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Effects of a semi-synthetic N-,O-sulfated glycosaminoglycan K5 polysaccharide derivative in a rat model of cerebral ischemia/reperfusion injury.

Massimo Collino[1], Sara Castiglia[1], Marco Manoni[2], Liana Salsini[2], Jacopo Chini[2], Emanuela Masini[3], Roberto Fantozzi[1]

1 Department of Anatomy, Pharmacology and Forensic Medicine, University of Turin, Turin, Italy

2 INALCO RSM S.p.A, Research Center, Montale, Pistoia, Italy

3 Department of Preclinical and Clinical Pharmacology, University of Florence, Florence, Italy.

Corresponding author:

Massimo Collino, Department of Anatomy, Pharmacology and Forensic Medicine, University of Turin, via P. Giuria 9, 10125 Torino, Italy. Tel: +39 011 6707955. Fax: +39 011 2367955. E-mail address: massimo.collino@unito.it

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The heparin-like derivative K5-N,OSepi prevents cerebral I/R injury

ABSTRACT

Heparin and low molecular weight heparins may reduce brain damage evoked by ischemia/reperfusion (I/R) injury, although their use is hampered by the risk of haemorrhage. Chemical and enzymatic modifications of K5 polysaccharide have shown the possibility to produce heparin-like compounds with low anticoagulant activity and strong anti-inflammatory effects. Using a rat model of transient cerebral I/R, we investigated the effects of an epimerised N-,O-sulfated K5 polysaccharide derivative, K5-N,OSepi, on the infarct size, motor activity and injury caused by ischemia (30 min) and reperfusion. Reperfusion was allowed for 60 min or 1-5 days. Rats reperfused for 5 days showed an infarct volume of 30.7±3.1% and K5-N,OSepi (0.1-1 mg/kg) caused dose-dependent reduction in infarct size (maximum at 1 mg/kg: 13.1±2.1% infarct volume). This effect was associated with a significant improvement in motor performance. In the rat hippocampus, one of the brain areas most sensitive to I/R injury, I/R induced a robust increase in myeloperoxidase (MPO) activity, a marker of neutrophil infiltration, that was halved by K5-N,OSepi administration (66.38±7.75 µU MPO/tissue g, 30.78±5.67 µU MPO/tissue g, respectively). K5-N,OSepi drastically reduced the expression of cyclooxygenase-2, inducible-nitric-oxide-synthase and intercellular-adhesion-molecule-1. I/R-induced activation of nuclear factor-kB was attenuated by drug treatment. Furthermore, K5-N,OSepi administration was associated with a significant modulation of apoptosis markers, such as Bid and Bcl-2. In conclusion, the results demonstrated that the sulfated semi-synthetic K5 derivative K5-N,OSepi protects the brain against I/R injury by disrupting multiple levels of the apoptotic and inflammatory cascade, including inhibition of NF-κB activation.

Keywords: heparin-like derivative, hippocampus, cerebral ischemia/reperfusion, inflammation

Introduction

Ischemic cerebrovascular diseases have a major impact on the public health of every nation. Cerebral ischemia is defined as a reduction in cerebral blood flow, sufficient to cause a metabolic or functional deficit. The characteristics of brain injury depend on the severity and the duration of cerebral blood flow (CBF) reduction. Although reperfusion following transient ischemia leads to restoration of CBF, there is compelling evidence to support the notion that reperfusion may exacerbate the injury initially caused by ischemia, producing a so-called "cerebral ischemia/reperfusion (I/R) injury". Inflammation and neutrophil infiltration significantly contribute to the tissue injury evoked by reperfusion of the ischemic organ (1). The release of pro-inflammatory cytokines, increased expression of endothelial adhesion molecules and chemotactic factors, activation of microglia, and infiltration of leukocytes have recently emerged as important determinants of post-ischemic inflammation, which contributes to the progression of brain damage (2). Therefore, interventions aimed at suppressing post-ischemic inflammatory reactions are an emerging therapeutic strategy for the treatment of cerebral I/R injury.

Heparin, low molecular weight heparins and heparin-like compounds have been widely employed in acute stroke and their ability to reduce brain injury after I/R has been clearly demonstrated. However, their potential for causing hemorrhage continues to arouse caution (3, 4). Although heparin's mechanism of action for brain protection has been thought to be dependent on its anticoagulant property, recent findings suggest that heparin's anti-inflammatory properties may play a more important role than its anticoagulant property (5-7). In recent years chemical and/or enzymatic modifications of K5 polysaccharide have shown the possibility to produce biotechnological heparin-like compounds of bacterial origin with defined and selected chemical-biological features, also designed as bioheparin (8). In particular, considerable efforts have been made to develop heparin derivatives with a low anticoagulant activity, while retaining strong anti-inflammatory effects. O-desulfated non-anticoagulant biotechnological heparins have been

demonstrated to inhibit translocation of the transcription nuclear factor-kappaB (NF-κB) from the cytoplasm to the nucleus in human endothelial cells (9) and to the production of interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α by LPS-stimulated mononuclear cells, with no effect on the anti-inflammatory cytokine IL-10 (10). In addition, non-anticoagulant heparin-like derivatives have been demonstrated to be effective in reducing organ injury in a few in vivo models of coronary, hepatic and renal injury (11-13). However, the effects of heparin-like semi-synthetic derivatives on cerebral I/R injury have not yet been investigated. Thus, this study was designed to investigate the effects of an epimerised N,O-sulfated K5 polysaccharide derivative, K5-N,OSepi, on infarct size, the degree of inflammation, and apoptosis caused by cerebral I/R in the rat. K5-N,OSepi was obtained by N-desacetylation/N-sulfation of K5 polysaccharide and epimerization of K5 with the enzyme glucuronyl C5 epimerase and O-sulfation (10). This compound has been previously demonstrated to be endowed with anti-inflammatory and anti-adhesive effects but it is devoid of any anti-coagulant activity (8, 10). In addition, we investigated whether the protective actions of K5-N,OSepi are partially mediated through the reduction of the inflammatory response associated with cerebral I/R injury.

Materials and Methods

Animals and surgery.

