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Nadroparine inhibits the hypersensitivity response in the conjunctiva

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Abstract

This study sought to investigate the effects of nadroparine on an in vivo experimental model of type I hypersensitivity response in the rat conjunctiva. Following drug application onto the eye, either before or after challenge with the mast cell degranulator, basic polyamine compound 48/80, the conjunctival histamine content and the nitrite levels in the conjunctival lavage fluid were quantified fluorometrically and spectrophotometrically, respectively. Instillation into the eye of nadroparine inhibited the C48/80-induced decreases in conjunctival histamine and the delayed increases in nitrite levels, without influencing basal mediator levels. Protamine did not induce histamine release and only partially reversed the effects of nadroparine post-challenge, yet it had no effect on the protective action of the drug when administered prior to degranulation. The results showed that nadroparine was equally effective in attenuating the effects of compound 48/80 in the eye when administered topically either before or after challenge.

Keywords: Histamine; Eye; Nitric oxide (NO); Nadroparine; Hypersensitivity

1. Introduction

Among the mast cell-derived multifunctional mediators, which regulate immunoglobulin E (IgE)- and non-IgE-dependent hypersensitivity reactions, histamine plays a pivotal role by activating the early- and late-phase inflammatory cascades (Mita et al., 1994; Baddeley et al., 1995; Bacon et al., 2000), the delayed nitric oxide (NO) release being part of this response (Meijer et al., 1996; Ko et al., 2000). Moreover, the granule-associated heparin is released from mast cells together with histamine and it has been reported to have different fate than the exogenously administered heparin (Jaques et al., 1977), which exhibits anti-inflammatory, immunoregulatory and antiallergic activities, distinct from the well-established anticoagulant properties of the molecule (Bowler et al., 1993; Seeds et al., 1993; Beltran et al., 1999; Lever and Page, 2001). The heterogeneity of mast cells (Tainsh and Pearce, 1992; Baddeley et al., 1995) as well as the type of molecules triggering the response may differentially modulate mediator release in various models of type I hypersensitivity reactions (Shefler and Sagi-Eisenberg, 2001), although the distinct biochemical processes merge into common clinical manifestations (Bacon et al., 2000). The differences in responses justify the use of particular experimental models for the investigation of the effectiveness of potentially therapeutic preparations.

An in vivo model of ocular anaphylaxis based on the topical application of the non-immunogenic cationic polyamine compound 48/80 (C48/80), a condensation product of *N*-methyl-*p*-methoxyphenethylamine with formaldehyde (Koibuchi et al., 1985), has been shown to be clinically relevant and experimentally practical (Allansmith et al., 1989; Abelson and Schaefer, 1993; Mita et al., 1994; Li et al., 1996; Tiligada et al., 2000). By activating pertussis toxin-sensitive Gi proteins, this commonly used basic polyamine induces mediator release from mast cells, similar to that evoked by neomycin (Aridor and Sagi-Eisenberg, 1990), the wasp venom peptide mastoparan (Aridor et al., 1990), peptidergic stimuli such as bradykinin and substance P and other non-immunologic stimuli (Emadi-Khiav et al., 1995). Application of C48/80 onto the eye has been reported to induce biochemical (Tiligada et al., 2000, in press), histologic and clinical changes, resembling those seen in some common forms of IgE- and non-IgE-mediated (Johansson et al., 2001) allergic conjunctivitis (Allansmith et al., 1989; Abelson and Schaefer, 1993; Mita et al., 1994; Li et al., 1996; Tiligada et al., in press). Most of the in vivo

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experiments have focused on microscopic observations, significant degranulation having been detected 1 to 6 h following challenge with C48/80 (Allansmith et al., 1989; Li et al., 1996; Tiligada et al., in press). Regarding the kinetics of conjunctival histamine concentration, a peak has been reported at 30 min post-allergen challenge in Sprague–Dawley rats (Rankov et al., 1990), while in the conjunctiva of Wistar rats, reduced histamine levels have been demonstrated 1 h after challenge with C48/80, this reduction being maintained for at least 24 h (Tiligada et al., 2000).

