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Interactive contribution of NK_1 and kinin receptors to the acute inflammatory oedema observed in response to noxious heat stimulation: studies in NK_1 receptor knockout mice

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1 Scald injury in Sv129 + C57BL/6 mice induced a temperature and time dependent oedema formation as calculated by the extravascular accumulation of [^{125}I]-albumin. Oedema formation was suppressed in NK_1 knockout mice compared to wildtypes at 10 ($P<0.01$) and 30 min ($P<0.001$). However, at 60 min a similar degree of extravasation was observed in the two groups.

2 Kinin B_1 (des-Arg^{10} Hoe 140; 1 μmol kg^{-1}) and B_2 (Hoe 140; 100 nmol kg^{-1}) antagonists caused an inhibition of oedema in wildtype mice at 10 and 30 min ($P<0.001$), but not at 60 min or at 30 min in NK_1 receptor knockout mice.

3 The inhibition of thermic oedema by des-Arg^{10} Hoe 140 was reversed by des-Arg^{9} bradykinin (0.1 μmol kg^{-1}; $P<0.01$) and also observed with a second B_1 receptor antagonist (des-Arg^{9} Leu^{8} bradykinin; 3 μmol kg^{-1}; $P<0.01$). Furthermore des-Arg^{10} Hoe 140 had no effect on capsaicin (200 μg ear^{-1}) ear oedema, but this was significantly reduced with Hoe 140 ($P<0.05$).

4 Scalding induced a large neutrophil accumulation at 4 h, as assessed by myeloperoxidase assay ($P<0.001$). This was not suppressed by NK_1 receptor deletion or kinin antagonists.

5 These results confirm an essential role for the NK_1 receptor in mediating the early, but not the delayed phase of oedema formation or neutrophil accumulation in response to scalding. The results also demonstrate a pivotal link between the kinins and sensory nerves in the microvascular response to burn injury, and for the first time show a rapid involvement of the B_1 receptor in murine skin. *British Journal of Pharmacology* (2001) **134,** 1805–1813

Keywords: Thermal injury; NK_1 receptor; kinins; oedema; neutrophils

Abbreviations: HBSS, Hank's buffered salt solution; H_2O_2, hydrogen peroxide; HTAB, hexadecyltrimethylammonium; MPO, myeloperoxidase; NK_1, neurokinin 1; ((S)1-(2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl) piperidin-3-yl]ethyl)-4-phenyl-1-azoniabicyclo[2.2.2]octane chloride), SR14033; TMB, tetramethylbenzidine

Introduction

Swelling, due to the formation of tissue oedema, is observed soon after noxious heat stimulation of the skin, for example after accidental scalding or burn injury. Several lines of evidence support the suggestion that tachykinin NK_1 receptors play a major role in the early acute oedema formation. NK_1 receptor antagonists and capsaicin depletion of sensory nerves substantially inhibit the oedema formation in a variety of noxious heat models in the rat (Saria, 1984; Jonsson *et al*., 1986; Lofgren *et al*., 1999). However, our recent studies suggest that NK_1 receptor antagonists attenuate the early, but not the later stages of the ongoing inflammatory swelling (Siney & Brain, 1996) and have little effect on the subsequent neutrophil accumulation (Pinter *et al*., 1999).

The mouse is now routinely used in animal models of inflammation because of the comparative ease of genetic manipulation in this species. Tachykinin NK_1 receptor knockout mice have been developed, and study of these animals has confirmed the importance of NK_1 receptors in inflammation (Bozic *et al*., 1996; Ahluwalia *et al*., 1998). In our laboratory, using wildtype and NK_1 receptor knockout mice, we have confirmed that substance P increases microvascular permeability *via* the NK_1 receptor (Cao *et al*., 1999). However, interestingly neither substance P nor selective NK_1 agonists were able to induce neutrophil accumulation in the naïve untreated normal skin of the wildtype mouse (Cao *et al*., 2000). By comparison, we found that if microvascular inflammation was induced in skin, then neutrophil accumulation was NK_1 receptor dependent, as observed in experiments using NK_1 antagonists or NK_1 knockout mice (Cao *et al*., 2000), indicating that the NK_1 receptor contributes to neutrophil accumulation, in addition to oedema formation in the inflamed microvasculature.

