal Care and Use Committees of Emory University and Carolinas Medical Center approved the study protocols.

Twenty-four heartworm-free adult dogs of either sex were anesthetized with pentobarbital sodium (20 mg/kg) and endotracheally intubated. Anesthesia was supplemented with fentanyl citrate (0.3 μg · kg⁻¹ · min⁻¹) and diazepam (0.03 μg · kg⁻¹ · min⁻¹) was administered intravenously as needed to maintain deep anesthesia. Each dog was ventilated with a volume-cycled respirator using oxygen-enriched room air. A rectal temperature probe was inserted to measure core body temperature. The right femoral artery and vein were cannulated with polyethylene catheters for arterial blood sampling and for intravenous access, respectively. Serial arterial blood gases were measured to maintain the arterial oxygen tension >100 mmHg, Arterial carbon dioxide tension was maintained between 30 and 40 mmHg, and arterial pH was maintained between 7.35 and 7.45 by adjustment of the ventilatory rate (acidemia was counteracted with intravenous sodium bicarbonate).

After median sternotomy, the superior and inferior venae cavae were looped with umbilical tapes, and the heart was suspended using a pericardial cradle. Millar catheter-tipped pressure transducers (Millar Instruments, Houston, TX) were placed in the proximal aorta and in the left ventricular (LV) cavity to measure aortic and LV pressure, respectively. A polyethylene catheter was inserted into the left atrium for colored microsphere injection. A 1-cm portion of the left anterior descending (LAD) coronary artery distal to the first diagonal branch was dissected and loosely encircled with a 2-0 silk suture. A pair of opposing ultrasonic crystals were placed intramyocardially within the proposed ischemic area at risk (AAR) within the LAD coronary artery distribution and were used to assess regional function within the AAR (13).

Experimental protocol. The dogs were randomized to one of three groups (n = 8 in each group): 1) control (saline), 2) unmodified HEP (3 mg/kg), and 3) modified HEP (ODS-HEP, 3 mg/kg). The LAD was occluded for 90 min producing ischemia and then released for 4 h of reperfusion. Each pharmaceutical agent (saline, HEP, ODS-HEP) was infused as an intravenous bolus 10 min before initiation of reperfusion and at 90 and 180 min during reperfusion. Analog hemodynamic and cardiodynamic data were sampled by a personal computer using an analog-to-digital converter (Data Translation, Marlboro, MA), as described previously (12). Hemodynamic and cardiodynamic data were averaged from no fewer than 10 cardiac cycles. Percent systolic shortening, segmental work, and the characteristics of segmental stiffness described by exponential curve-fitting analysis were determined as described (13).

Activated clotting time (in seconds) was measured throughout the experiment using the Hemochron 401 Whole Blood Coagulation System (International Techidyne, Edison, NJ).

Arterial blood creatine kinase activity was analyzed using a kit from Sigma Diagnostics and expressed as international units per gram of protein (13). The experiment was terminated with a bolus administration of intravenous pentobarbital sodium (100 mg/kg). The heart was immediately excised for further analysis and placed into ice-cold KH buffer of the following composition (mmol/l): 118 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄·7H₂O, 2.5 CaCl₂·2H₂O, 12.5 NaHCO₃, and 11 glucose at pH 7.4.

Determination of AAR, infarct size, and regional myocardial blood flow. After postexperimental excision of the heart, the myocardial AAR and infarct size were determined as previously described (13) using Unisperse pigment exclusion and 1% triphenyltetrazolium chloride, respectively. The AAR and infarct size were calculated gravimetrically as previously described (13). Regional myocardial blood flow in the ischemic-reperfused and nonischemic myocardium were obtained by spectrophotometric analyses of dye-released colored microspheres (Triton Technology, San Diego, CA). Left atrial injections of microspheres and reference blood sampling were performed at baseline, at the end of 90 min of ischemia, and at 15 min and 4 h of reperfusion.

