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Anti-inflammatory effects of low molecular weight heparin derivative in a rat model of carrageenan-induced pleurisy

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Summary

Low molecular weight heparin derivatives are characterized by low anti-coagulant activity and marked anti-inflammatory effects that allow for these molecules to be viewed as a new class of non-steroidal anti-inflammatory drugs (NSAIDs). We show here that K5NOSepiLMW, an O-sulphated heparin-like semi-synthetic polymer of the D-glucuronic acid–N-acetyleparoson disaccharide unit with low molecular weight, has marked anti-inflammatory effects in a rat model of acute inflammation, the carrageenan-induced pleurisy, commonly used to test NSAID efficacy. A 30-min. pre-treatment with K5NOSepiLMW (0.1, 0.5 and 1 mg/kg b.wt., given intrapleurally) attenuated the recruitment of leucocytes in the lung tissue and the pleural exudate, inhibited the induction of inducible nitric oxide synthase and cyclooxygenase-2 (COX-2), thereby abating the generation of nitric oxide and pro-inflammatory prostaglandins such as PgE_2 and $PGF_{1\alpha}$, reduced the inflammation-induced nitroxidative lung tissue injury, as shown by tissue thiobarbituric acid-reactive substances and nitrotyrosine, and blunted the local generation of cytokines such as interleukin-1β and tumour necrosis factor-α. All these parameters were markedly increased by intrapleural carrageenan in the absence of any pre-treatment. The anti-inflammatory action of K5NOSepiLMW is specific, as judged by the lack of therapeutic effects of B4/110, a biologically inactive cognate polysaccharide, given in the place of the authentic molecule. Moreover, K5NOSepiLMW showed similar effects as celecoxib (1 mg/kg b.wt), a COX-2 inhibitor and well-known NSAID. This study provides further insight into the mechanisms underlying the beneficial effects of heparin derivatives in inflammation and identifies K5NOSepiLMW as a novel, promising anti-inflammatory drug.

Keywords: low molecular weight heparin • nitric oxide synthase • cyclooxygenase-2 • leucocytes • cytokines • pleurisy

Introduction

The major hallmark of inflammation is the recruitment and activation of leucocytes, especially polymorphonuclear neutrophils (PMNs), which results from a complex interplay among these cells and the vascular endothelium [1–3] and accounts for destruction of foreign pathogens on one hand, and breakdown and remodelling of inflamed tissues on the other hand [4]. Inflammation is triggered and sustained by pro-inflammatory cytokines as well as prostaglandins and gaseous radicals, such as nitric oxide [4–6], which are produced locally by the inflammation-induced enzymes cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS). Most of the classical anti-inflammatory drugs target the endogenous production of these pro-inflammatory mediators.

In this context, the sulphated proteoglycan heparin can be also regarded as a non-conventional anti-inflammatory molecule, as it has several biological actions independent of its well-known anti-coagulant activity, including the modulation of extracellular matrix synthesis, cell proliferation, angiogenesis and inflammation [7–11]. In particular, heparin has been shown to inhibit pro-inflammatory cytokine production by human monocytes stimulated

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by lipopolysaccharide or interferon-γ [12]. On these grounds, in recent years, considerable efforts have been made to identify the molecular domains of heparin responsible for its anti-inflammatory properties and to develop synthetic derivatives with low or no anti-coagulant properties and clear-cut anti-inflammatory effects [13]. In animal models of inflammation, such as acute peritonitis and organ-specific ischemia reperfusion, these anti-inflammatory heparin-like molecules effectively inhibited leucocyte adhesion and migration [13].

We have recently studied the anti-inflammatory properties of K5 polysaccharide obtained from *Escherichia coli* strain 010:K5:H4. This is a polymer of the D-glucuronic acid and N-acetyleparoson disaccharide unit, the precursor of heparin and heparan sulphate. This structural affinity makes the K5 polysaccharide an optimal precursor for the synthesis of sulphated heparin semi-synthetic derivatives. In particular, the low molecular weight compound K5-N, OS epi (K5NOSepiLMW) lacks appreciable anti-thrombotic activity and is able to inhibit the production of pro-inflammatory cytokines from human lipopolysaccharide-stimulated mononuclear cells, without influencing other anti-inflammatory cytokines such as interleukin (IL)-10 [14]. The aim of the present study is to evaluate the effects of K5NOSepiLMW in a widely used *in vivo* model of acute inflammation in the rat, namely carrageenan-induced pleurisy, whose cellular and molecular mechanisms are well characterized [15–21].

Materials and methods

Materials

Primary rabbit polyclonal anti-nitrotyrosine (NT) antiserum, biotin-conjugated goat anti-rabbit IgG, and avidin-biotin peroxidase complex were obtained from Upstate Biotech (DBA, Milan, Italy). Rabbit polyclonal anti-COX-2 and anti-iNOS antisera were from Cayman Chemicals (Ann Arbor, MI, USA). Celecoxib was from LC Laboratories (Woburn, MA, USA). Unless otherwise stated, all other reagents and compounds used were obtained from Sigma (Milan, Italy). The semi-synthetic low molecular weight heparin polysaccharide K5-N,OSepi (K5NOSepiLMW) was provided by INALCO RSM Research Center, Montale, Italy, as reported previously [14].

Carrageenan-induced pleurisy and drug treatments

Male Wistar albino rats (Charles River, Milan, Italy), weighing 250–300 g, were housed in a controlled environment and provided with standard rodent chow and water. The experimental protocols were designed in compliance with the Italian and the European Community regulations on animal experimentation for scientific purposes (D.M. 116192; O.J. of E.C. L358/1 12/18/1986).