The increasing number of reports regarding the interaction between pro-inflammatory mediators and heparins, particularly their low molecular weight counterparts, during the inflammatory responses point to interesting, yet unresolved and thus largely unexploited characteristics of these substances (Lever and Page, 2001). Being heterogeneous with respect to molecular weight, anticoagulant activity and pharmacokinetic properties, unfractionated heparin, an anionic glycosaminoglycan of a mean molecular weight of 15,000 daltons, as well as its 4500–5000-dalton depolymerization fragments are widely used in antithrombotic therapy (Hirsh et al., 2001). Compared to unfractionated heparin, which inhibits mainly thrombin and factor Xa, low molecular weight heparins, such as nadroparine (mean molecular weight of 4500 daltons), inhibit factor Xa and minimally affect thrombin (Hirsh et al., 2001). Evidence has now accumulated that heparins can affect the immune response including allergic inflammation (Lever and Page, 2001). Most related in vitro, animal and human studies have been performed in the airways and argue that heparins exert their anti-inflammatory effects in a molecular weight-dependent manner by yet largely speculative mechanisms (Lucio et al., 1992; Inase et al., 1993; Lever and Page, 2001). In particular, in canine mastocytoma cells, the concentration-dependent inhibition of C48/80-induced histamine release by heparin has been attributed to its negative charge density (Inase et al., 1993), while in sheep, heparin inhibited the mast cell-related cutaneous reaction and acute bronchoconstrictor response without attenuating the effects of histamine (Lucio et al., 1992). Unfractionated and low molecular weight heparins have been shown to prevent the response induced by C48/80, to attenuate exercise-induced acute bronchoconstriction and to inhibit antigen-induced histamine release from isolated mast cells (Molinari et al., 1998).

Despite the number of pharmacological agents currently used to prevent the clinical manifestations of ocular immediate hypersensitivity, there are still continuing efforts aiming at the development of more efficacious topical medications to control the most severe episodes of the disease (Yanni et al., 1999). In the present study, we report the inhibitory effects of the low molecular weight nadroparine on the C48/80-induced mast cell degranulation in the rat conjunctiva, by determining the tissue histamine content and NO levels in the conjunctival lavage fluid.

2. Materials and methods

2.1. Animals and drug application

Male Wistar rats of 200–250-g body weight were maintained under controlled light and temperature conditions. They received a standard diet and water ad libitum. They were divided into groups of 3–10 animals each, as shown in Table 1. C48/80 was purchased from Sigma (St. Louis, MO, USA), dissolved in phosphate buffer saline (PBS), immediately prior to use and a single 0.01 ml drop of 0.1 g ml^{-1} C48/80 was applied topically. Protamine sulphate was obtained from Leo and nadroparine calcium (Fraxiparine®) was kindly offered by Sanofi-Synthelabo, Greece. Since C48/80 was dissolved in PBS at pH 7.4, the topical application of the buffer, pH 7.4, alone was considered as control treatment. Instillation of the drugs into the lower conjunctival fornix was performed under light ether anesthesia, as shown in Table 1, and the animals were then permitted to move freely.

2.2. Clinical and cytological examination

Rats were evaluated clinically in room light without the aid of magnification. All procedures were performed by fully trained and experienced personnel and complied with ethical codes and regulations (license no K/4358/01). Cytological examination of the unchallenged and challenged conjunctivae was performed under the light microscope at ×400 and ×1000 magnification, following staining of tissue specimens and scrapings with 0.25% (w/v) toluidine blue in 70% (v/v) ethanol, pH 1.6, and with May-Grünwald-Giemsa.

Table 1
Application of drugs onto the eyes of experimental animals

Group	One eye	Contralateral eye
1	C48/80	PBS
2	NDRP 235 IU	PBS
3	NDRP 470 IU	PBS
4	NDRP 705 IU	PBS
5	NDRP 235 IU → C48/80	PBS → C48/80
6	C48/80 → NDRP 235 IU	C48/80 → PBS
7	PROT 500	PBS
8	PROT 500 → C48/80	PBS → C48/80
9	C48/80 → PROT 500	C48/80 → PBS
10	PROT 500 → NDRP 235 IU	PBS → NDRP 235 IU
11	NDRP 235 IU → PROT 500	NDRP 235 IU → PBS
12	PROT 500 → C48/80 → NDRP 235 IU	PBS → C48/80 → NDRP 235 IU
13	NDRP 235 IU → C48/80 → PROT 500	NDRP 235 IU → C48/80 → PBS
14	[NDRP 235 IU + PROT 500] → C48/80	PBS → C48/80
15	C48/80 → 1 h → NDRP 235 IU	C48/80 → 1 h → PBS

The administration of drugs was performed in 15 min intervals, unless otherwise stated. $n = 3–10$.