Measurement of myocardial neutrophil accumulation. Tissue samples of 0.4 g were taken from the nonischemic zone and from the nonnecrotic and necrotic regions of the AAR for spectrophotometric analysis of myeloperoxidase (MPO) activity (Δ absorbance/min) and for assessment of polymorphonuclear neutrophil (PMN) accumulation in the myocardium, as described previously (13).

PMN adherence to postexperimental coronary artery endothelium. PMN adherence to postexperimental coronary artery endothelium was used as a bioassay of basal endothelial function. Canine PMNs were isolated from arterial blood and fluorescently labeled as previously described (35). After excision of the heart, ischemic-reperfused LAD and nonischemic left circumflex (LCx) segments were isolated, cut into 3-mm segments, opened to expose the endothelium while being submerged in ice-cold KH buffer, and then placed in dishes containing KH buffer at 37°C. After unstimulated, fluorescently labeled PMNs (6 x 10⁶ cells/dish) were incubated with postexperimental segments for 15 min, the coronary segments were washed of nonadherent PMNs and mounted on glass slides, and adherent PMNs were counted under epifluorescence microscopy (490-nm excitation, 504-nm emission), as described previously (35).

Agonist-stimulated macrovascular relaxation. Agonist-stimulated vasoreactivity in epicardial macrovessels from ischemic LAD and nonischemic LCx arteries was studied using the organ chamber technique (30). Indomethacin (10 μmol/l) was used to inhibit prostaglandin release. Coronary rings were preconstricted with the thromboxane A₂ mimetic U-46619 (5 nmol/l). Endothelial function was assessed by comparing the vasorelaxation responses to incremental concentrations of acetylcholine (1–686 μmol/l) and A-23187 (1–191 μmol/l), whereas smooth muscle function was assessed with sodium nitroprusside (1–381 μmol/l).
In Vitro Studies

PMN degranulation. Supernatant MPO activity was measured as the product of canine PMN degranulation using the method by Ely as modified by Jordan et al. (12). Canine PMNs (20 × 10⁶ cells/ml) were incubated in the presence or absence of ODS-HEP and stimulated to degranulate with platelet-activating factor (PAF, 10 μmol/l) and cytochalasin B (5 μg/ml). MPO activity in supernatants was assayed spectrophotometrically (12).

PMN adherence to normal coronary artery endothelium. Adherence of PMNs to normal canine epicardial arteries was assessed using coronary segments and PMNs from normal animals. Unstimulated PMNs and coronary artery segments prepared and labeled as described for adherence studies were incubated in the presence or absence of HEP or ODS-HEP. After PAF (100 nmol/l) stimulation for 15 min, adherent PMNs were counted as outlined earlier.

Experiments with human umbilical vein endothelial cells. HUVECs were isolated according to the method of Jaffe et al. (11), cultured on coverslips using endothelial cell growth medium (Clonetics), and tested for expression of von Willebrand factor. HUVECs were washed twice with PBS and incubated in Neuman-Tytell medium alone for 24 h, followed by incubation with lipopolysaccharide (1 μg/ml) plus 10–20 ng/ml tumor necrosis factor-α (TNF-α) for 2 h, or in HEP or ODS-HEP (200 μg/ml) for 4 h with the addition of lipopolysaccharide and TNF-α after 2 h. HUVECs were fixed for 20 min on ice with 4% paraformaldehyde in cell extraction buffer (CEB; in mmol/l: 10 Tris-HCl, pH 7.9, 60 KCl, 1 EDTA, and 1 dithiothreitol) with protease inhibitors (PI) (1 mmol/l Pefabloc, 50 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 40 μg/ml bestatin, 3 μg/ml E-64, and 100 μg/ml chymostatin), permeabilized for 2 min with 0.1% NP-40 in CEB/PI, washed once with cold CEB, and fixed as before for 10 min. Coverslips were incubated in 3% H₂O₂ for 30 min to suppress peroxidase, washed three times in cold PBS, blocked for 2 h with 2% BSA in PBS on ice, and incubated overnight at 4°C with 1 μg/ml of anti-p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 0.1% BSA/PBS. Unbound anti-p65 was removed using a Sephadex G-25 column. The probe (0.5 g/ml) was incubated with 10 μg HUVEC nuclear protein (Bio-Rad method) in 20 μl of buffer containing a final concentration of (in mmol/l) 10 HEPES, (pH 7.5), 50 KCl, 5 MgCl₂, 1 dithiothreitol, and 1 EDTA and 5% glycerol plus 5 μg of poly(dI-dC) to reduce nonspecific binding. Incubations were carried out at room temperature for 20 min. Reactions were electrophoresed at 14 V/cm for 1.5–2.0 h on a 6% nondenaturing polyacrylamide gel in 0.5× (in mmol/l) 45 Tris borate, 25 boric acid, and 1 EDTA at 4°C and autoradiographed at ~80°C.