Rats were anaesthetized with sodium pentobarbital (Abbott, Latina, Italy; 50 mg/kg b.wt., intraperitoneally) and submitted to a skin incision at the sixth left intercostal space to access the pleural cavity. The animals were then treated as described below.

Briefly, 48 rats were divided in seven groups, eight animals each:

(1) Sham-operated control animals, injected intrapleurally with 0.2 ml sterile saline;
(2) Carrageenan-treated animals, injected intrapleurally with λ-carrageenan, 1% w/v in 0.2 ml sterile saline;
(3)–(5) Animals pre-treated with K5NOSepiLMW, given intrapleurally at the doses of 0.1, 0.5 or 1 mg/kg b.wt. 30 min. before carrageenan;
(6) Animals treated with B4/110, a biologically inactive non-sulphated polysaccharide compound used as negative control molecule, given intrapleurally at the dose of 1 mg/kg b.wt. 30 min. before carrageenan;
(7) Animals treated with celecoxib, a COX-2 inhibitor used as reference anti-inflammatory drug, given intrapleurally at the dose of 1 mg/kg b.wt. 30 min. before carrageenan. This dose was consistent with that required to abrogate COX-2 activity [19].

After the injections, the skin incision was closed with a suture. Four hours later, the animals were killed with a lethal dose of sodium pentobarbital. The chest was carefully opened and the pleural cavity was rinsed with 2 ml saline containing heparin (5 IU/ml) and indomethacin (10 μg/ml). The total volume of exudate and washing solution was collected. The exudates contaminated with blood were discarded. The amount of exudate was calculated by subtracting the volume injected (2 ml) from the total volume recovered. Leucocytes in the exudate were stained with the vital dye Trypan Blue and counted with a Bürker chamber. Lung tissue specimens were also collected and immediately processed as described below.

Evaluation of iNOS and COX-2 expression by Western blot analysis

Lung tissue fragments were quickly minced and homogenized in 500 μl cold lysis buffer containing 10 mmol/l Tris/HCl pH 7.4, 10 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, 2 mmol/l Na_2ethylenediaminetetraacetic acid (EDTA), 1% v/v Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mg/ml pefabloc and 2.5 μg/ml aprotinin. Upon centrifugation at 13,000 × *g* at 4°C for 10 min., the supernatants were collected and the total protein content was measured spectrophotometrically using micro BCA Protein Assay Reagent Kit (Pierce, IL, USA). The samples, each containing 100 μg of total proteins, were electrophoresed by SDS-PAGE (200 V, 1 hr) using a denaturating 7.6% v/v polyacrylamide gel and blotted onto nitrocellulose membranes (Amersham, Buckinghamshire, UK) (150 V, 1 hr). After thorough washings in PBS containing 0.1% v/v Tween (T-PBS), the membranes were blocked with T-PBS containing 5% w/v bovine serum albumin at room temperature for 1 hr and incubated overnight at 4°C with rabbit polyclonal antisera against iNOS and COX-2, both diluted 1:1000 in T-PBS containing 1% w/v bovine serum albumin. After washing in T-PBS the membranes were incubated with peroxidase-labelled goat anti-rabbit antibody (Vector, Burlingame, CA, USA), diluted 1:10,000 in T-PBS containing 1% w/v bovine serum albumin, for 1 hr at room temperature. Immune reaction was revealed by incubation with the enhanced chemiluminescent (ECC) substrate (Amersham) followed by exposure to high sensitivity photographic film (Biomax ML, Kodak, Rochester, NY, USA). As internal control, membranes immunostained with rabbit polyclonal anti β-actin antibody (1:20,000) were used. For iNOS and COX-2, quantitative evaluations of the band intensities were performed by computer-assisted densitometry,

using the Scion Image Beta 4.0.2 image analysis program (Scion, Frederick, MD, USA). Each band was then normalized to that of actin.

Determination of nitric oxide synthase activity

The calcium-independent conversion of L-arginine to L-citrulline was assumed as iNOS activity, according to Cuzzocrea *et al.* [21]. Lung tissue specimens were homogenized in a buffer composed of: 50 mmol/l Tris-HCl, 0.1 mmol/l EDTA, and 1 mmol/l phenylmethylsulfonyl fluoride, pH 7.4, and then used for the L-[^{3}H]arginine to L-[^{3}H]citrulline conversion assay. Briefly, 50 μl of each sample were incubated in the presence of 10 μmol/l L-[^{3}H]arginine (5 kBq per tube), 1 mmol/l $NADPH^+$, 30 nmol/l calmodulin, 5 μmol/l tetrahydrobiopterin, and 2 mmol/l ethylene glycol tetro-acetic acid (EGTA) for 20 min. at 22°C. The reaction was stopped by 0.5 ml ice-cold HEPES buffer, pH 5.5, containing 2 mmol/l EGTA and 2 mmol/l EDTA. Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted L-[^{3}H]citrulline activity was measured by a scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA).

Measurement of nitrites

Total nitrites, the stable end products of nitric oxide, were measured in the exudate as described previously [18]. Briefly, nitrates in the sample were first reduced to nitrites by incubation with 280 mU/ml nitrate reductase and 160 μmol/l $NADPH^+$ at room temperature for 3 hrs. The total nitrite concentration in the samples was then measured using the Griess reaction by adding 100 μl of Griess reagent (0.1% w/v naphthylethylendiamide dihydrochloride in water and 1% w/v sulfanilamide in 5% v/v H_3PO_4) to 100 μl of the samples. Optical density was measured at 550 nm wavelength using a microplate reader (SLT-Lab Instruments, Salzburg, Austria). Nitrite concentrations were calculated by comparison with a standard curve of sodium nitrite.