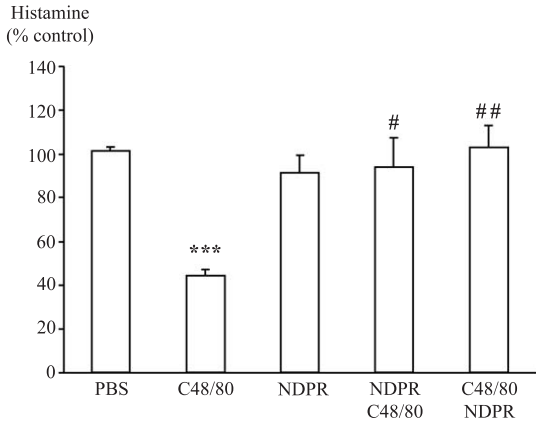

Fig. 1. Effect of the topical administration of 235 IU nadroparine (NDPR) on the histamine content of the rat conjunctiva, under basal and degranulation conditions. ***$P<0.001$ vs. conjunctival histamine content following administration of the vehicle (PBS), $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. challenge with compound 48/80 (C48/80).

2.3. Quantification of histamine

The animals were sacrificed by intraperitoneal injection of 1 ml of 0.05 g ml^{-1} ketamine hydrochloride (Ketalar®, Warner Lambert) 45 min or 6 h after the challenge with C48/80. The conjunctiva was rapidly removed using standard microsurgical techniques, placed on ice and the wet weight of the tissue was recorded. Removal of the tissue was performed at least 45 min after challenge, when the animals had fully recovered from treatment and their eyes showed no abnormal clinical features. The conjunctival histamine was extracted and quantified fluorometrically as described previously (Tiligada et al., 2000).

2.4. Quantification of nitrite

At 6 h following challenge with C48/80, lavage fluid was collected from the eye surface as follows. Instillation of 0.02-ml sterile PBS in the lower conjunctival fornix was followed by three forced blinks and the lavage fluid was collected using a micropipette, avoiding any touch with the ocular surface. The concentration of nitrite, stable endproduct of NO, was determined photometrically by the Griess reaction (Granger et al., 1996; Peponis et al., 2002).

2.5. Statistical evaluation of the results

Histamine and nitrite were quantified as ng/mg of tissue and μM, respectively, expressed as percentage of the levels in the eye receiving PBS and presented as the mean ± S.E.M. Significant differences between means were located using the independent samples test and one-way analysis of variance (ANOVA) followed by Scheffé or Dunnett test, $P<0.05$ being regarded as acceptable levels of significance for all statistical analyses.

3. Results

3.1. Clinical and cytological evaluation

On recovery, following administration of C48/80, the rats rubbed their eyes, but these movements were not considered sufficiently vigorous and persisting to cause any alteration in the conjunctiva. Five to ten minutes after the topical challenge with C48/80, animals developed palpebral edema, slight redness of the conjunctiva and signs of itching. The contralateral eyes showed no abnormal clinical features. Moreover, infiltration of eosinophils was observed in conjunctival specimens, 45 min and 6 h after challenge with C48/80 (Tiligada et al., in press).

3.2. Conjunctival histamine content

In group 1 rats, 45 min following challenge with C48/80, the conjunctival histamine levels were significantly ($P<0.001$) reduced to 45 ± 3%, compared to the control (Fig. 1). Topical administration of 235 IU nadroparine in groups 5–6, either before or after challenge with C48/80, completely reversed the effect of the degranulator (Fig. 1), the conjunctival histamine levels being significantly different from those detected following degranulation alone ($P<0.05$) and nonsignificantly different from the control ($P>0.6$). The topical administration of 235–705 IU nadroparine alone in groups 2–4 did not induce any significant alterations in the conjunctiva ($P>0.2$), the histamine content being 91 ± 8%, 98 ± 7% and 115 ± 9% of the control, respectively.