Experiments with isolated perfused rat hearts. Male Sprague-Dawley rats (300–400 g) were anesthetized with pentobarbital sodium (40 mg/kg ip), and the hearts were quickly excised and perfused in a Langendorff apparatus as previously described (31) with modified KH bicarbonate buffer consisting of (in mmol/l): 118 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄·7H₂O, 3.0 CaCl₂·2H₂O (yielding 2.5 mmol/l free Ca²⁺ in the presence of EDTA), 0.5 EDTA, 11 dextrose, and 25 NaCHO₃. Three groups were studied: 1) nonischemic control hearts were perfused 45 min; 2) ischemic-reperfused hearts were subjected to 15 min of warm global ischemia and 15 min of reperfusion; and 3) ODS-HEP hearts from rats injected with 6 mg/mg iv ODS-HEP 120 min before heart excision were subjected to 15 min each of global ischemia and reperfusion with 100 μg/ml ODS-HEP in perfusion buffer. After perfusion, ventricles were frozen with Wollenberger clamps precooled in liquid N₂ and pulverized under liquid N₂. Nuclear proteins were immediately isolated from frozen myocardial powders by the method of Li et al. (22). EMSAs were performed using 15 μg of nuclear protein (Pierce protein assay) in each binding reaction. Competition experiments were performed by incubation of nuclear proteins with 10× unlabeled NF-κB or CAMP responsive element oligonucleotides (AGAGATTGCTGACGTCA-GAGGCTG) for 5 min before addition of 32P-labeled NF-κB probe. Supershift assays were performed by adding 0.5 μg of antibodies to p65 and p50 components of NF-κB (Santa Cruz Biotechnology) to the binding reaction after labeled probe. Reactions were electrophoresed at 100 V for 2 h at room temperature on a 5% nondenaturing polyacrylamide gel in 0.5× 120 mmol/l glycine, 1 mmol/l EDTA, and 25 mmol/l Tris, pH 8.5, and autoradiographed.

Statistical Analysis

The data were analyzed by one-way analysis of variance or repeated measures two-way analysis of variance for analysis of group, time, and group-time interactions. If significant interactions were found, Tukey's or Student-Newman-Keuls post hoc multiple comparisons tests were applied to locate the sources of differences. Differences in the densities of the p65-containing NF-κB gel band between treated and untreated ischemic-reperfused rat hearts were compared using the t-test. A P < 0.05 was considered significant, and means ± SE are reported.

RESULTS

HEP and ODS-HEP Reduce Infarct Size

HEP and ODS-HEP significantly reduced myocardial infarct size (Fig. 1). HEP or ODS-HEP treatment decreased infarct size (area of necrosis, AN), expressed as a percentage of the area at risk (AN/AAR), by 35% and 38%, respectively, compared with controls. There was no statistical difference in the size of infarcts between the HEP and ODS-HEP groups, and the AAR from LAD occlusion, expressed as a percentage of the LV mass (AAR/LV), was comparable among groups. Plasma creatine kinase activity was used to confirm histological measurement of infarct size. There were no significant differences in plasma creatine kinase activ-
Heparin (HEP) and o-desulfated (ODS)-HEP reduce infarct size (AN/AAR). Area at risk (AAR) is expressed as a percentage of the left ventricle (LV). Infarct size (area of necrosis, AN) is expressed as a percentage of the AAR. Columns represent group means ± SE for n = 8 in each experimental group. *P < 0.05 vs. control.