Bradykinin has been suggested to be involved in the inflammatory oedema observed in response to noxious heat stimulation in the rat as determined by the study of normal and kininogen-deficient rats (Yonehara *et al*., 1995). Furthermore in this study, it was suggested that kinin generation was linked to the release of substance P from sensory nerves. We have also observed a link between the kinin and tachykinin system in our NK_1 knockout mice, in that both B_1 and B_2 receptor antagonists reduced neutrophil accumulation in response to carrageenan in wildtype mice.

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However in NK_1 receptor knockout mice, the kinin antagonists were without effect (Cao *et al*., 2000). Indeed, evidence for a link between the tachykinin and kinin receptors has been produced in several models of inflammation (Ricciardolo *et al*., 1994; Lindstrom & Andersson, 1997; Schuligoi *et al*., 1998; Ferreira *et al*., 2000). In addition, studies in the microvasculature of neutral endopeptidase knockout mice have revealed that spontaneous plasma leakage is inhibited by treatment with NK_1 and kinin B_2 antagonists (Lu *et al*., 1997).

Thus, NK_1 receptors contribute to the vascular and cellular phases of inflammation and have differential effects in inflamed skin and naïve skin. In addition, a link between the inflammatory activities of the tachykinin NK_1 and kinin, B_1 and B_2 receptors is suggested from a range of studies. We therefore decided to examine the contribution that these receptors play in mediating the vascular and cellular responses to noxious heat using NK_1 receptor knockout mice and selective kinin antagonists. In addition, the potential for interaction between the tachykinin NK_1 and kinin B_1 receptors in thermal injury was investigated.

Methods

Induction of thermal injury

All experiments were performed in accordance with the Animal (Scientific Procedures) Act 1986. Male/female Sv129 + C57BL/6 wildtype and NK_1 knockout mice (20–30 g) were generated at the Perlmutter Laboratory, Children's Hospital, Boston, U.S.A. (Bozic *et al*., 1996) and bred at King's College London. The mice were housed in a light (07:00–19:00) and temperature controlled (18–22°C) environment, and food and water were provided for consumption *ad libitum*. The animals were anaesthetized with urethane (2.5 μg g^{-1}; i.p.; Sigma, U.K.) and the depth of anaesthesia was assessed by the pedal reflex with maintenance doses administered as required. Initially, a local cutaneous thermal injury was induced by submersion of one ear in ultrapure water heated to 40–60°C for 10 s to obtain a temperature-response curve. For subsequent experiments, a temperature of 55°C was selected as this was observed to induce a highly significant, but non-maximal increase in plasma extravasation. The control, contralateral ear was submersed in ultrapure water at room temperature for the same period. Ears selected for thermal injury or collection of control tissue were alternated to remove any bias relating to skin heterogeneity (e.g. thickness, vascular density). At 10 min–4 h post-burn the animals were killed by cervical dislocation and the ears removed for analysis and weighing (wet weight).

Capsaicin-induced ear oedema

Animals were prepared as described above. Capsaicin (Sigma, U.K.) was dissolved in ethanol to give a stock solution of 10 μg μl^{-1}. Ten μl of stock solution was topically applied to each side of the ear selected for capsaicin treatment to give a total dose of 200 μg ear^{-1}. The control, contralateral ear was treated with ethanol in the same manner. Ears selected for capsaicin or vehicle treatment were alternated from animal to animal. At 30 min after application of capsaicin or vehicle, the mice were killed by cervical dislocation and the ears removed for analysis.

Measurement of plasma extravasation

Plasma extravasation was measured as previously described (Cao *et al*., 1999). Briefly, the mice received [^{125}I]-albumin (45 kBq; ICN Biochemicals, U.S.A.) i.v. at least 5 min prior to the induction of thermal injury or capsaicin treatment. Microvascular extravasation in the ear following thermal injury or capsaicin treatment was calculated by comparison to a known volume of blood plasma which was collected *via* cardiac puncture immediately prior to cervical dislocation.