Male Wistar rats (Harlan-Italy; Udine, Italy) weighing 210 to 230 g were housed in a controlled environment at 25±2 °C with alternating 12-h light and dark cycles. They were provided with a Piccioni pellet diet (n.48, Gessate Milanese, Italy) and water *ad libitum*. All rats were acclimatised in our animal facility for at least 1 week prior to experiments and stressful stimuli were avoided. Animal care was in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (D.M. 116/92) as well as with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. The experimental protocol, approved by the Turin University Ethics Committee, was performed as described elsewhere (14, 15). Briefly, rats were anaesthetised with i.p. injection (30 mg/kg) of Zoletil 100 (mixture of tiletamine and zolazepam, Laboratoires Virbac, France), which was supplemented as needed. Anaesthetised rats were placed onto a thermostatically controlled heating pad, a rectal temperature probe was inserted and body temperature was monitored and maintained at 37 °C. Both common carotid arteries were exposed over a midline incision and a dissection was made between the sternocleidomastoid and the sternohyoid muscles, parallel to the trachea. Each carotid artery was freed from its adventitial sheath and vagus nerve, which was carefully separated and maintained. Ischemia was achieved by clamping the bilateral common carotid arteries for 30 min using non-traumatic artery clamps (Micro Bulldog Clamps, Harvard Apparatus Ltd., Kent, U.K.). During ischemia, the animals were monitored for body temperature, respiration pattern, loss of righting reflex, unresponsiveness, corneal reflexes, and fixed and dilated pupils. Recirculation of blood flow was established by releasing the clips and restoration of blood flow in the carotid arteries was confirmed by careful observation. Reperfusion was allowed for 1h, 24 h or 5 days. Post-surgery, the animals were kept for at least 3 h in a 37°C incubator to ensure that postoperative

recovery was satisfactory. Thereafter, they were group-housed under temperature- and light-controlled conditions with food and water *ad libitum*. At the end of the reperfusion, the anaesthetised rats were killed by decapitation after aortic exsanguination. Sham-operated rats underwent identical surgical procedures except that no artery clamps were applied. After decapitation, the forebrain was rapidly dissected at 0 °C and the whole hippocampus from both hemispheres was removed and transferred to an appropriate ice-chilled homogenising medium for biochemical assays.

Preparation of the N-, O-sulfated K5 polysaccharide.

The precursor of the compound K5-N,OSepi is the capsular K5 polysaccharide obtained from E.coli strain 010:K5:H4, a polymer with the structure [-4)-GlcA β1-4 GlcNAc-(1-]n in which the disaccharidic unit formed by D-glucuronic acid and N-acetylglucosamine is linked by a α 1→4 bond. This structure is alike to N-acetylheparosan, the natural precursory polymer of heparin and of heparan sulphate from mast cells. The purified K5 polysaccharide was prepared as described by Manzoni et al. (16). N, O-sulphated K5 polysaccharide was obtained by chemical N- and O-sulfation of the K5 polysaccharide followed by the enzymatic epimerization with the enzyme glucuronyl C5 epimerase according to the method described by Gori at al. (10). Briefly, the K5 polysaccharide was treated with 2M sodium hydroxide at 50°C for 18 h and, after neutralization, the solution was added with sodium carbonate and pyridine sulphur trioxide and maintained at 55°C for 6 h. The reaction product was treated with the enzyme glucuronyl C5 epimerase immobilized on a CNBr Sepharose 4B resin (Pharmacia, Uppsala, Sweden) in 25 mM Hepes buffer, pH 6,5 50 mM $CaCl_2$ at 37°C. The epimerized product was then purified by ultrafiltration, precipitated with ethanol and passed through a cation-exchange resin. The O-sulfation was performed by treating the product with tetrabutylamonium hydroxyde in N-N-dimetylformamide and with pyridine sulphur

trioxide at 50°C for 24 h. The product was finally depolymerised to the wanted molecular weight by controlled nitrous acid deamination as described by Horton and Philips (17).

Biochemical characterization of the K5 derivative.

The structural profile of the compound was characterized by nuclear magnetic resonance (1H-NMR and 13C-NMR) analysis; the sulfate content was obtained by sulphate/carboxyl ratio analysis and molecular weight determination was performed by HPLC analysis according to described methods (18). The compound used in these experiments had an average molecular weight of 6,000 Da, a sulphate/carboxyl ratio of 4.0 and an iduronic acid/glucuronic acid ratio of 0.8. K5-N,OSepi was also tested for their antithrombotic/anticoagulant activity, showing no anti-factor Xa activity.

Drug treatments.

Animals were randomly allocated into different groups: I/R groups (n = 8 per group); K5-N,OSepi groups (the drug was administered in the dose-range 0.1 – 1 mg/kg at beginning of reperfusion, after 6 h reperfusion and until day 5, n = 8 per group); Sham (the common carotid arteries were exposed but not occluded, n = 8). An additional group of rats received K5-N,OSepi (1 mg/kg i.v.) prior to the sham operation (n = 4). Ischemia lasted 30 min, while reperfusion was allowed for 1 hour, 24 hours or 5 days.

Determination of infarct volume.

At 1 or 5 days of reperfusion, the rats were killed with an overdose of Zoletil 100 (mixture of tiletamine and zolazepam) and decapitated. The rats' brains were immediately removed and placed in ice-cold saline for 5 min. Each brain was then placed in a brain matrix and coronal sections were

cut into 2-mm slices. Brain slices were immediately immersed in 2% 2,3,5-triphenyltetrazolium chloride monohydrate (TTC) solution (in saline) at 37 °C for 30 min, followed by 4% paraformaldehyde solution. The infarct area and hemisphere area of each section were traced and quantitated by an image analysis system (Inquiry; Loats, Westminster, MD, U.S.A.) and expressed as the percentage infarct area of the whole brain.

Physical performance test: Rota-rod (motor coordination).

An Omni rotor (Omnitech Electronics Inc., Columbus, Ohio) was used to evaluate the rats' motor coordination by testing the ability of mice to remain on a revolving rod. The rotor consisted of a rotating rod (75mm diameter), which was divided into four compartments, permitting testing of four rats at a time. The apparatus automatically recorded the time to 0.1 seconds when the rats fell off the rotating shaft. The speed was set at 10 rpm and the cut off time was 180 seconds.

Tissue extracts.

Cytosolic and nuclear extracts were prepared by the Meldrum method (19). Briefly, rat hippocampi were homogenised at 10% (w/v) in a Potter Elvehjem homogeniser (Wheaton, Millville, NJ, USA) using a homogenisation buffer containing 20 mM HEPES, pH 7.9, 1 mM MgCl2, 0.5 mM EDTA, 1% NP-40, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM Phenylmethyl Sulphonyl Fluoride (PMSF), 5 μg/ml aprotinin, 2.5 μg/ml leupeptin. Homogenates were centrifuged at 1,000 g for 5 min at 4°C. Supernatants were removed and centrifuged at 15,000 g at 4°C for 40 min to obtain the cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer containing 20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 300 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml aprotinin, 2.5 μg/ml leupeptin. Next, the suspensions were incubated on ice for 30 min for high-salt extraction followed by centrifugation at 15,000 g for 20

min at 4°C. The resulting supernatants containing nuclear proteins were carefully removed and the protein content was determined using a BCA protein assay following the manufacturers' instructions. Samples were stored at -80°C until use.