Fig. 1. HEP and ODS-HEP reduce PMN influx in reperfused myocardium. PMN influx is a major mechanism underlying lethal reperfusion injury. Treatment with HEP or ODS-HEP significantly reduced MPO activity in necrotic myocardium by 50% compared with the control group (Fig. 3). PMN accumulation within normal myocardium was significantly lower in HEP- and ODS-HEP-treated hearts than in the control group.

HEP and ODS-HEP Reduce PMN Accumulation in Reperfused Myocardium

PMN influx is a major mechanism underlying lethal reperfusion injury. Treatment with HEP or ODS-HEP significantly reduced MPO activity in necrotic myocardium by 50% compared with the control group (Fig. 3). PMN accumulation within normal myocardium was significantly lower in HEP- and ODS-HEP-treated hearts than in the control group.
low and comparable among control, HEP, and ODS-HEP groups (16 ± 6, 18 ± 11, and 18 ± 8 absorbance units/min, respectively). HEP and ODS-HEP both decreased MPO activity in the nonnecrotic AAR, but these changes did not achieve significance (P > 0.10).

ODS HEP Does Not Produce Anticoagulation

Despite reducing infarct size, ODS-HEP did not produce anticoagulation. At 4 h of reperfusion, activated clotting time was increased greater than 10-fold after HEP treatment compared with control (1,425 ± 38 s vs. 123 ± 10 s, respectively). In contrast, activated clotting time in the ODS-HEP group (145 ± 10 s) was not different from controls (123 ± 10 s, P = 0.768). Thus ODS-HEP was able to effect the same benefits as HEP without anticoagulation.

HEP and ODS-HEP Reduce Neutrophil Adherence and Endothelial Dysfunction in Coronary Arteries

ODS-HEP did not significantly reduce PAF-stimulated PMN degranulation (data not shown), suggesting that ODS-HEP has little direct effect on PMN activity. However, PAF-stimulated PMN attachment to coronary endothelium was significantly reduced by both HEP and ODS-HEP in a dose-dependent manner (Fig. 4). Inhibition of PMN adherence to PAF-stimulated coronary endothelium was charge dependent, as suggested by reversal of the inhibiting effects of the polyanions HEP or ODS-HEP on attachment by the polycation protamine (PMNs/mm² endothelium = 66 ± 3 with 100 µg/ml HEP vs. 180 ± 8 with HEP + 1 mg/ml protamine; 86 ± 4 with 100 µg/ml ODS-HEP vs. 136 ± 4 with ODS-HEP + 1 mg/ml protamine; P < 0.05 for both).

HEP and ODS-HEP also reduced PMN adherence to ischemic-reperfused coronary endothelium in vivo. PMN adherence to the ischemic-reperfused LAD coronary artery was increased by 300% in the untreated control group compared with the nonischemic-reperfused LCx artery (Fig. 5). HEP or ODS-HEP reduced PMN adherence to the ischemic-reperfused LAD by 51 and 42%, respectively, compared with untreated controls (Fig. 5).

