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

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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.

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-acetylated heparin, 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

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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 ~10,500 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 pentobarbital sodium (20 mg/kg) and enflurane (3% for maintenance and 2% for induction). 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 pharmacological 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 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 Technidyne, 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-release 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 nonentric and necrotic regions of the AAR for spectrophotometric analysis of myeloperoxidase (MPO) activity (Δ absorbance/min) and for assessment of polymorphonuclear (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 × 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 Jordán et al. (12). Canine PMNs (20 × 10^6 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. Primary 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 (CEF; 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 aprotinin, 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 described previously (31). 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 washed away with 2% BSA/PBS, and bound antibody was incubated with biotinylated swine anti-rabbit immunoglobulin (1:1,000) in 0.1% BSA/PBS for 45 min on ice, followed by three washes with 2% BSA/PBS. Coverslips were then incubated with streptavidin biotin peroxidase at room temperature for 1 h, washed again, incubated in 0.03% wt/vol 3-3’diaminobenzidine with 0.003% H2O2 until a brown reaction product could be seen, counterstained with eosin, and then viewed under light microscopy.

Electrophoretic mobility shift assays (EMSAs) were also used to study the translocation of NF-κB from the cytoplasm to the nucleus. Nuclear proteins were obtained from HUVEC as described by Digman et al. (6) with the addition of the following protease inhibitors: 1 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 0.5 μg/ml chymostatin, 1 μg/ml antipain, 1 μg/ml leupeptin, and 4 μg/ml aprotin. The double-stranded oligonucleotide DNA probe (Santa Cruz Biotechnology) of the NF-κB consensus sequence AGAGGCTAG for 5 min before addition of 32P-labeled NF-κB probe. Supershift assays were performed by adding 0.5 μg of antibodies to probe 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 glycerine, 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 36%, 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-
ity at baseline among groups (1.6 ± 0.5 for control; 1.2 ± 0.2 for HEP; and 1.0 ± 0.1 international units/g protein for ODS-HEP) and no increases in creatine kinase activity after regional ischemia (3.2 ± 0.6 for control; 2.2 ± 0.4 for HEP; and 2.0 ± 0.2 international units/g protein for ODS-HEP). Hearts in the control group showed a steep rise in creatine kinase activity within the initial hour of reperfusion, which was significantly reduced by HEP or ODS-HEP treatment, consistent with the smaller infarct sizes in these groups (creatine kinase after 4 h reperfusion = 43.4 ± 3.7 for control; 27.6 ± 5.3 for HEP; and 21.9 ± 4.0 international units/g protein for ODS-HEP).

Despite their favorable effects on infarct size, HEP and ODS-HEP produced no significant changes in myocardial blood flow. Subendocardial blood flow in the ischemic-reperfused LAD coronary artery region was statistically comparable among the three groups at baseline (Fig. 2). Transmural blood flow in the AAR was significantly decreased during ischemia, with no group differences. All groups showed a comparable hyperemic response in the AAR at 15 min of reperfusion, after which blood flow was diminished to similar levels in all groups by 4 h. In the nonischemic-reperfused LCx coronary artery region, transmural blood flow was comparable in all groups throughout the protocol (data not shown).

Differences in infarct size were also not from hemodynamic or cardiodynamic differences. Hemodynamics at baseline and during ischemia and reperfusion were comparable among groups (data not shown). Heart rate was significantly increased during ischemia and reperfusion in all animals, and LV end-diastolic pressure was comparably elevated during ischemia in all three groups. After ischemia, hearts in all groups demonstrated dyskinesis in the AAR. All hearts showed poor recovery of percent systolic shortening throughout the 4 h of reperfusion (−6 ± 2% for control hearts; −7 ± 3% for HEP-treated hearts; −6 ± 4% for ODS-HEP-treated hearts at 4 h reperfusion), and diastolic stiffness (as measured by the unitless β-coefficient) increased following ischemia to comparable levels in all groups (from 0.2 ± 0.05 at baseline to 0.7 ± 0.1 units after 4 h reperfusion in control hearts; from 0.2 ± 0.04 at baseline to 1.0 ± 0.2 units after 4 h reperfusion in HEP-treated hearts; from 0.2 ± 0.04 at baseline to 0.5 ± 0.2 units after 4 h reperfusion in ODS-HEP-treated hearts).

**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
low and comparable among control, HEP, and ODS-HEP groups (16.6 ± 8, 18.6 ± 11, and 18.6 ± 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 ± 638 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\textsubscript{50}; –log [M]) was significantly greater for the control (–6.98 ± 0.06) compared with the HEP (–7.38 ± 0.06) or ODS-HEP (–7.17 ± 0.09) 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, 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).

ODS-HEP Prevents Activation of NF-kB

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-kB. NF-kB has recently been shown to be activated as a consequence of myocardial ischemia-reperfusion (22). To study whether HEP could inhibit activation of NF-kB, we studied the effect of nonanticoagulant ODS-HEP treatment of cultured HUVECs and perfused myocardium on NF-kB DNA-binding activity in nuclear protein. Figure 7 shows EMSAs in HUVECs stimulated with TNF-α. TNF-α stimulates endothelial DNA binding of NF-kB (Fig. 7, lane 2) compared with untreated controls (lane 1). Pretreatment with 200 µg/ml ODS-HEP eliminates NF-kB binding activity (Fig. 7, lane 3), indicating that ODS-HEP prevents activation of NF-kB. Interruption of endothelial NF-kB 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-kB 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-kB (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-kB oligonucleotide probe (Fig. 8B, lane 2). Supershift experiments identified complex I as the band containing the p65 component of NF-kB (Fig. 8A, lane 5). ODS-HEP treatment reduced ischemia-reperfusion-related stimulation of NF-kB 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-kB 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 $= 36,780 \pm 2,589$ for sham vs. $4,575 \pm 1,856$ for 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 $\beta$-blockers and angiotensin-converting enzyme inhibitors, no medical therapies exist for minimizing myocardial injury following reperfusion. 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.
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). HEP 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, exposing 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 neutrophilic influx into necrotic myocardium, and preserves endothelial vasodilator function within the ischemic-reperfused 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 anti-inflammatory 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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