Western blot analysis.

About 15 µg total proteins were loaded. Proteins were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyldenedifluoride (PVDF) membrane, which was then incubated with SuperBlock blocking buffer. Membranes were incubated with primary antibody (rabbit anti-iNOS, rabbit anti-COX-2, rabbit anti-Bcl-2, rabbit anti-Bax, goat anti-Bid, goat anti-S100B, goat anti-ICAM-1, mouse anti-NF-κB p65). Blots were then incubated with a secondary antibody conjugated with horseradish peroxidase and developed with the ECL detection system. The immunoreactive bands were visualised by autoradiography and the density of the bands was evaluated densitometrically using Gel Pro®Analyser 4.5, 2000 software (Media Cybernetics, Silver Spring, MD, USA). The membranes were stripped and incubated with β-actin monoclonal antibody and subsequently with an anti-mouse antibody to assess gel-loading homogeneity.

Myeloperoxidase activity.

Myeloperoxidase (MPO) activity was used as an indicator of polymorphonuclear leukocytes infiltration into the hippocampus. At the specified reperfusion time, hippocampi were obtained and weighed and each piece homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H_2O_2. The rate of change in absorbance was measured

spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 µmol of peroxide per min at 37°C and was expressed in milliunits per gram of wet tissue.

Materials.

Unless otherwise stated, all compounds were purchased from the Sigma-Aldrich Company Ltd. (St. Louis, Missouri, USA). The K5 derivative K5-N,OSepi was kindly provided by INALCO RSM S.p.A (Montale, Pistoia, Italy). The BCA Protein Assay kit and SuperBlock blocking buffer were from Pierce Biotechnology Inc. (Rockford, IL, USA) and PVDF was from the Millipore Corporation (Bedford, Massachusetts, USA). Goat polyclonal antibody against ICAM-1 and S100B, horseradish peroxidase-conjugated donkey anti-goat IgG, mouse monoclonal antibody against NF-κB p65 and rabbit polyclonal antibodies against Bcl-2 and Bax were from Santa Cruz Biotechnology (Santa Cruz, California, USA). Rabbit polyclonal antibody against COX-2 was from the Cayman Chemical Company (Ann Arbor, MI, USA). The anti-mouse and anti-rabbit Ig horseradish peroxidase-linked whole antibodies and Luminol ECL detection reagents were from Amersham (Buckinghamshire, UK).

Statistical analysis.

All values in both the text and figures are expressed as mean ± standard error of the mean (S.E.M.) for n observations. One-way analysis of variance with Dunnett's post test was performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, California, USA) and p values below 0.05 were considered to be significant.

Results

Effect of K5-N,OSepi on cerebral infarction and motor performance.

In comparison with the brain sections obtained from sham-operated rats, those rats that had been reperfused for 5 days showed an infarct volume of 30.7±3.1%. As shown in Figure 1A, administration of K5-N,OSepi during reperfusion induced a dose-dependent reduction in the I/R-induced infarct volume in the range of 0.1-1 mg/kg, with a maximum effect at 1 mg/kg (13.1±2.1 %). The mean size of ischemic lesions in vehicle-treated animals was larger after 5 days of reperfusion than 24 h reperfusion (Figure 1B). Interestingly, the mean size of the ischemic area in the rats treated with K5-N,OSepi (1 mg/kg) was reduced by 30% when measured after 24 h reperfusion and almost 60% when measured at 5 days reperfusion. Consequently, 1 mg/kg, the most effective dose, was taken as the reference dose and endpoints were determined at 5 days of reperfusion in all subsequent experiments.

S100B, a calcium binding protein, which has been recognized as a marker of neuronal damage (20), was scantily detectable in the hippocampi of sham-operated animals. Animals subjected to cerebral I/R exhibited a two-fold increase in this protein marker when measured at 5 days reperfusion (Figure 1C). Treatment of animals with K5-N,OSepi almost completely prevented this rise in S100B levels, so that values of S100B measured in animals treated with K5-N,OSepi were similar to those measured in sham-operated animals.

Consistent with the results of cerebral infarction, treatment with K5-N,OSepi resulted in a significantly better motor performance at 5 days of reperfusion (Figure 2), when compared with I/R controls. The results obtained from the rota-rod showed a drastic impairment in motor activity in the I/R group over sham values. K5-N,OSepi administration evoked a significant improvement in balance and coordination compared to the I/R group.

Effects of K5-N,OSepi on neutrophil infiltration.

The improvement in the outcome of I/R injury was associated with a reduced neutrophil infiltration measured in reperfused hippocampi at 5 days of reperfusion. As shown in Figure 3A, cerebral I/R caused a robust increase in hippocampal MPO activity, a specific marker of local neutrophil activity, in comparison with sham-operated rats (66.38±7.75 µU MPO/tissue g, 12.04±4.42 µU MPO/tissue g, respectively, $p<0.05$). In K5-N,OSepi-treated animals, the MPO activity was halved (30.78±5.67 µU MPO/tissue g, $p<0.05$). The adhesion molecule ICAM-1, which is the endothelial ligand for the neutrophil receptor CD11b/CD18, was scarcely detectable in the hippocampus from sham-operated diabetic animals and its expression was strongly induced by 5 days of reperfusion (Figure 3B). Administration of K5-N,OSepi drastically reduced the increase in ICAM-1 expression afforded by transient cerebral I/R ($p<0.05$).

Administration of K5-N,OSepi to sham-operated rats had no significant effect on all the markers measured in the present study when compared to sham-operated rats only (data not shown).

K5-N,OSepi reduced the expression of inflammatory markers evoked by cerebral I/R.

Densitometric analysis of the autoradiograms detected low COX-2 and iNOS protein levels in the hippocampi obtained from sham-operated animals (Figure 4). Rats that had undergone transient cerebral ischemia followed by 5 days of reperfusion exhibited a significant ($p<0.05$) increase in the expression of COX-2 and iNOS (Figure 4A and 4B, respectively). Administration of K5-N,OSepi did not affect basal COX-2 and iNOS protein levels, but prevented the increase in expression caused by I/R, bringing the hippocampal protein levels of these inflammatory markers back to values similar to those measured in sham-operated animals.

K5-N,OSepi prevented the nuclear translocation of the NF-κB p65 subunit induced by cerebral I/R injury.