HEP and ODS-HEP also preserved receptor-mediated vasodilator responses of coronary endothelium following ischemia and reperfusion. To quantify agonist-stimulated endothelial dysfunction in epicardial coronary arteries, we studied the vascular response to incremental concentrations of the vasodilators acetylcholine (endothelial dependent; receptor dependent), A-23187 (endothelial dependent; receptor independent), and sodium nitroprusside (direct smooth muscle) in postischemic coronary vascular ring preparations. Figure 6 illustrates vasodilator responses to acetylcholine in isolated coronary rings from the ischemic-reperfused LAD, expressed as a percentage of U-46619-...
induced precontraction. In the control group, there is a statistically significant shift to the right in the concentration-response curve, representing reduced relaxation to acetylcholine. In contrast, the relaxant effect of coronary vessels to acetylcholine was preserved by HEP or ODS-HEP treatment. The concentration of acetylcholine required to effect 50% relaxation ($EC_{50}$; $-\log [M]$) was significantly greater for the control ($-6.98 \pm 0.06$) compared with the HEP ($-7.30 \pm 0.06$) or ODS-HEP ($-7.20 \pm 0.05$) groups ($P < 0.05$). There were no differences in nonischemic-reperfused ring preparations from LCx (data not shown). In addition, there were no differences between LAD versus LCx preparations from LCx (data not shown). In addition, there were no differences in nonischemic-reperfused coronary vessels to acetylcholine. In contrast, the relaxant effect of acetylcholine required to effect 50% relaxation (EC$_{50}$; $-\log [M]$) was significantly greater for the control ($-6.98 \pm 0.06$) compared with the HEP ($-7.18 \pm 0.06$) and ODS-HEP or ODS-HEP treatment. The concentration of acetylcholine required to effect 50% relaxation ($EC_{50}$; $-\log [M]$) was significantly greater for the control ($-7.17 \pm 0.09$) for LAD and LCx, respectively) or sodium nitroprusside (maximal relaxation = $129 \pm 5$ and $121 \pm 4$% and $EC_{50}$ $-\log [M]$ = $-7.31 \pm 0.02$ and $-7.29 \pm 0.04$ for LAD and LCx, respectively), and responses were unaffected by HEP or ODS-HEP.

**ODS-HEP Prevents Activation of NF-κB**

The increase in PMN adherence following ischemia-reperfusion is from enhanced expression of endothelial cell adhesion molecules (ECAM), the transcription of which are strongly influenced by activation of the NF-κB. NF-κB has recently been shown to be activated as a consequence of myocardial ischemia-reperfusion (22). To study whether HEP could inhibit activation of NF-κB, we studied the effect of nonanticoagulant ODS-HEP treatment of cultured HUVECs and perfused myocardium on NF-κB DNA-binding activity in nuclear protein. Figure 7 shows EMSAs in HUVECs stimulated with TNF-α. TNF-α stimulates endothelial DNA binding of NF-κB (Fig. 7, lane 1) compared with untreated controls (lane 1). Pretreatment with 200 μg/ml ODS-HEP eliminates NF-κB binding activity (Fig. 7, lane 3), indicating that ODS-HEP prevents activation of NF-κB. Interruption of endothelial NF-κB activation was confirmed immunohistochemically by the demonstration of reduced anti-p65 nuclear staining in TNF-α-stimulated HUVECs pretreated with HEP or ODS-HEP (data not shown).

ODS-HEP also reduced DNA binding of NF-κB in ischemic-reperfused myocardium. Exposure of rat hearts to 15 min of warm global ischemia and 15 min of reperfusion increased DNA binding of myocardial nuclear protein to oligonucleotide sequences for NF-κB (Fig. 8A, lane 2). Three distinct bands of increased DNA binding were observed, all of which were eliminated by the addition of excess unlabeled NF-κB oligonucleotide probe (Fig. 8B, lane 2). Supershift experiments identified complex I as the band containing the p65 component of NF-κB (Fig. 8A, lane 5). ODS-HEP treatment reduced ischemia-reperfusion-related stimulation of NF-κB binding to DNA in all three bands (Fig. 8A, lane 3). DNA binding of the p65-containing complex I was nearly eliminated by ODS-HEP, with a reduction of 54 ± 6% as measured by densitometry in comparison to complex I of untreated ischemic-reperfused rat hearts ($P < 0.05$, n = 4). Thus, in addition to directly attenuating vascular adherence of PMNs to coronary endothelium, decreasing PMN accumulation in the AAR and reducing myocardial necrosis, HEP, or ODS-HEP also interrupt NF-κB activation and possibly adhesion molecule expression.