Measurement of neutrophil accumulation

The ears were snap frozen and stored at −70°C prior to homogenization and subsequent analysis. The tissues were homogenized in ice cold phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). The resulting homogenate was centrifuged at 25,000 × *g* for 25 min at 4°C. Neutrophil accumulation in tissues was determined *via* myeloperoxidase (MPO) activity (Schierwagen *et al*., 1990; Cao *et al*., 2000) which was calculated *via* the hydrogen peroxide (H_2O_2) dependent oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB). The assay was performed in a 96 well microtitre plate. Tissue homogenates were diluted 1 : 2 in phosphate buffer (pH 6.0) to give a total volume of 50 μl. The resultant mixture was incubated with 100 μl of 'K-Blue' (a commercial preparation of H_2O_2 and TMB; Bionostics, U.K.) for 60 min at room temperature. Following incubation, the absorbance of the plate was recorded at 620 nm. The neutrophil content in the homogenates was calculated by comparing their absorbance with that of a standardized preparation of mouse neutrophils. All samples were assayed in duplicate and the data expressed as neutrophils g tissue^{-1} (10^6).

Preparation of mouse neutrophil standards

Mixed leukocytes were collected from the peritoneal cavity of Sv129 + C57BL/6 mice by inducing a sub-acute peritonitis (Moroney *et al*., 1988; Cao *et al*., 2000). For this, 6% oyster glycogen (Sigma, U.K.) was dissolved in 1 ml isotonic saline and injected i.p. After 16 h, the mice were killed by cervical dislocation and 5 ml ice-cold modified HBSS (Hank's buffered salt solution) free of Ca^{2+} and Mg^{2+} (Sigma, U.K.) was injected into the peritoneal cavity. After 1 min of massage, the peritoneal fluid was aspirated and centrifuged at 4°C for 10 min at 400 × *g*. The supernatant was removed and erythrocytes lysed by brief exposure to hypotonic saline (0.2% sodium chloride). After tonicity was restored, the preparation was centrifuged in a similar manner and the supernatant discarded. The pellet was dissolved in 2 ml HBSS containing 1.26 mM Ca^{2+} and 0.9 mM Mg^{2+}. A sample of the resultant mixture was diluted 1 : 10 with complete HBSS and exposed to 0.4% Trypan blue prior to haemocytometry to determine the total leukocyte count in the preparation. For determination of the number of neutrophils present, cell smears were prepared by cytospin (Shandon Scientific, U.K.) and slides were fixed in acetone, air dried and exposed to Mayer's haematoxylin (BDH, U.K.) for 10 min. The slides were washed with tap water and placed in 1% chromotrope

2R, 1% phenol. Standard mouse neutrophil preparations ($0.078-2.5\times10^6$ cells ml^{-1}) were aliquoted and stored at $-70°C$ prior to use.

Drug treatments

The tachykinin NK_1 antagonist, SR140333 ((S)1-(2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl) piperidin-3-yl]ethyl)-4-phenyl-1-azoniabicyclo[2.2.2]octane chloride) was a gift from Dr X. Emonds-Alt (Sanofi Recherche, France). The drug was dissolved in a minimal volume of ethanol and diluted to a final volume with physiological saline. SR140333 was administered at a dose of 480 nmol kg^{-1}. The kinin B_1 receptor antagonists des-Arg[10] Hoe 140 and des-Arg[9] Leu[8] bradykinin were purchased from Sigma, U.K. and Bachem, U.K. respectively. Both drugs were dissolved in physiological saline. des-Arg[10] Hoe 140 was administered at a dose of 1 μmol kg^{-1} and des-Arg[9] Leu[8] bradykinin at 3 μmol kg^{-1}. The B_2 receptor antagonist, Hoe 140 was obtained from Sigma, U.K., dissolved in physiological saline and administered at a dose of 100 nmol kg^{-1}. The B_1 receptor agonist, des-Arg[9] bradykinin was purchased from Bachem, U.K., dissolved in physiological saline and administered at a dose of 0.1 μmol kg^{-1}. All drugs were administered to mice as i.v. pretreatments, 5 min prior to the induction of thermal injury or the topical application of capsaicin.