Western blot analyses were performed on rat hippocampi to elucidate whether NF-κB activation was involved in K5-N,OSepi protective mechanisms. Measurement of the nuclear translocation of the p65 subunit NF-κB from the cytosolic to the nuclear fraction of tissue extracts obtained from rats subjected to I/R injury showed higher levels of p65 subunit in the nucleus in comparison with sham-operated rats, thus suggesting NF-κB activation secondary to I/R (Figure 5). The rise in the nucleus/cytosol ratio of the NF-κB p65 subunit was observed at 1 h but not at 24 h of reperfusion (data not shown). K5-N,OSepi administration attenuated the rise in the nucleus/cytosol ratio hence, indicating reduced translocation of p65 to the nucleus ($p < 0.05$; Figure 5).

Effects of K5-N,OSepi on markers of apoptosis.

Using an antibody against the intact form of the pro-apoptotic protein Bid, Western blot analysis revealed that hippocampi obtained from rats subjected to I/R showed a significant reduction in Bid expression, when compared to sham-operated rats (Figure 6A), thus demonstrating its activation by cleavage of intact Bid into truncated forms of Bid. The administration of K5-N,OSepi prevented the I/R-induced activation of Bid, when compared to control rats. The basal expression of Bcl-2 protein, a well-known suppressor of apoptosis, was significantly reduced by I/R and this effect was abolished by exogenous K5-N,OSepi administration. On the contrary, the levels of the mitochondrial apoptotic protein Bax showed no significant quantitative differences among different groups, not being changed either by I/R or by K5-N,OSepi pre-treatment. Therefore, the Bcl-2-to-Bax ratio, which was calculated as an index of apoptosis signalling, was significantly reduced in animals subjected to I/R in comparison with sham-operated rats and this effect was attenuated by treatment with K5-N,OSepi (Figure 6B).

Discussion

The effects of heparin for patients with ischemic cerebrovascular diseases have been investigated in clinical trials, however, the efficacy and safety of heparin use in cerebral I/R injury remain debatable and depend on a balance between benefits, such as reduction of infarct volume, and risks, such as cerebral haemorrhage (3, 4). Thus, heparin-like derivatives without a bleeding tendency could represent an effective pharmacological strategy to reduce the brain damage induced by I/R. K5-N,OSepi is an epimerised N-desacetylated and N- and O-sulfated heparin-like molecule obtained by chemical and enzymatic modifications of the capsular K5 polysaccharide from Escherichia coli. This K5 derivative shows a limited anticoagulant/antithrombotic activity in comparison to heparin (8). This may be due to the influence of the O-sulfated glucuronic acid residues present in sulfated K5 derivatives but not in extractive heparin. On the other hand, the enzymatic modification of K5 by C5-epimerase produces a conformational change in the molecule and enables its anti-inflammatory activity, suggesting that epimerization is necessary for the anti-inflammatory activity of the O-sulfated molecules (10). The results here reported demonstrated that the heparin-like derivative K5-N,OSepi attenuates cerebral I/R injury in vivo, reducing infarct size. This protective effect is further confirmed by data on hippocampal expression of S100B, a member of the S100 family of calcium-binding proteins, which is mainly expressed in the brain (21). Clinical studies indicate that an increase in the levels of S100B correlates with impairment of hippocampal function as well as cerebral infarct size (20). In our study, K5-N,OSepi administration was able to reverse the significant increase in S100B protein level seen in rats that had undergone cerebral I/R injury. We also document a marked correlation between infarct size and impairment of motor coordination and, most notably, we show that the decrease in infarct size evoked by K5-N,OSepi was associated with improved motor coordination.

In search of the mechanism(s) underlying the protective action of K5-N,OSepi, we investigated whether K5-N,OSepi may affect the inflammatory response associated with cerebral I/R injury, as its pivotal role in the pathogenesis of ischaemic cerebrovascular diseases is well known (22). We focused our investigation on the hippocampus, one of the brain areas most sensitive to I/R injury (23). We recently observed (14) that 30 min ischemia followed by 1 h reperfusion causes significant oxidative stress, whereas the inflammatory response is partially delayed (by at least 24 h) in the rat hippocampus. During cerebral reperfusion, expression of adhesion molecules such as ICAM-1 in endothelial cells is a fundamental requirement for the recruitment of neutrophils into cerebral tissue (24). A number of reports have shown that inhibition of ICAM-1 expression during reperfusion is accompanied by a suppression of recruited leukocytes into the ischemic brain parenchyma (25-27). We have already reported that heparin and partially desulfated heparins reduce neutrophil adhesion to vascular endothelium (28). The present results demonstrate that administration of K5-N,OSepi can suppress the expression of ICAM-1 and, thus, significantly reduce neutrophil migration out of the vessels. The measurement of MPO activity was selected as a marker of tissue neutrophil infiltration and, in agreement with previous reports (29, 30), the results show that cerebral I/R evoked a strong increase in MPO activity. The elevated activity of MPO was inhibited by K5-N,OSepi treatment. Therefore, we can speculate that the decreased cerebral neutrophil infiltration may have significantly contributed to the reduced cerebral injury after 5 days reperfusion in rats treated with K5-N,OSepi. Two important enzymes involved in the ischemic inflammatory cascade, iNOS and COX-2, were up-regulated by I/R and, most notably, K5-N,OSepi administration protected the rat hippocampus from I/R-induced iNOS and COX-2 over-expression. The current findings are in keeping with a recent paper showing that an O-sulphated heparin-like K5 derivative blunted inflammatory markers, including the generation of prostaglandins and cytokines, in a rat model of carrageenan-induced pleurisy (31). In the present study, the reduction of the inflammatory response evoked by K5-N,OSepi at 24 h reperfusion was associated with the early inhibition of the proinflammatory nuclear transcription factor NF-κB measured at 1 h reperfusion. Experimental

evidence indicates that NF-κB plays a fundamental role in the development of I/R injury (32) and we and others have recently reported NF-κB to be activated in the hippocampi of rats undergoing I/R (33, 34). Here, we show that K5-N,OSepi attenuates the nuclear translocation of NF-κB p65, which may account for the observed reduction in the expression of COX-2, iNOS and ICAM-1, all of which are NF-κB-dependent proteins. As previously suggested (9, 35), these results are consistent with the possibility that nonanticoagualnt heparin-like molecules may bind electrostatically to cell membranes of different cells and internalize into the cytosolic compartment, thus preventing translocation of NF-κB from cytoplasm to the nucleus. Therefore, the observed reduction of inflammatory responses and the improvement of cerebral injury after K5-N,OSepi treatment are due, at least in part, to the reduction of NF-κB activation. However, further study is required to determine the mechanism(s) more precisely.