**ODS-HEP Reduces Contractile Dysfunction After Ischemia and Reperfusion of Isolated Rat Hearts**

After 15 min of both ischemia and reperfusion, hearts recovered high contractile function (95% of baseline, ischemia-reperfusion; and 93% of baseline, ODS-HEP ischemia-reperfusion). Therefore, in additional studies, the period of ischemia was increased 30 min. Both untreated and ODS-HEP-treated hearts had
reduced contractile function after 30 min of ischemia and 15 min of reperfusion (pressure rate product = 36,780 ± 2,589 for sham vs. 4,575 ± 1,856 for ischemic-reperfused and 10,965 ± 2,908 mmHg/min for ODS-HEP-treated ischemic-reperfused hearts, n = 4 each), but hearts treated with ODS-HEP had significantly improved recovery of contractile function, which was 2.4 times better than that observed in hearts that did not receive ODS-HEP (P < 0.05). Thus, in this severe model, ODS-HEP reduces both molecular and physiological consequences of ischemia and reperfusion.

**DISCUSSION**

Treatment of acute myocardial infarction has been revolutionized by modalities for clot dissolution. However, except for β-blockers and angiotensin-converting enzyme inhibitors, no medical therapies exist for minimizing myocardial injury following relief of ischemia. Developing such therapies will be key to reducing the size of infarcts and the long-term hemodynamic consequences of infarction, because cardiac myocytes do not regenerate. The potential for salvaging myocardium has been underscored by recent recognition that a large portion of myocytes are still viable immediately after relief of coronary occlusion but undergo progressive necrosis during reperfusion as a consequence of reperfusion injury (7).

At doses greatly exceeding those needed for anticoagulation, HEP substantially reduces reperfusion injury both in the isolated perfused heart (8) and intact whole animal models of myocardial infarction (6, 19). This protective effect is independent of the activity of HEP as an anticoagulant (2, 8, 18, 19) and has been attributed largely to inhibition of complement (2, 8). Undoubtedly, activation of complement contributes to myocardial injury following ischemia and reperfusion.