Statistical analysis

All data are presented as mean ± s.e.mean and was analysed by ANOVA and the Bonferroni's test of multiple comparisons where appropriate. Plasma extravasation is expressed as ll g tissue-1 and neutrophil accumulation as cells g $tissue^{-1}$. $P<0.05$ was taken as being significant.

Results

Effect of scald injury on plasma extravasation: role of NK_1 receptors

Scalding of the ears of wildtype mice induced plasma extravasation which was seen to be both dependent on the temperature of burn and time post-burn. Thermic oedema was raised compared to controls at 45°C and became significantly different at 55 (Figure 1; $P<0.001$) and 60°C (Figure 1; $P<0.001$). Plasma extravasation did not change in the contralateral, control ears at any of the temperatures (Figure 1) or timepoints investigated (Figure 2). Oedema formation was rapid in wildtype animals and was significantly greater than controls at 10 min post-burn (Figure 2; $P<0.05$). Oedema formation continued to increase at 30 (Figure 2; $P<0.001$) and 60 min (Figure 2; $P<0.001$) post-burn. In contrast, scald oedema formation was markedly suppressed in NK_1 knockout mice compared to wildtype animals at 10 (Figure 2; $P<0.05$) and 30 min (Figure 2; $P<0.01$) post-burn. However, at 60 min the degree of plasma extravasation in knockout mice was similar to that seen in wildtype mice (Figure 2). To confirm a role for the NK_1 receptor in mediating the early phase of vascular leakage described, wildtype mice were pretreated (−5 min) with the NK_1 antagonist, SR140333 (480 nmol kg^{-1}, i.v.;) and oedema assessed at 10 min post-burn. SR140333 pretreated mice exhibited a similar pattern of plasma extravasation to that seen in NK_1 knockout animals and were significantly different from vehicle treated controls (Figure 3; $P<0.05$).

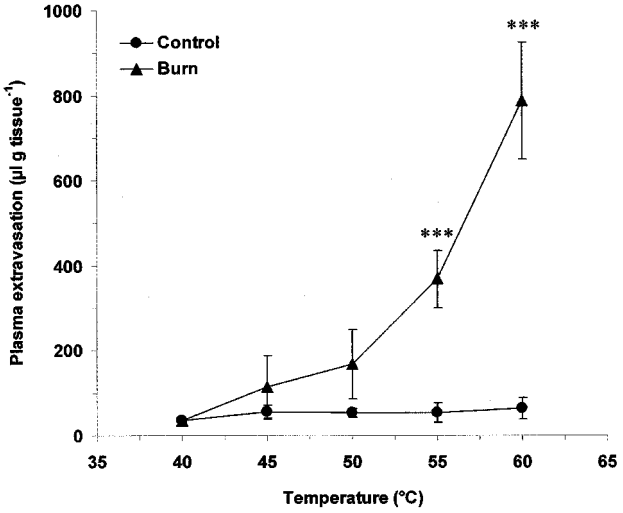

Figure 1 Temperature-dependency of thermic oedema. Plasma extravasation in the ears of wildtype mice at 60 min following thermal injury (40–60°C). All data are presented as mean ± s.e.mean. ***$P<0.001$ vs control at the same temperature; $n=4-5$.

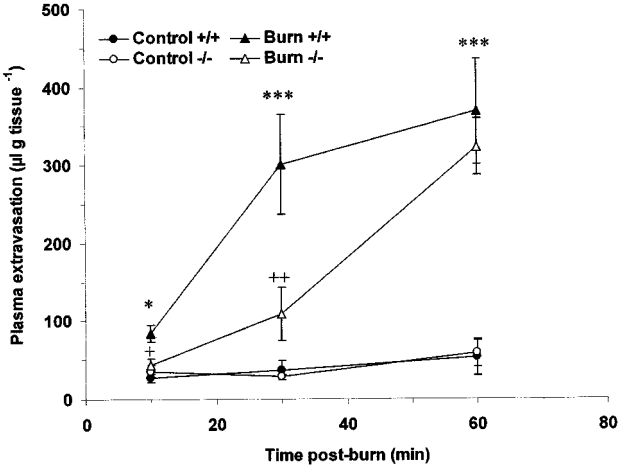

Figure 2 Comparison of thermic oedema in wildtype and NK_1 knockout mice. Plasma extravasation in the ears of wildtype and knockout mice over time (10–60 min) following thermal injury (55°C). All data are presented as mean ± s.e.mean. *$P<0.05$ vs control in the same animal at the same time point, ***$P<0.001$ vs control in the same animal at the same time point, +$P<0.05$ vs wildtype burn at the same time point, ++$P<0.01$ vs wildtype burn at the same time point; $n=6-8$.