Evaluation of cyclooxygenase activity

Lung tissue specimens were homogenized in a buffer composed of: 20 mmol/l HEPES, pH 7.2, 320 mmol/l sucrose, 1 mmol/l dithiothreitol, 10 μg/ml styrosporin, 2 μg/ml aprotinin and 10 μg/ml leupeptin. The homogenates were incubated at 37°C for 30 min. in the presence of excess arachidonic acid (30 μmol/l). The samples were boiled and centrifuged at 10,000 × *g* for 5 min. The concentration of 6-keto-PGF1α in the supernatant was then measured by radioimmunoassay as previously described [22]. Protein concentration in each homogenate was measured using the Bradford assay using a standard curve of bovine serum albumin [23].

Myeloperoxidase (MPO) activity

MPO activity, an indicator of PMN accumulation, was determined as described previously [24]. Lung tissue specimens were weighed and homogenized in a buffer composed of: 0.5% w/v hexadecyltrimethylammonium bromide dissolved in 10 mmol/l potassium phosphate buffer, pH 7.0, and centrifuged for 30 min. at 20,000 × *g* at 4°C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mmol/l tetramethylbenzidine and 0.1 mmol/l hydrogen peroxide. The rate of change in absorbance was measured spectrophotometrically at 650 nm wavelength. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per min. at 37°C and was expressed in mU/g wet tissue.

Thiobarbituric acid-reactive substance (TBARS) measurement

TBARS levels in the lung tissue, an indicator of lipid peroxidation, were determined as described previously [25]. Lung tissue specimens were homogenized in 1.15% w/v KCl solution. A 100 μl aliquot of the homogenate was added to a reaction mixture containing 200 μl of 8.1% w/v SDS, 1.5 ml of 20% v/v acetic acid, pH 3.5, 1.5 ml of 0.8% w/v thiobarbituric acid and 700 μl of distilled water. Samples were then boiled for 1 hr at 95°C and centrifuged at 3000 × *g* for 10 min. The absorbance of the supernatant was measured spectrophotometrically at 532 nm wavelength.

Immunohistochemical localization of nitrotyrosine

NT, an index of protein nitrosylation by harmful oxidants generated during inflammation, such as peroxynitrite, was determined by immunohistochemistry as described previously [21]. Sections from formaldehyde-fixed, paraffin-embedded lung tissue specimens were rehydrated, endogenous peroxidase was quenched with 0.3% v/v hydrogen peroxide in 60% v/v methanol for 30 min., the sections were permeabilized with 0.1% w/v Triton X-100 in PBS for 20 min., non-specific labelling was minimized by incubating the section in 2% v/v normal goat serum in PBS for 20 min., the sections were incubated with rabbit polyclonal anti-NT antiserum (Upstate Biotechnology, Buckingham, UK; 1:100) at 4°C overnight, immune reaction was revealed by goat anti-rabbit IgG conjugated with biotin (Vector Lab, Burlingame, CA, USA; 1:200) followed by incubation with ABC complex (Vector Lab; 1:200). Negative controls were carried out by omitting the primary or the secondary antibodies. The sections were counterstained with haematoxylin before mounting.

Evaluation of nitrotyrosine expression by dot blot analysis

Samples of lung tissue homogenates, each containing 200 μg of total proteins, were spotted on nitrocellulose membrane strips (Amersham). They were air-dried for 30 min. and then saturated with T-PBS containing 5% w/v bovine serum albumin for 1 hr at room temperature. The strips were subsequently incubated with rabbit polyclonal antiserum against NT (Upstate), diluted 1:300 in T-PBS containing 1% w/v bovine serum albumin, for 1 hr at room temperature. After washing in T-PBS the membranes were incubated with peroxidase-labelled goat anti-rabbit antibodies diluted 1:10,000 in T-PBS containing 1% w/v bovine serum albumin, for 1 hr at room temperature. Immunoreactivity was revealed by chemiluminescence using the ECL system.

Histological examination

Lung specimens were fixed in 10% w/v PBS-buffered formaldehyde and embedded in paraffin. Histopathological examination was carried out on tissue sections, 7 μm thick, stained with haematoxylin and eosin.