Fig. 8. ODS-HEP decreases DNA binding of NF-κB in ischemic-reperfused myocardium. A: electrophoretic mobility shift assays (EMSAs) of nuclear protein from ischemic-reperfused rat myocardium. Langendorff perfused rat hearts were subjected to 15 min of warm global ischemia followed by 15 min of reperfusion. Nuclear protein was then harvested for EMSAs to measure DNA binding of NF-κB. Compared with sham-perfused control hearts (lane 1), ischemia and reperfusion typically increased DNA binding of myocardial nuclear protein to oligonucleotide sequences for NF-κB (lanes 2 and 4). Three distinct complexes were identified. Supershift experiments performed with antibody to p65 (lane 5), antibody to p50 (lane 6), or both antibodies (lane 7) demonstrated complex I to be shifted (arrowhead), identifying it as the band containing the p65 component of NF-κB. Pretreatment and perfusion with ODS-HEP (6 mg/kg iv 2 h before heart perfusion; 100 µg/ml in perfusate) prevented the ischemia-reperfusion-related stimulation of NF-κB DNA binding of the p65-containing complex I (lane 3). DNA binding of the p65-containing complex I was nearly eliminated by ODS-HEP, with a reduction of 54 ± 6% as measured by densitometry in comparison to untreated ischemic-reperfused rat hearts (P < 0.05, n = 4). B: competition experiments were performed by incubation of nuclear proteins with 10^{-3} unlabeled NF-κB (lane 2) or cAMP responsive element oligonucleotides (CRE, AGAGATTGCCTGACGTCAGAGAGCTAG, lane 3) for 5 min before addition of 32P-labeled NF-κB probe. Compared with binding reactions without excess unlabeled probe (lane 1), addition of unlabeled NF-κB blocked DNA binding in all three complexes.
NF-κB is activated in the heart by ischemia alone or ischemia plus reperfusion (22), with subsequent up-regulation of adhesion molecules on the myocyte surface (14). Nuclear translocation of NF-κB is prevented by synthetic cell permeable peptides containing the NF-κB NLF, which competes for nuclear uptake (23). NF-κB is readily bound and internalized into the cytosolic compartment by endothelium, vascular and airway smooth muscle, mesangial cells, and even cardiac myocytes (1, 32). We therefore postulated that, once internalized into the cytoplasm, the polyanion HEP might bind electrostatically to the positively charged amino acids of the NLF and prevent it from targeting NF-κB to the nuclear pore. HEP and ODS-HEP prevented TNF-α-induced endothelial cell translocation of NF-κB from cytoplasm to the nucleus, studied immunohistochemically, and reduced binding of NF-κB to DNA in electrophoretic mobility shift assays performed with HUVEC nuclear protein (Fig. 7). ODS-HEP also prevented enhanced DNA binding of NF-κB in ischemic-reperfused myocardium (Fig. 8). In contrast, the glycosaminoglycan dermatan sulfate has recently been shown to activate NF-κB and induce expression of ICAM-1 in cultured human dermal microvascular endothelium (26). Thus inhibition of NF-κB activation appears specific for HEP. These results are consistent with the possibility that HEP electrostatically blocks the NLF, exposed when NF-κB dissociates from its inhibitor I-κB. Because HEP can inhibit mitogen-activated protein kinase (4), we cannot presently rule out an inhibitory effect of HEP on I-κB kinase-mediated phosphorylation of I-κB. Nevertheless, inhibition of NF-κB activation would reduce the enhanced expression of endothelial adhesion molecules (17) and chemotaxins such as IL-8 (20) following myocardial ischemia and reperfusion, forming an additional barrier to PMN influx other than inhibition of selectin-mediated leukocyte rolling and transmigration across the basement membrane. Complement has recently been shown to induce endothelial IL-8 and monocyte chemoattractant protein-1 through NF-κB activation (15). Thus NF-κB inhibition is another level at which HEP can blunt effects of the complement cascade.

In addition to preventing activation of NF-κB, ODS-HEP also significantly improved ventricular function during reperfusion in a severe 30-min model of warm ischemia. This functional benefit of ODS-HEP is unlikely from inhibition of complement or neutrophil influx, because rat hearts were perfused with plasma-free KH buffer. It is possible that inhibition of NF-κB by ODS-HEP reduces TNF-α production by ischemic-reperfused myocardium. TNF-α is a potent myocardial depressant, and levels of TNF-α mRNA and protein are elevated in myocardium after global ischemia-reperfusion (3). NF-κB is a prominent transcriptional promoter of TNF-α, and inhibition of NF-κB reduces circulating TNF-α following myocardial ischemia-reperfusion (3). Thus ODS-HEP may improve the function of reperfused rat myocardium by blocking NF-κB-related production of TNF-α by the myocardium itself following ischemia.
The studies described in this paper present the novel finding that HEP and ODS-HEP prevent activation of the proinflammatory transcription factor NF-κB in both cultured human endothelium and perfused myocardium. We propose that this effect may be from charge neutralization of the cationic NF-κB nuclear localization sequence, uncovered when the inhibitor 1-kB is removed from the transcription factor complex during activation. When given at the time of coronary reperfusion, this novel nonanticoagulant HEP decreases myocardial infarct size, reduces neutrophil influx into necrotic myocardium, and preserves endotelial vasodilator function within the ischemic-reperfusion coronary AAR without producing anticoagulation. Inhibition of NF-κB activation and myocardial reperfusion injury is unlikely from the previously reported anticomplement activity of HEP, because the nonanticoagulant HEP we used has low inhibitory activity against complement. We believe that these findings provide new insight into the mechanisms of antiinflammatory activity of HEP and disclose a true nonanticoagulant HEP with potential for interrupting both the pathophysiological consequences of ischemia-reperfusion syndromes and NF-κB-mediated inflammation.

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