Another important factor that contributes to the development of I/R-induced cerebral injury is apoptosis. In response to oxidative load in the mitochondria, the outer membrane of mitochondria becomes permeabilized, resulting in the translocation of Bax from cytosol to the mitochondria and the release of cytochrome c normally confined to the mitochondrial intermembrane space. The translocation of those proapoptotic proteins is controlled by the Bcl-2 family proteins (36). In examining the expression of key apoptotic related molecules in the hippocampi of rats at 5 days of reperfusion, we found that Bid (a pro-apoptotic marker) is activated during cerebral I/R injury and is attenuated with K5-N,OSepi administration. Activation of Bid depends on its proteolytic processing into truncated forms of tBid. Bid activation has been shown both in vivo following cerebral I/R and in vitro in primary cultured mouse neurons (37), whereas deletion of this molecule by gene targeting has been demonstrated to provide significant neuroprotection (38). We also determined the effects of K5-N,OSepi administration on the ratio of Bcl-2-to-Bax expression, a reliable index of apoptotic signalling. Bcl-2 acts as an antiapoptotic regulator by preventing or delaying the release of cytochrome c, perhaps through its ability to influence the creation,

maintenance, and function of mitochondrial membrane channels (39). In contrast, Bax (a 21-kDa protein coimmunoprecipitated with Bcl-2) is a promoter of cell death, and its pro-apoptotic function is directly antagonised by Bcl-2 through formation of the Bcl-2/Bax heterodimer (40). Overexpression of Bcl-2 has been demonstrated to lessen the impact of various neurological insults, including I/R injury (41) and several reports have shown a decreased Bcl-2-to-Bax ratio after I/R injury (42). Our results confirmed that cerebral I/R injury caused the suppression of Bcl-2 without associated change in Bax expression, resulting in a decreased Bcl-2-to-Bax ratio to 0.37-fold of the control. Most notably, we observed a significant increase in Bcl-2-to-Bax ratio in the hippocampi of K5-N,OSepi-treated rats, indicating the antiapoptotic capacity of the heparin derivative after cerebral I/R injury.

In conclusion, our results show that post-ischemic administration of K5-N,OSepi attenuates cerebral I/R injury. This protective effect can be attributed, at least in part, to its ability to inhibit neutrophil infiltration and suppress the inflammatory response by preventing activation of the proinflammatory transcription factor NF-κB. The mitochondrial apoptotic pathway may represent a further potential target for heparin-like compounds. Overall, our results provide a new understanding of the effects of new heparin derivatives, with low anticoagulant and high anti-inflammatory activities, in post-ischemic injury and may offer a potential new therapeutic strategy for acute brain injury. However, further investigation is warranted for a better assessment of the pharmacodynamics and safety of a therapy with heparin-like derivatives in conditions associated with cerebral I/R injury.

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REFERENCES

1. Mehta SL, Manhas N, Raghubir R. Molecular targets in cerebral ischemia for developing novel therapeutics. Brain Res Rev 2007 Apr;54(1):34-66.
2. Frangogiannis NG. Chemokines in ischemia and reperfusion. Thromb Haemost 2007 May;97(5):738-47.
3. Sandercock P. Immediate anticoagulation for acute stroke in atrial fibrillation: no. Stroke 2006 Dec;37(12):3054-5.
4. Bousser MG. Antithrombotic strategy in stroke. Thromb Haemost 2001 Jul;86(1):1-7.
5. Floris S, van den Born J, van der Pol SM, et al. Heparan sulfate proteoglycans modulate monocyte migration across cerebral endothelium. J Neuropathol Exp Neurol 2003 Jul;62(7):780-90.
6. Attanasio M, Gori AM, Giusti B, et al. Cytokine gene expression in human LPS- and IFNgamma-stimulated mononuclear cells is inhibited by heparin. Thromb Haemost 1998 May;79(5):959-62.
7. Hochart H, Jenkins PV, Preston RJ, et al. Concentration-dependent roles for heparin in modifying lipopolysaccharide-induced activation of mononuclear cells in whole blood. Thromb Haemost 2008 Mar;99(3):570-5.
8. Rusnati M, Oreste P, Zoppetti G, et al. Biotechnological engineering of heparin/heparan sulphate: a novel area of multi-target drug discovery. Curr Pharm Des 2005;11(19):2489-99.
9. Thourani VH, Brar SS, Kennedy TP, et al. Nonanticoagulant heparin inhibits NF-kappaB activation and attenuates myocardial reperfusion injury. Am J Physiol Heart Circ Physiol 2000 Jun;278(6):H2084-93.
10. Gori AM, Attanasio M, Gazzini A, et al. Cytokine gene expression and production by human LPS-stimulated mononuclear cells are inhibited by sulfated heparin-like semi-synthetic derivatives. J Thromb Haemost 2004 Sep;2(9):1657-62.
11. Wan JG, Mu JS, Zhu HS, et al. N-desulfated non-anticoagulant heparin inhibits leukocyte adhesion and transmigration in vitro and attenuates acute peritonitis and ischemia and reperfusion injury in vivo. Inflamm Res 2002 Sep;51(9):435-43.
12. Kouretas PC, Kim YD, Cahill PA, et al. Nonanticoagulant heparin prevents coronary endothelial dysfunction after brief ischemia-reperfusion injury in the dog. Circulation 1999 Mar 2;99(8):1062-8.
13. Zhou T, Chen JL, Song W, et al. Effect of N-desulfated heparin on hepatic/renal ischemia reperfusion injury in rats. World J Gastroenterol 2002 Oct;8(5):897-900.
14. Collino M, Aragno M, Mastrocola R, et al. Oxidative stress and inflammatory response evoked by transient cerebral ischemia/reperfusion: effects of the PPAR-alpha agonist WY14643. Free Radic Biol Med 2006 Aug 15;41(4):579-89.
15. Collino M, Aragno M, Castiglia S, et al. Insulin reduces cerebral ischemia/reperfusion injury in the hippocampus of diabetic rats: a role for glycogen synthase kinase-3{beta}. Diabetes 2008 Oct 7.
16. Manzoni M. BS, Cavazzoni V. Extracellular K5 polysaccharide of Escherichia coli: production and characterization. Journal of Bioactive and compatible polymers 1993;8(3):251-7.
17. Horton DP, K.D. . The nitrous acid deamination of glycosides and acetates of 2-amino-2-deoxy-. D. -glucose. . Carbohydr Res 1973;30:367-73.
18. Guerrini M, Bisio A, Torri G. Combined quantitative (1)H and (13)C nuclear magnetic resonance spectroscopy for characterization of heparin preparations. Semin Thromb Hemost 2001 Oct;27(5):473-82.
19. Meldrum DR, Shenkar R, Sheridan BC, et al. Hemorrhage activates myocardial NFkappaB and increases TNF-alpha in the heart. J Mol Cell Cardiol 1997 Oct;29(10):2849-54.
20. Heizmann CW, Fritz G, Schafer BW. S100 proteins: structure, functions and pathology. Front Biosci 2002 May 1;7:d1356-68.
21. Foerch C, Singer OC, Neumann-Haefelin T, et al. Evaluation of serum S100B as a surrogate marker for long-term outcome and infarct volume in acute middle cerebral artery infarction. Arch Neurol 2005 Jul;62(7):1130-4.
22. del Zoppo GJ. Stroke and neurovascular protection. N Engl J Med 2006 Feb 9;354(6):553-5.
23. Sharma BK, Kumar K. Role of proinflammatory cytokines in cerebral ischemia: a review. Metab Brain Dis 1998 Mar;13(1):1-8.