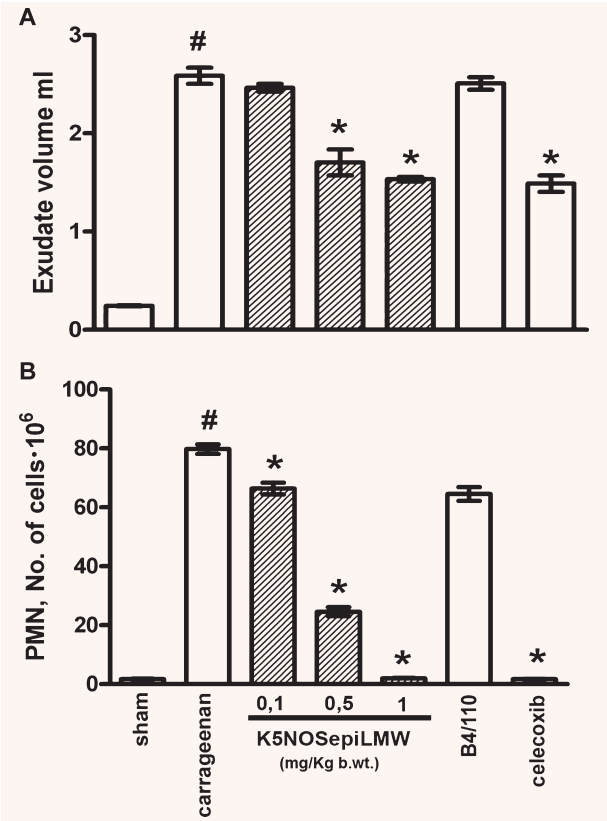

Fig. 1 Pleural exudate volume (**A**) and number of polymorphonuclear cells, PMN, in the pleural fluid (**B**) in the different experimental groups, collected 4 hrs after carrageenan injection. K5NOSepiLMW (0.1–1 mg/kg b.wt.) and celecoxib (1 mg/kg b.wt.), but not compound B4/110 (1 mg/kg b.wt.), given 30 min. before carrageenan, significantly reduced these parameters as compared with carageenan alone. Significance of differences (one-way ANOVA, $n = 8$): $^{\#}P < 0.001$ *versus* sham-operated controls; $^{*}P < 0.001$ *versus* carrageenan treated.

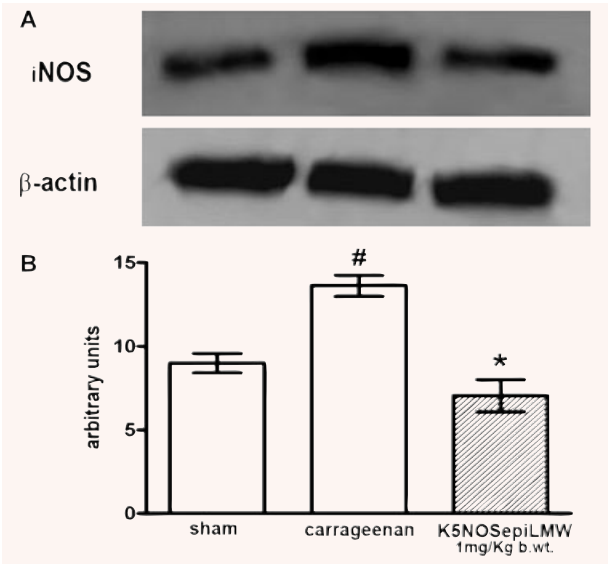

Fig. 2 iNOS protein expression evaluated by Western blot analysis in lung tissue samples from the different experimental groups, collected 4 hrs after carrageenan injection. Carrageenan increases iNOS expression, whereas pre-treatment with K5NOSepiLMW (1 mg/kg b.wt.) inhibits this effect (**A**). These data are confirmed by densitometric analysis (**B**). Significance of differences (one-way ANOVA, $n = 8$): $^{\#}P < 0.001$ *versus* sham-operated controls; $^{*}P < 0.001$ *versus* carrageenan treated.

Measurement of pro-inflammatory cytokines and PGE_2

Tumour necrosis factor (TNF)-α and IL-1β levels in the pleural exudate were evaluated using a commercial enzyme-linked immunosorbent assay kit (Calbiochem-Novabiochem, Milan, Italy) with a lower detection limit of 10 pg/ml. PGE_2 levels were measured by radioimmunoassay (Amersham) without prior extraction, as described previously [26].

Measurement of oxygen free radical scavenging properties of K5NOSepiLMW

To evaluate whether the observed effects of K5NOSepiLMW could be attributable to intrinsic oxygen free radical scavenging properties, this substance was tested in a cell-free system using the xanthine-xanthine oxidase superoxide anion generating system [27]. Briefly, xanthine oxidase metabolizes *in vitro* its substrate xanthine and generates superoxide anion, which can be measured spectrophotometrically by the oxidation of the chromogen nitro blue tetrazolium (NBT) to formazan. K5NOSepiLMW was added to 10, 100 and 1000 μg/ml (concentrations in the range of the dose administered to the rats) to a reaction solution containing 5 mU/ml xanthine oxidase, 250 μg/ml xanthine and 100 μM NBT bromide in phosphate buffer, pH 7.4. The samples were placed in a thermostat at 37°C and the reduction of NBT was evaluated spectrophotometrically at 570 nm wavelength after 5 and 15 min. of incubation.

Statistical analysis

Values are expressed as mean ± S.E.M. Statistical significance of the differences between the experimental groups was checked by of one- or two-way ANOVA, as appropriate, followed by *post hoc* test for comparison between individual groups. A *P*-value ≤ 0.05 was considered significant.

Results

All the rats treated with carrageenan developed an acute pleurisy characterized by the production of turbid exudate (Fig. 1A). Compared with the number of PMN collected from the pleural space of the sham-operated controls, injection of carrageenan induced a significant increase in the cell number (Fig. 1B). Pre-treatments of rats with K5NOSepiLMW dose-dependently reduced