Effect of kinin antagonists on scald and capsaicin induced ear oedema

Scald induced plasma extravasation in wildtype mice pretreated with the B_1 (des-Arg[10] Hoe 140; 1 μmol kg^{-1}, i.v.) and B_2 (Hoe 140; 100 nmol kg^{-1}, i.v.) receptor antagonists progressed in a manner akin to that observed in NK_1 knockout animals. Both

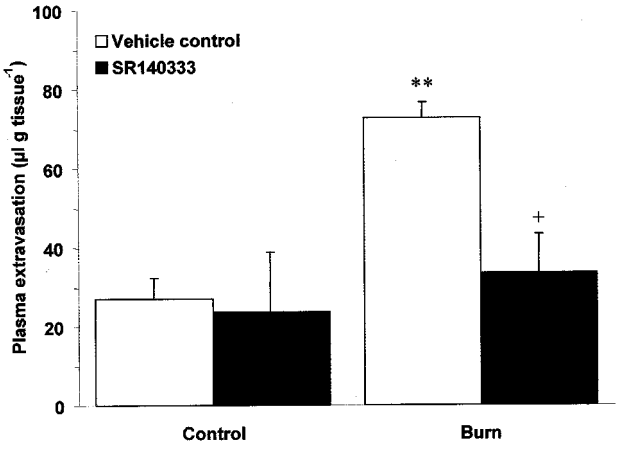

Figure 3 Effect of the NK_1 antagonist, SR140333 on thermic oedema. Plasma extravasation in the ears of wildtype mice pretreated (−5 min; i.v.) with SR140333 (480 nmol kg^{-1}) or vehicle control at 10 min following thermal injury (55°C). All data are presented as mean ± s.e.mean. $^{**}P<0.01$ vs control in the same animal, $+P<0.05$ vs burn in animals treated with vehicle; $n=6$.

antagonists significantly reduced vascular leakage at 10 (Figure 4a; $P<0.001$) and 30 min (Figure 4b; $P<0.001$) post-burn, but were ineffective at 60 min post-burn (Figure 4c). In addition, the inhibition of thermic oedema by des-Arg^{10} Hoe 140 was reversed by des-Arg^{9} bradykinin (0.1 µmol kg^{-1}) when compared to animals pretreated with des-Arg^{10} Hoe 140 alone at 30 min post-burn (266.15 ± 30.18 vs 149.74 ± 24.22, $n=4$; $P<0.01$). A second B_1 receptor antagonist, des-Arg^{9} Leu^{8} bradykinin (3 µmol kg^{-1}) was applied to the model and was also seen to significantly attenuate post-burn oedema formation at 30 min when compared to vehicle treated controls (152.06 ± 20.49 vs 333.61 ± 61.10, $n=6$; $P<0.01$). In an effort to learn more about the role that kinin receptors play in mediating plasma extravasation in the mouse ear, the effect of des-Arg^{10} Hoe 140 (1 µmol kg^{-1}) and Hoe 140 (100 nmol kg^{-1}) on capsaicin ear oedema was examined at 30 min (Figure 5). Topical application of capsaicin was observed to cause a significant rise in plasma extravasation when compared to vehicle treated control ears in the same animal ($P<0.001$). This was significantly reduced by pretreatment with Hoe 140 ($P<0.001$), but not des-Arg^{10} Hoe 140 when compared to vehicle treated controls. In contrast to the profound effect of the kinin antagonists on thermic oedema in wildtype mice at 30 min, neither des-Arg^{10} Hoe 140 (1 µmol kg^{-1}) or Hoe 140 (100 nmol kg^{-1}) had any effect on modulating plasma extravasation caused by scald injury in NK_1 knockout mice (Figure 6).