24. Frijns CJ, Kappelle LJ. Inflammatory cell adhesion molecules in ischemic cerebrovascular disease. Stroke 2002 Aug;33(8):2115-22.
25. Liu SJ, Zhou SW, Xue CS. Effect of tetrandrine on neutrophilic recruitment response to brain ischemia/reperfusion. Acta Pharmacol Sin 2001 Nov;22(11):971-5.
26. Storini C, Rossi E, Marrella V, et al. C1-inhibitor protects against brain ischemia-reperfusion injury via inhibition of cell recruitment and inflammation. Neurobiol Dis 2005 Jun-Jul;19(1-2):10-7.
27. Wang YH, Wang WY, Chang CC, et al. Taxifolin ameliorates cerebral ischemia-reperfusion injury in rats through its anti-oxidative effect and modulation of NF-kappa B activation. J Biomed Sci 2006 Jan;13(1):127-41.
28. Silvestro L, Viano I, Macario M, et al. Effects of heparin and its desulfated derivatives on leukocyte-endothelial adhesion. Semin Thromb Hemost 1994;20(3):254-8.
29. Suzuki Y, Takagi Y, Kawano K, et al. A novel guinea pig model with cyclic flow reductions following thrombotic cerebral ischemia. Brain Res Brain Res Protoc 2002 Oct;10(2):55-9.
30. Stevens SL, Bao J, Hollis J, et al. The use of flow cytometry to evaluate temporal changes in inflammatory cells following focal cerebral ischemia in mice. Brain Res 2002 Apr 5;932(1-2):110-9.
31. Ceccarelli M, Bani D, Cinci L, et al. Anti-Inflammatory Effects of Low Molecular Weight Heparin Derivative in a Rat Model of Carrageenan-Induced Pleurisy. J Cell Mol Med 2009 Jan 16.
32. Nichols TC. NF-kappaB and reperfusion injury. Drug News Perspect 2004 Mar;17(2):99-104.
33. Shen WH, Zhang CY, Zhang GY. Antioxidants attenuate reperfusion injury after global brain ischemia through inhibiting nuclear factor-kappa B activity in rats. Acta Pharmacol Sin 2003 Nov;24(11):1125-30.
34. Collino M, Thiemermann C, Mastrocola R, et al. Treatment with the glycogen synthase kinase-3beta inhibitor, TDZD-8, affects transient cerebral ischemia/reperfusion injury in the rat hippocampus. Shock 2008 Sep;30(3):299-307.
35. Hochart H, Jenkins PV, Smith OP, et al. Low-molecular weight and unfractionated heparins induce a downregulation of inflammation: decreased levels of proinflammatory cytokines and nuclear factor-kappaB in LPS-stimulated human monocytes. Br J Haematol 2006 Apr;133(1):62-7.
36. Antonsson B, Conti F, Ciavatta A, et al. Inhibition of Bax channel-forming activity by Bcl-2. Science 1997 Jul 18;277(5324):370-2.
37. Plesnila N, Zinkel S, Le DA, et al. BID mediates neuronal cell death after oxygen/ glucose deprivation and focal cerebral ischemia. Proc Natl Acad Sci U S A 2001 Dec 18;98(26):15318-23.
38. Yin XM, Luo Y, Cao G, et al. Bid-mediated mitochondrial pathway is critical to ischemic neuronal apoptosis and focal cerebral ischemia. J Biol Chem 2002 Nov 1;277(44):42074-81.
39. Lam M, Bhat MB, Nunez G, et al. Regulation of Bcl-xl channel activity by calcium. J Biol Chem 1998 Jul 10;273(28):17307-10.
40. Sato T, Hanada M, Bodrug S, et al. Interactions among members of the Bcl-2 protein family analyzed with a yeast two-hybrid system. Proc Natl Acad Sci U S A 1994 Sep 27;91(20):9238-42.
41. Zhao H, Yenari MA, Cheng D, et al. Bcl-2 overexpression protects against neuron loss within the ischemic margin following experimental stroke and inhibits cytochrome c translocation and caspase-3 activity. J Neurochem 2003 May;85(4):1026-36.
42. Brambrink AM, Schneider A, Noga H, et al. Tolerance-Inducing dose of 3-nitropropionic acid modulates bcl-2 and bax balance in the rat brain: a potential mechanism of chemical preconditioning. J Cereb Blood Flow Metab 2000 Oct;20(10):1425-36.

Figure legends

Figure 1. Effects of K5-N,OSepi administration on infarct volume and hippocampal S100B expression. Dose-response effects of K5-N,OSepi against infarct volume induced by 30 min of cerebral ischemia followed by 5 days of reperfusion (Panel A). Effects evoked by K5-N,OSepi treatment (1 mg/kg) on cerebral infarct volume were evaluated at both 24 h and 5 days reperfusion (Panel B). Data are means ± S.E.M. of four animals/group. Panel C shows the effects of K5-N,OSepi (1 mg/kg) on S100B protein expression, measured subsequent to I/R (30 min/5 days) on fresh cytosolic fractions of hippocampus. The immunoblot of S100B protein expression and the corresponding β-actin are representative of three separate experiments. ★ $p < 0.05$ versus I/R.

Figure 2. Effects of K5-N,OSepi administration on motor performance measured by Rota-rod Test at 5 days reperfusion. Rats that received K5-N,OSepi (1 mg/kg) during reperfusion (I/R+K5-N,OSepi) showed better motor coordination than rats that underwent ischemia and reperfusion only (I/R). Data are means ± S.E.M. of five animals/group. ★ $p < 0.05$ versus I/R.