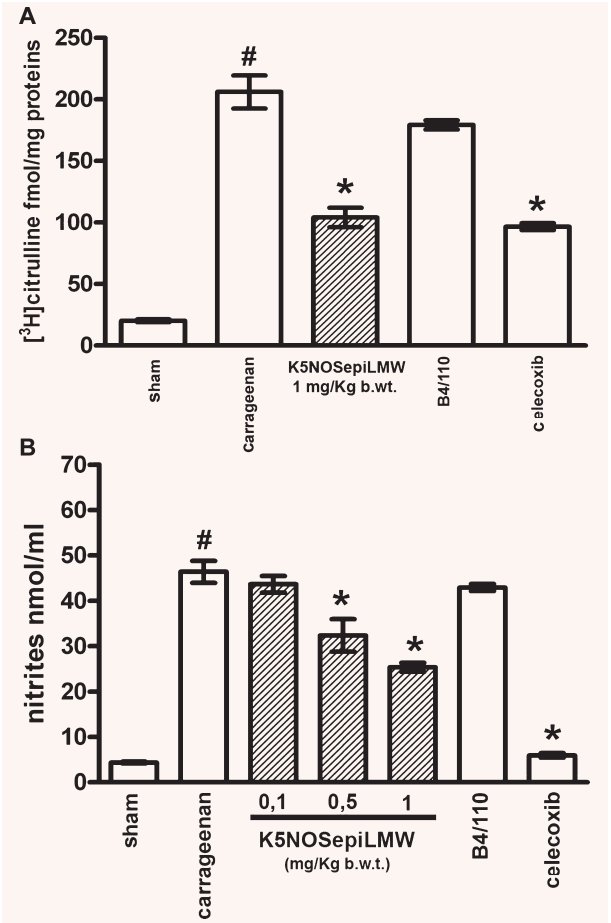

Fig. 3 iNOS enzyme activity, evaluated as L-[^{3}H]citrulline generation (**A**) and nitrite production (**B**), in lung tissue samples from the different experimental groups, collected 4 hrs after carrageenan injection. Carrageenan increases iNOS activity whereas pre-treatment with K5NOSepiLMW inhibits this effect (**A**, **B**). Moreover, K5NOSepiLMW (0.1–1 mg/kg b.wt.) and celecoxib (1 mg/kg b.wt.), but not compound B4/110 (1 mg/kg b.wt.), significantly reduce iNOS activity as compared with carageenan alone (**A**, **B**). Significance of differences (one-way ANOVA, $n = 8$): $^{\#}P < 0.001$ *versus* sham-operated controls; $^{*}P < 0.001$ *versus* carrageenan treated.

the volume of the pleural exudate and the number of PMN within the exudate. These inhibitory effects were also observed when the rats were pre-treated with celecoxib (1 mg/kg b.wt.) but not with compound B4/110 (1 mg/kg b.wt.) (Fig. 1A and B).

A significant increase in lung iNOS expression and activity was observed 4 hrs after administration of carrageenan (Fig. 2A and B). Pre-treatment of rats with K5NOSepiLMW significantly reduced iNOS expression, evaluated by Western blot analysis (Fig. 2A), pre-treatment of rats with K5NOSepiLMW (1 mg/kg b.wt.) or celecoxib (1 mg/kg b.wt.) significantly reduced iNOS activity, measured as citrulline formation (Fig. 3A) and nitrite production (Fig. 3B). B4/110 (1 mg/kg b.wt.) did not cause any changes in iNOS activity (Fig. 3A and B).

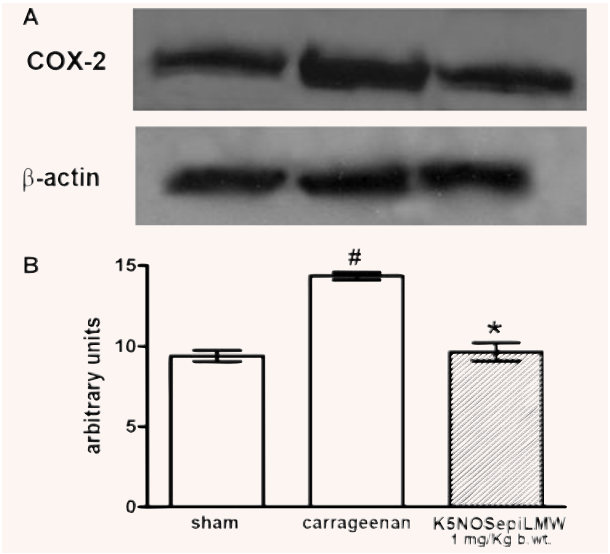

Fig. 4 COX-2 protein expression evaluated by Western blot analysis in lung tissue samples from the different experimental groups, collected 4 hrs after carrageenan injection. Carrageenan increases COX-2 expression, whereas pre-treatment with K5NOSepiLMW (1 mg/kg b.wt.) inhibits this effect (**A**). These data are confirmed by densitometric analysis (**B**). Significance of differences (one-way ANOVA, $n = 8$): $^{\#}P < 0.001$ *versus* sham-operated controls; $^{*}P < 0.001$ *versus* carrageenan treated.

COX-2 expression was evaluated by Western blotting (Fig. 4), whereas COX-2 activity was determined by measuring the levels of PGE_2 in the exudates (Fig. 5A) and of 6keto-$PGF_{1\alpha}$ in the supernatant of lung tissue homogenates (Fig. 5B). In comparison with the sham-operated controls, the amount of COX-2 protein and the levels of both PGE_2 and 6keto-$PGF_{1\alpha}$ were greatly increased in the carrageenan-treated rats, an effect significantly and dose-dependently blunted by pre-treatment of the rat with K5NOSepiLMW and celecoxib (1 mg/kg b.wt.), but not with B4/110 (1 mg/kg b.wt.).