Administration of 0.5 mg of protamine, in group 7 rats, resulted in a nonsignificantly different response from the control (Fig. 2) and it had no effect on the challenged eyes of groups 8–9, when instilled either before or after C48/80, the conjunctival histamine content being 59 ± 9% and

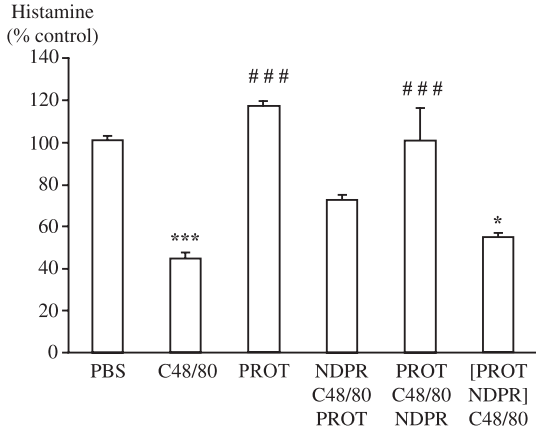

Fig. 2. Effect of the topical administration of protamine (PROT) on the protective effect of 235 IU nadroparine (NDPR) upon degranulation in the rat conjunctiva. *$P<0.05$ and ***$P<0.001$ vs. conjunctival histamine content following administration of the vehicle (PBS), $^{\#\#\#}P<0.001$ vs. challenge with compound 48/80 (C48/80).

50 ± 8% of the control, respectively ($P>0.4$). Additionally, protamine had no significant effect on the action of 235 IU nadroparine in groups 10–11 ($P>0.9$). In the challenged eyes, protamine partially reversed the protective effect of nadroparine (Fig. 2), when administered after the challenge, in group 13 rats ($P<0.06$ vs. control, $P>0.8$ vs. nadroparine administration before or after challenge), but it was unable to reverse this protection when administered before challenge, in group 12 rats (Fig. 2). Moreover, the chemical neutralization of nadroparine by protamine, before co-instillation in group 14 rats, had no effect on the degranulating action of C48/80 ($P>0.2$). Finally, the conjunctival histamine content 6 h after the challenge was still significantly reduced, as reported previously (Tiligada et al., 2000), and this decrease was not affected by the administration of nadroparine 1 h after challenge in group 15 ($P>0.3$ vs. histamine levels following administration of C48/80 alone).

3.3. Nitrite levels in the conjunctival lavage fluid

Based on the above results regarding the early-phase response, the late-phase response was preliminary studied, by determining the nitrite levels in the lavage fluid of the conjunctiva, 6 h following challenge with C48/80, in experimental groups 1, 2, 5 and 15. Nitrite levels in the conjunctival lavage fluid were significantly ($P<0.01$) increased compared to the control (Fig. 3), 6 h after the topical instillation with C48/80. Topical administration of 235 IU nadroparine 1 h after challenge with C48/80 (Fig. 3) reversed the effect of the degranulator ($P<0.05$ vs. nitrite levels following administration of C48/80 alone). Nadroparine alone did not significantly alter the nitrite levels in the lavage fluid, 6 h post-administration ($P>0.1$ vs. control), while when instilled prior to challenge, the amount of nitrite in the lavage fluid was found to be significantly reduced ($P<0.01$) compared to the challenged eye.

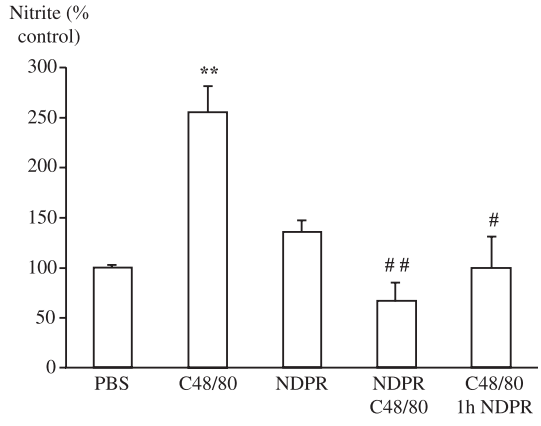

Fig. 3. Effect of the topical administration of 235 IU nadroparine (NDPR) on the nitrite levels in the rat conjunctival lavage fluid, under basal conditions and 6 h following degranulation. $^{**}P<0.01$ vs. nitrite levels following administration of the vehicle (PBS), $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. challenge with compound 48/80 (C48/80).

4. Discussion

This study sought to investigate the effects of the low molecular weight nadroparine on an established experimental model of non-IgE-dependent hypersensitivity response in the eye, where the reduction of the conjunctival histamine content and the increased nitrite levels documented the inflammatory changes associated with topical C48/80 challenge (Allansmith et al., 1989; Li et al., 1996; Meijer et al., 1996; Ko et al., 2000; Tiligada et al., 2000, in press).