Figure 3. K5-N,OSepi prevents I/R-induced neutrophil infiltration in the rat hippocampus. Myeloperoxidase (MPO) activity (Panel A) and ICAM-1 expression (Panel B) were measured in hippocampi homogenates of sham-operated rats (Sham) and rats that underwent 30 min ischemia and 5 days reperfusion (I/R). K5-N,OSepi (1 mg/kg) was administered during reperfusion (I/R+ K5-N,OSepi). Each immunoblot is from a single experiment and is representative of three separate experiments. Densitometric analysis of the bands is expressed as relative optical density (O.D.), corrected for the corresponding β-actin contents and normalized using the related sham-operated band. Data are means ± S.E.M. of three separate experiments for Western Blot and four animals/group for MPO. ★ $p < 0.05$ versus I/R.

Figure 4. Alterations in hippocampal expression of COX-2 (Panel A) and iNOS (Panel B) induced by K5-N,OSepi administration. Rats were subjected to 30 min ischemia and 5 days reperfusion (I/R). K5-N,OSepi (1 mg/kg) was administered during reperfusion (I/R + K5-N,OSepi). Each immunoblot is from a single experiment and is representative of three separate experiments. Densitometric analysis of the bands is expressed as relative optical density (O.D.), corrected for the corresponding β-actin contents and normalized using the related sham-operated band. Data are means ± S.E.M. of three separate experiments. ★ $p < 0.05$ versus I/R.

Figure 5. K5-N,OSepi prevents the nuclear translocation of p65 NF-κB evoked by cerebral I/R injury. NF-κB translocation from the cytosol to the nucleus was evaluated in the hippocampus of rats subjected to the surgical procedure alone (Sham) or rats subjected to 30 min ischemia and 1 h reperfusion and treated with vehicle (I/R) or K5-N,OSepi (1 mg/kg, I/R + K5-N,OSepi). NF-κB p65 subunit levels were measured in both cytosol and nuclear fractions and the results are expressed as nucleus/cytosol ratio. Densitometric analysis is expressed as relative optical density (O.D.) of the bands, corrected for the corresponding β-actin contents and normalized using the related sham-operated band. Data are means ± S.E.M. of three separate experiments. ★ $p < 0.05$ versus I/R.

Figure 6. Effects of K5-N,OSepi on expression of apoptosis markers in the hippocampus of rats that underwent I/R injury. Representative Western blot and corresponding densitometric analysis of the bands showing expression of Bid (Panel A), Bcl-2 and Bax (Panel B) at 5 days reperfusion in the presence or absence of K5-N,OSepi treatment (1 mg/kg). The reduction in Bid expression as well as Bcl-2 to Bax ratio evoked by I/R was completely reversed by K5-N,OSepi. Each immunoblot is from a single experiment and is representative of three separate experiments. The data from bands densitometric analysis are means ± S.E.M. of three separate experiments. ★ $p < 0.05$ versus I/R.

Extra table

What is known on this topic:

- ✓ Ischemic cerebrovascular diseases have a major impact on the public health of every nation and, despite advances in understanding their pathophysiology, little progress has been made in their pharmacological treatment.
- ✓ The beneficial effects of heparin and low molecular weight heparins against cerebral ischemia/reperfusion injury are hampered by their potential for causing hemorrhage.
- ✓ Heparin-like molecules, endowed with anti-inflammatory effects but devoid of anti-coagulant activities, have been recently developed.

What this paper adds:

- ✓ The heparin-like K5 polysaccharide derivative, K5-N,OSepi, protects the brain against I/R injury, reducing infarct size and improving motor performance *in vivo* in the rat.
- ✓ K5-N,OSepi exerts protective effects when administered after the onset of ischemia, thus confirming its potential efficacy as therapeutic treatment.
- ✓ Semi-synthetic, low molecular weight heparin derivatives, endowed with anti-inflammatory activity but lacking of substantial effects on the coagulation system, may represent a potential new therapeutic strategy to acute cerebral I/R injury.

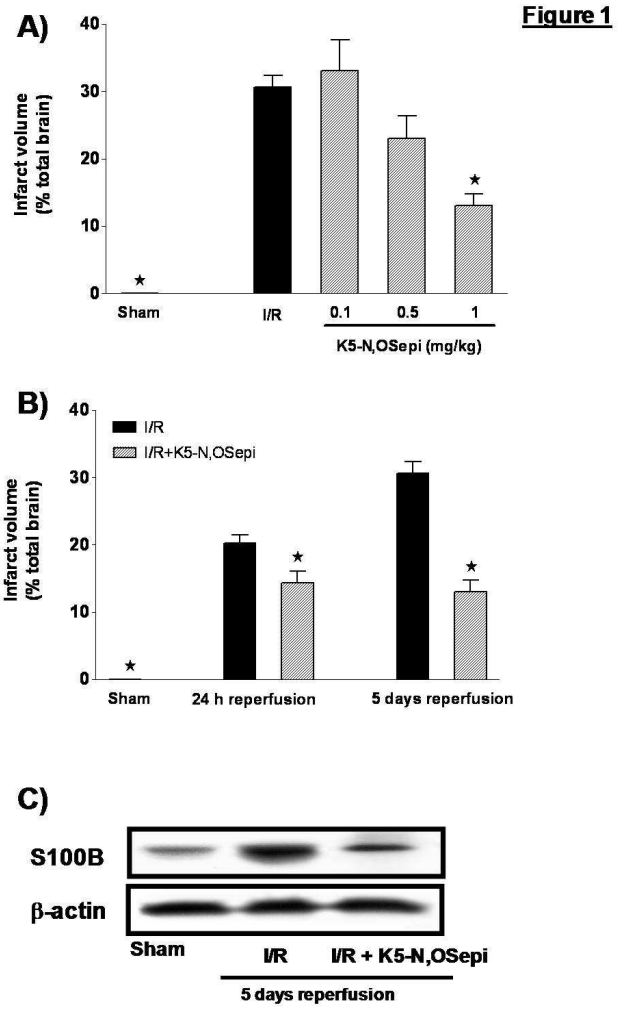

Figure 1

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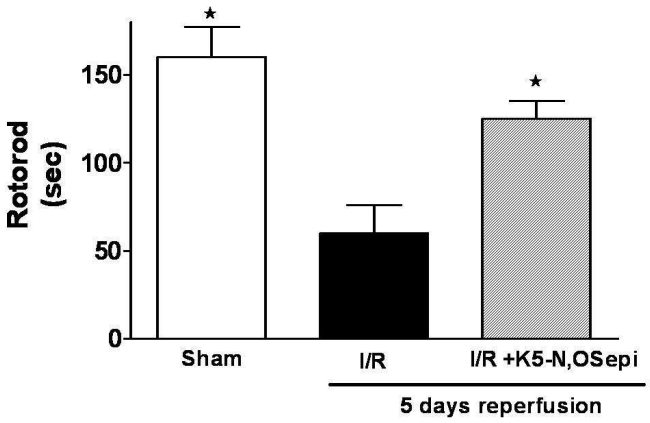