Carrageenan-treated rats exhibited a substantial increase in lung tissue MPO and TBARS, assumed as markers for PMN infiltration [24] and lipid peroxidation [25], respectively, as compared with the sham-operated controls (Fig. 6A and B). Pre-treatment of rats with K5NOSepiLMW attenuated in a dose-dependent fashion the increase in MPO and TBARS induced by carrageenan; celecoxib (1 mg/kg b.wt.) had a similar effect, while B4/110 (1 mg/kg b.wt.) was completely inactive (Fig. 6A and B).

Immunohistochemical analysis of lung sections obtained from carrageenan-treated rats revealed a positive staining for NT, which was absent in lung samples taken from the sham-operated controls (Fig. 7A and B). In contrast, no staining for NT was found in the lungs of carrageenan-treated rats that had been pre-treated with K5NOSepiLMW (1 mg/kg b.wt.) (Fig. 7C). Staining was absent in lungs from the sham-operated controls (not shown).

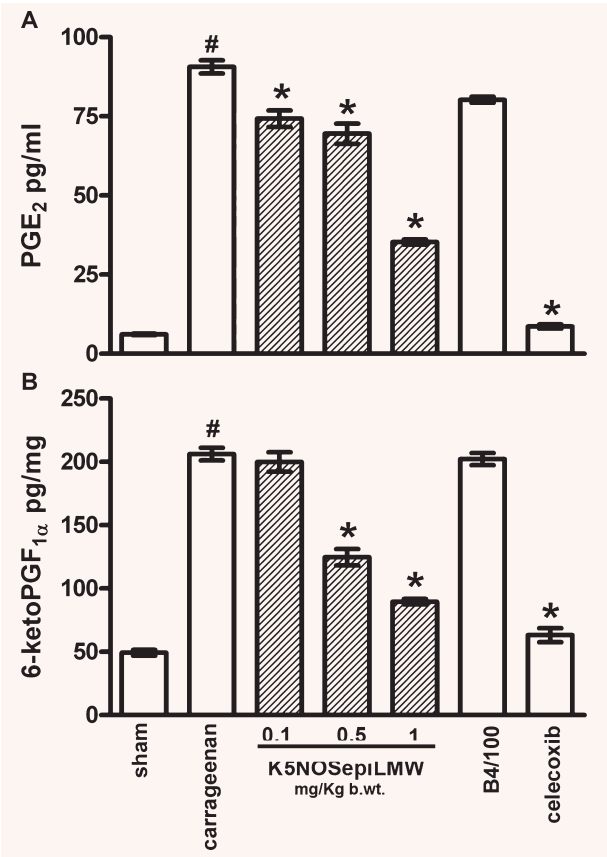

Fig. 5 COX-2 enzyme activity, evaluated as production of PGE_2 (**A**) and 6-keto-PGF1α (**B**), in lung tissue samples from the different experimental groups, collected 4 hrs after carrageenan injection. Carrageenan increases COX-2 expression, whereas K5NOSepiLMW (0.1–1 mg/kg b.wt.) and celecoxib (1 mg/kg b.wt.), but not compound B4/110 (1 mg/kg b.wt.), significantly reduced COX-2 activity as compared with carageenan alone (**A**, **B**). Significance of differences (one-way ANOVA, $n = 8$): $^{\#}P < 0.001$ *versus* sham-operated controls; $^{*}P < 0.001$ *versus* carrageenan treated.

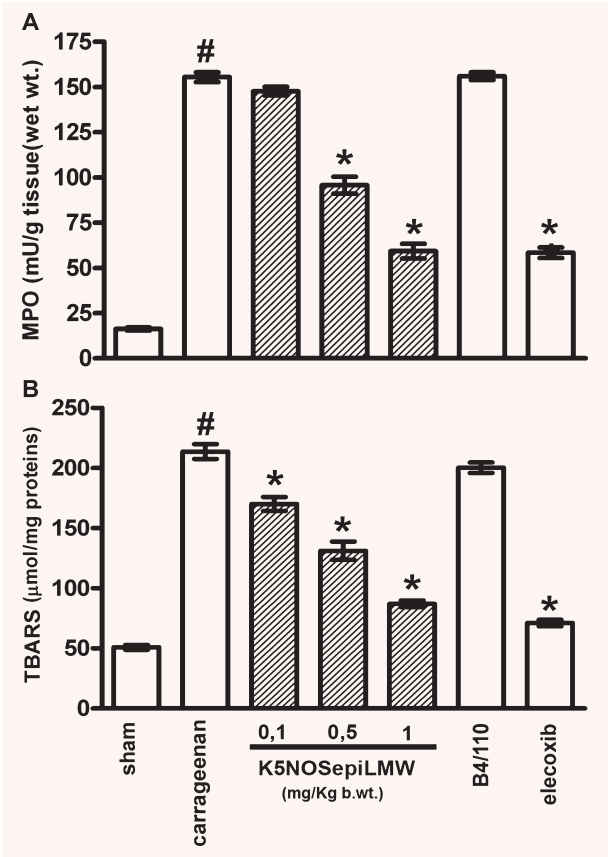

Fig. 6 Lung tissue myeloperoxidase, MPO, (**A**) and thiobarbituric acid-reactive substances, TBARS, (**B**) in lung tissue samples from the different experimental groups, collected 4 hrs after carrageenan injection. K5NOSepiLMW (0.1–1 mg/kg b.wt.) and celecoxib (1 mg/kg b.wt.), but not compound B4/110 (1 mg/kg b.wt.), significantly reduced these parameters as compared with carageenan alone. Significance of differences (one-way ANOVA, $n = 8$): $^{\#}P < 0.001$ *versus* sham-operated controls; $^{*}P < 0.001$ *versus* carrageenan treated.