The presented data showed that nadroparine inhibited both the early decreases in conjunctival histamine content and the subsequent delayed increases in nitrite levels following mast cell degranulation with C48/80, without influencing basal mediator levels. Interestingly, the effects of nadroparine were observed even when the drug was administered topically after challenge. These results are in accordance to previously reported data obtained from other tissues concerning modulation of immunologic and non-immunologic inflammatory processes and inhibition of histamine release by heparins (Lucio et al., 1992; Inase et al., 1993; Molinari et al., 1998). In a previous pilot study, unfractionated and low molecular weight heparins inhibited the effects of C48/80 in the same model of experimental allergic conjunctivitis (Tiligada et al., 2002). The present study extended these preliminary observations and strengthened the fact that nadroparine prevents the effects of pertussis toxin-sensitive Gi protein-mediated exocytosis (Aridor et al., 1990), while at the same time, it provided evidence for the inhibition of the propagation of the inflammatory response when applied topically in the eye after this initial event, an action comparable to that of glucocorticosteroids.

These in vivo effects of nadroparine could not be attributed to the counteraction between its anionic charge and the cationic C48/80, since administration was performed at intervals of 15 min, thus ensuring the absorption of one drug before the administration of the next. Moreover, the fact that nadroparine restored the NO levels in the lavage fluid during the delayed response, even when administered 1 h after challenge with C48/80, provides additional evidence for the biological rather than the chemical nature of its in vivo activity. Although related studies have suggested that the attenuation of post-antigen response by ultralow molecular weight heparins are mast cell-independent (Molinari et al., 1998), these results indicated that the attenuation of post-degranulation response in the conjunctiva, as far as the histamine and NO are concerned, may be mast cell-dependent. However, the underlying mechanisms remain elusive and the possible involvement of signalling pathways and cross-talk mechanisms between mast cells and coagulation factors (Dugina et al., 2002), particularly those mediated by the protease-activated receptors (PARs), cannot be excluded. In addition to being a low molecular weight heparin, which principally inhibits factor Xa and retains its in vivo activity upon

protamine administration (Racanelli and Fareed, 1992a), the reports that substantiate the use of nadroparine in this study include the interaction between mast cell PARs and factor Xa (Dugina et al., 2002), the correlation of tryptase levels to those of histamine in the lavage fluid following conjunctival provocation (Mita et al., 1994) and the nadroparine-dependent tryptase-mediated activation of the PARs of mast cells (Compton et al., 2001).

Furthermore, although there is experimental evidence that histamine may be released by protamine (Patella et al., 1997), in our model, protamine did not induce histamine release and it was indeed unable to reverse the effects of nadroparine in vivo, as it has been shown in certain previous reports (Levy et al., 1989). However, when the two compounds were allowed to react before administration, protamine neutralised nadroparine in a dose-dependent way. Since protamine completely neutralises the activities of unfractionated heparin (Racanelli and Fareed, 1992b), its partial effects on the action of nadroparine following degranulation may be due to counteraction of endogenously released heparin or heparin-like substances from conjunctival cells.

Finally, the effects of heparins on NO production have been a matter of controversy, partly attributed to the different isoforms of NO synthase (Bonmann et al., 1998). Inhibition of the late NO metabolite production following co-administration of nadroparine and C48/80 may be due to the early effects of the low molecular weight heparin on histamine release, thus arresting subsequent steps of signal transduction by preventing histamine H_1 receptor-mediated production of pro-inflammatory mediators by the conjunctival epithelial cells (Weimer et al., 1998; Yanni et al., 1999). In addition, although it is currently accepted that released histamine originates from mast cells, the possible contribution of histamine derived from other epithelial cell types to the observed action of nadroparine during the late-phase response cannot be excluded and deserves further consideration (Tiligada et al., in press). Since the effects of nadroparine were observed even when instilled after the elimination of conjunctival histamine (Allansmith et al., 1989; Tiligada et al., 2000), they could be attributed to the competition with sequestration of proinflammatory mediators and inflammatory cell migration during the late-phase response, which become significant at the transition between a type I response and the clinical manifestations of the reaction (Bacon et al., 2000; Suzuki and Freed, 2002).

In conclusion, this study reports for the first time that the low molecular weight nadroparine inhibits the effects of the histamine liberator C48/80, when administered topically in the eye, either before or after challenge. Further consideration and evaluation of these actions on early histamine liberation and late NO production may contribute both to the understanding of the mechanisms underlying the inflammatory response in the eye and to the development of novel therapeutic approaches.

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