Figure 2

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Figure 3

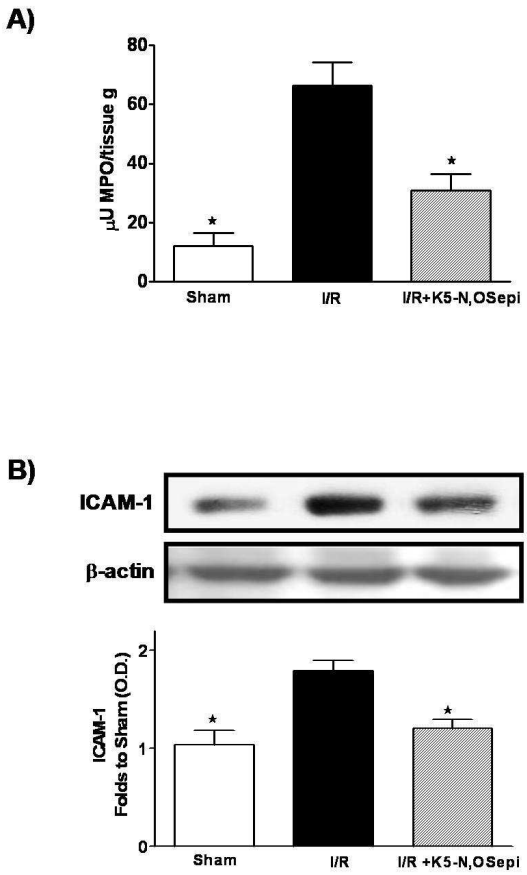

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Figure 4

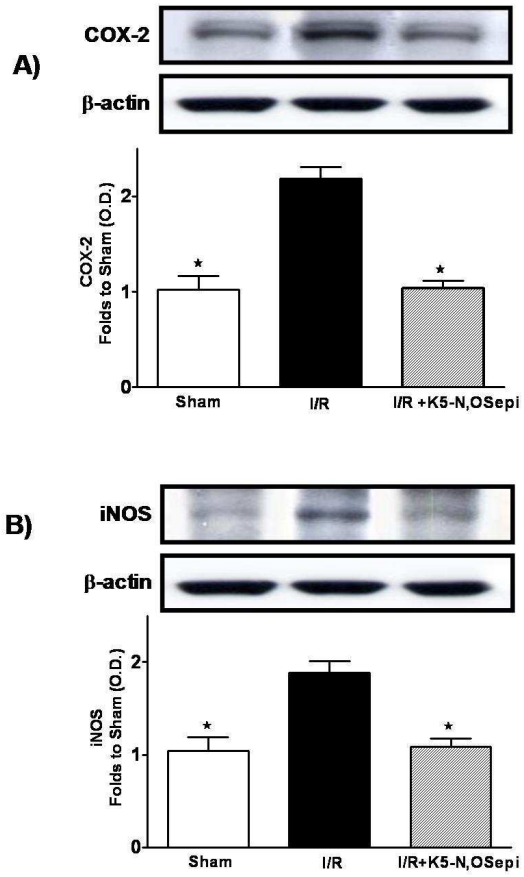

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Figure 5

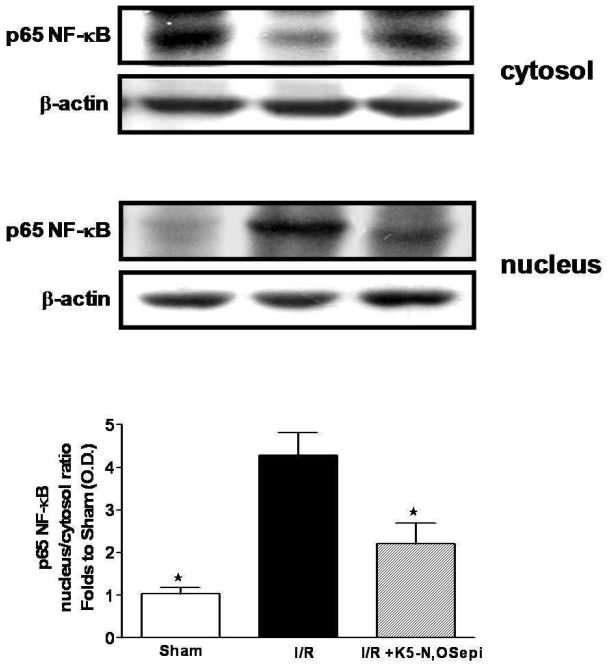

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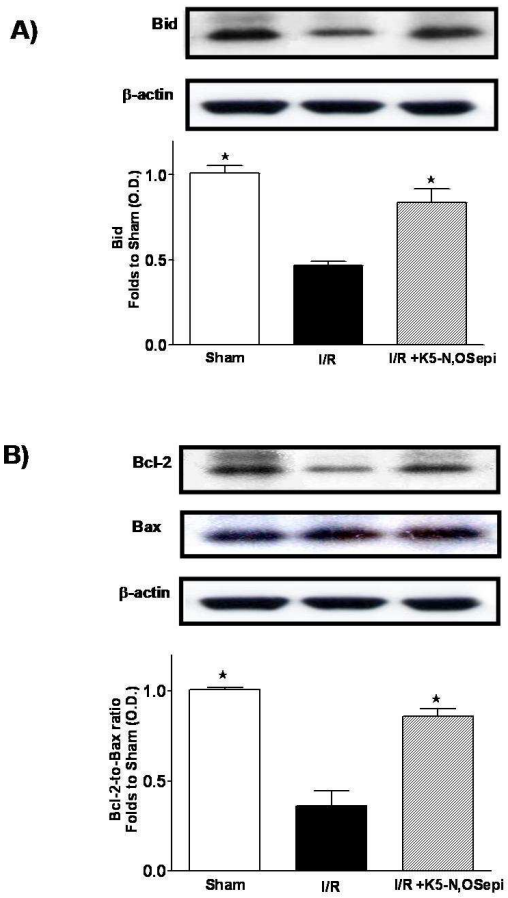
Figure 6



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