The immunohistochemical data was confirmed by dot blot analysis on lung tissue homogenates, showing that immunoreactive NT was almost undetectable in the sham-operated controls, was clearly present at high levels in the carrageenan-treated rats and became barely detectable upon pre-treatment with K5NOSepiLMW (1 mg/kg b.wt.) (Fig. 7D).

At variance with the normal histological appearance of the lungs from the sham-operated controls (Fig. 8A), the specimens from the rats treated with carrageenan showed tissue oedema, haemorrhage foci, infiltration with PMN and lymphocytes and reduced alveolar spaces (Fig. 8B). Pre-treatment K5NOSepiLMW (1 mg/kg b.wt.) reduced the above lung tissue abnormalities (Fig. 8C).

The levels of the pro-inflammatory cytokines IL-1β and TNF-α were increased in the pleural exudate of the carrageenan-treated rats compared with the sham-operated controls (Fig. 9A and B). The levels of both these cytokines were significantly and dose-dependently reduced upon pre-treatment with K5NOSepiLMW and celecoxib (1 mg/kg b.wt.), but not with B4/110 (1 mg/kg b.wt.) (Fig. 9A and B).

K5NOSepiLMW, at any time-point tested, did not influence the levels of superoxide anion, expressed as μmol of formazan, generated by the xanthine-xanthine oxidase system, thus indicating that this molecule has no intrinsic oxygen free radical scavenging properties (5-min. incubation: control, 5.1 + 1.2; K5NOSepiLMW: 10 μg/ml, 4.5 + 0.6; 100 μg/ml, 4.5 + 1; 1000 μg/ml, 3.2 + 0.3; $P > 0.05$. 15-min. incubation: control, 9.1 + 1.4; K5NOSepiLMW: 10 μg/ml, 9.5 + 0.5; 100 μg/ml, 7.4 + 1.5; 1000 μg/ml, 6.1 + 0.3; $P > 0.05$).

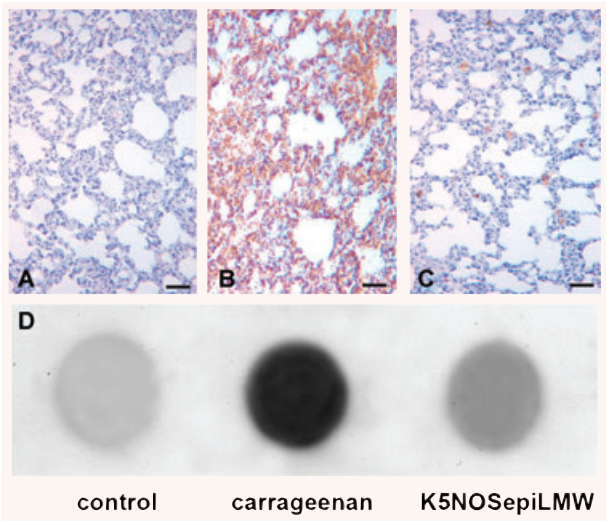

Fig. 7 Representative light micrographs of lung tissue immunostained to reveal nitrotyrosine (NT) in sham-operated rats (**A**), carrageenan-treated rats (**B**) and rats given K5NOSepiLMW (1 mg/kg b.wt.) 30 min. before carrageenan (**C**). Carrageenan causes the appearance of a clear-cut NT immunostaining, whereas K5NOSepiLMW nearly abolishes this effect. Bars = 50 μm. Dot blot analysis on lung tissue homogenates (**D**) shows that carrageenan treatment increases the amount of NT-containing proteins, whereas pre-treatment with K5NOSepiLMW inhibits this effect.

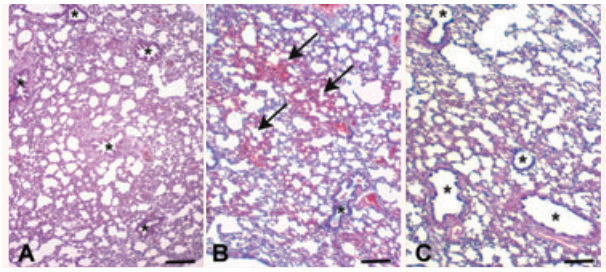

Fig. 8 Representative light micrographs of lung tissue in sham-operated controls (**A**), carrageenan-treated rats (**B**) and rats given K5NOSepiLMW (1 mg/kg b.wt.) 30 min. before carrageenan (**C**). Carrageenan causes the appearance of tissue oedema, haemorrhage foci, leucocyte infiltration and reduction of alveolar spaces (arrows), whereas K5NOSepiLMW blunts these histopathological changes. Asterisks label bronchial lumina. Bars = 100 μm.

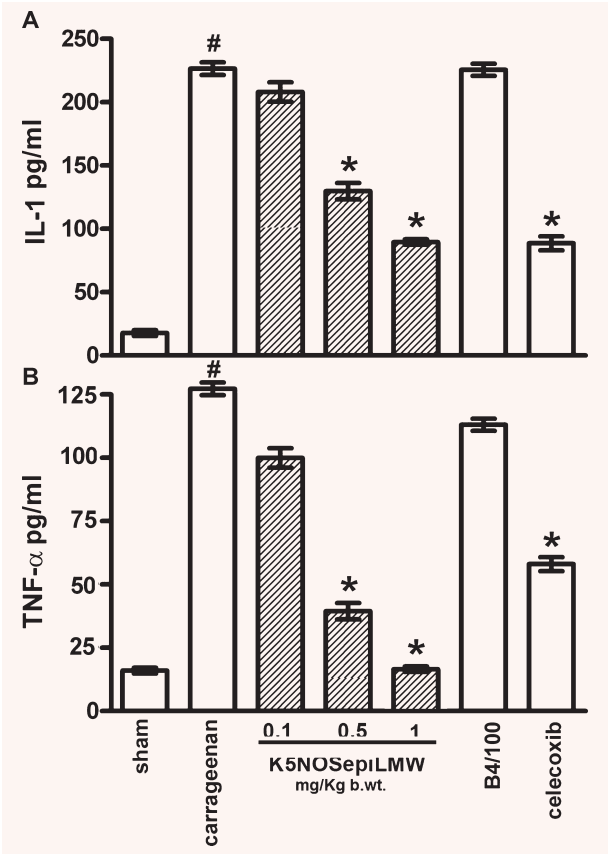

Fig. 9 Levels of IL-1β (**A**) and TNF-α (**B**) in the pleural exudates collected from the different experimental groups, collected 4 hrs after carrageenan injection.. K5NOSepiLMW (0.1–1 mg/kg b.wt.) and celecoxib (1 mg/kg b.wt.), but not compound B4/110 (1 mg/kg b.wt.), significantly reduced the amounts of these pro-inflammatory cytokines as compared with carageenan alone. Significance of differences (one-way ANOVA, $n = 8$): $^{\#}P < 0.001$ *versus* sham-operated controls; $^{*}P < 0.001$ *versus* carrageenan treated.

Discussion

This study provides evidence that a low molecular weight semi-synthetic O-sulphated heparin derivative (K5NOSepiLMW) markedly reduces the acute inflammatory response of carrageenan-induced pleurisy in the rat *in vivo*, a commonly used animal model to evaluate the efficacy of non-steroidal anti-inflammatory drugs (NSAIDs). A 30-min pre-treatment with K5NOSepiLMW, injected locally at doses of 0.1, 0.5 and 1 mg/kg b.wt., attenuated the recruitment of inflammatory leucocytes in the lung tissue and the pleural exudate, inhibited the induction of iNOS and COX-2, thereby abating the generation of harmful nitric oxide and pro-inflammatory PGs, such as PgE_2 and $PGF_{1\alpha}$ [16, 28], reduced the inflammation-induced nitroxidative stress, as shown by TBARS and NT, and blunted the local generation of cytokines such as IL-1β and TNF-α. As expected, all these parameters were markedly up-regulated by intrapleural injection of carrageenan in the absence of any pre-treatment. The anti-inflammatory effects of K5NOSepiLMW appear specific, as judged by the marked dose-response relationship in the 0.1–1 mg/kg b.wt. range; in this respect, K5NOSepiLMW is fairly potent, as its effects correspond to 1 mg/kg b.wt. of celecoxib, a selective COX-2 inhibitor assumed as reference NSAID. It is conceivable that the observed effect of K5NOSepiLMW to inhibit COX-2 and PG generation may be due,

at least in part, to inhibition of iNOS activity and nitric oxide production, as nitric oxide can activate COX-2 activity [29]. On the other hand, the current findings allow ruling out that the observed protection from lung inflammation afforded by K5NOSepiLMW may be related to oxygen free radical scavenging properties, as this molecule was unable to decrease the levels of superoxide anion generated by an *in vitro* free radical generating system, such as xanthine-xanthine oxidase.

The current findings are in keeping with previous studies showing that low molecular weight heparins are capable of functionally inhibiting the cells of acute and chronic inflammatory response, decreasing PMN and monocyte chemotaxis and activation [30–32] and inhibiting fibroblast proliferation, endothelial activation and neo-angiogenesis [33–35]. The low molecular weight of K5NOSepi is a useful feature for the anti-inflammatory activity of this substance, as it may allow this molecule to enter the target cells where it exerts its anti-inflammatory effects by interfering with the expression of pro-inflammatory cytokines [14]. This assumption is in keeping with previous reports that low molecular weight heparins and non-anticoagulant heparins blunt pro-inflammatory cytokine generation by inhibiting nuclear translocation of the transcription factor NF-κB [32, 36], although we have no direct evidence that such mechanism of action is also operating in K5NOSepiLMW. As IL-1β and TNF-α are known to mediate the induction of iNOS and COX-2 [37, 38], it is conceivable that the reduced levels of these inducible pro-inflammatory enzymes observed in the present study may be secondary to a reduced formation of endogenous IL-1β and TNF-α by K5NOSepiLMW. The possibility that K5NOSepiLMW could directly inhibit iNOS and COX-2 activity cannot be ruled out, but the present findings do not allow clarifying this issue. Moreover, the marked anti-inflammatory properties of K5NOSepiLMW appear to be related to O-sulphation, which enable it to bind to and inactivate endothelial L- and P-selectins, thus counteracting leucocyte rolling and adhesion [13].

In conclusion, this study provides support to the concept that semi-synthetic, low molecular weight heparin derivatives may represent a novel class of potent NSAIDs which, compared with unfractionated full-sized heparins, lack pro-angiogenic properties and substantial effects on the coagulation system [39]. Based on its specific pharmacological effects highlighted in the present study, K5NOSepiLMW could be suitable as an endothelial protecting drug for the treatment of vascular inflammation, potentially useful for the prevention of cardiovascular disease, which encompasses hypertension, atherothrombosis, myocardial infarction, stroke, peripheral vascular disease and renal failure.

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