Role of NK_1 and kinin receptors in mediating scald induced neutrophil accumulation

Induction of thermal injury caused a significant increase in neutrophils accumulation in wildtype mice when assessed at 4 h post-burn (Figure 7a; $P<0.001$). However, no significant difference was observed between wildtype and NK_1 knockout mice (Figure 7a). Pretreatment with des-Arg^{10} Hoe 140 (1 µmol kg^{-1}) and Hoe 140 (100 nmol kg^{-1}) also failed to prevent neutrophil accumulation in wildtype animals when compared to vehicle treated controls (Figure 7b). Similarly,

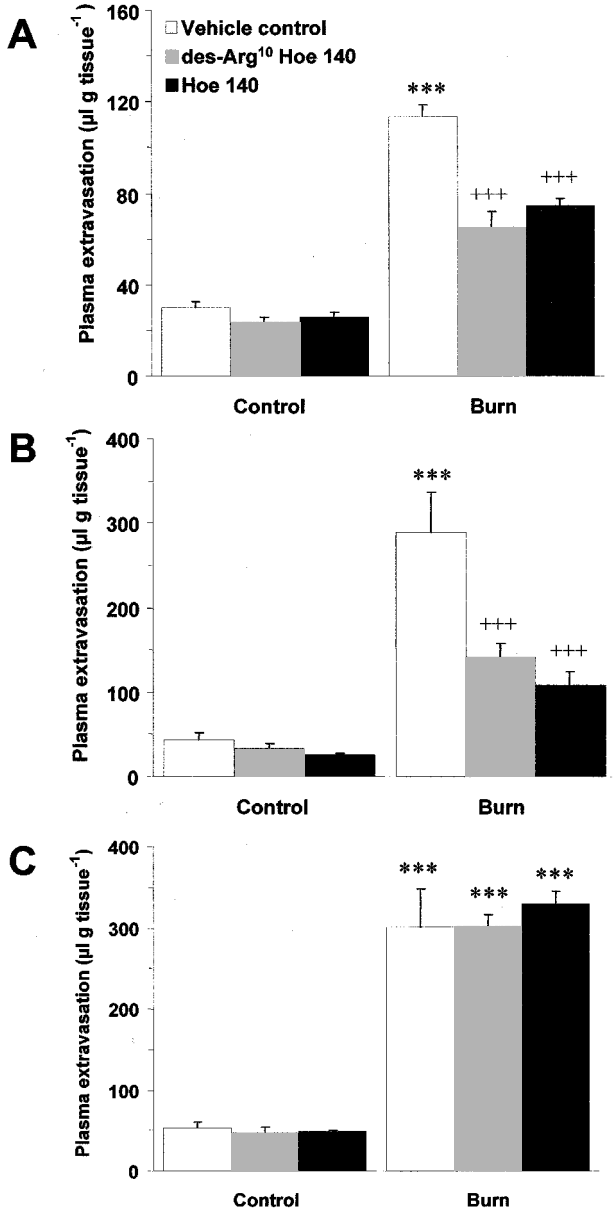

Figure 4 Effect of kinin antagonists on thermic oedema. Plasma extravasation in the ears of wildtype mice pretreated (−5 min; i.v) with vehicle control, des-Arg^{10} Hoe 140 (1 µmol kg^{-1}) or Hoe 140 (100 nmol kg^{-1}) at (A) 10 min, (B) 30 min and (C) 60 min post-burn (55°C). All data are presented as mean ± s.e.mean. $^{***}P<0.001$ vs control in the same animal at the same time point, $+++P<0.001$ vs burn in animals treated with vehicle; $n=5$.

neither NK_1 receptor deletion (26.46 ± 3.56 vs 28.69 ± 3.16, $n=5$), treatment with des-Arg^{10} Hoe 140 (13.63 ± 0.46 vs 13.94 ± 1.17, $n=5$) or Hoe 140 (16.04 ± 2.3 vs 13.94 ± 1.17, $n=5$) had any effect on neutrophil depletion compared to wildtype mice and vehicle treated controls respectively at 30 min post-burn.

Discussion

Previous studies have shown that local plasma extravasation following thermal injury is dependent on the temperature of

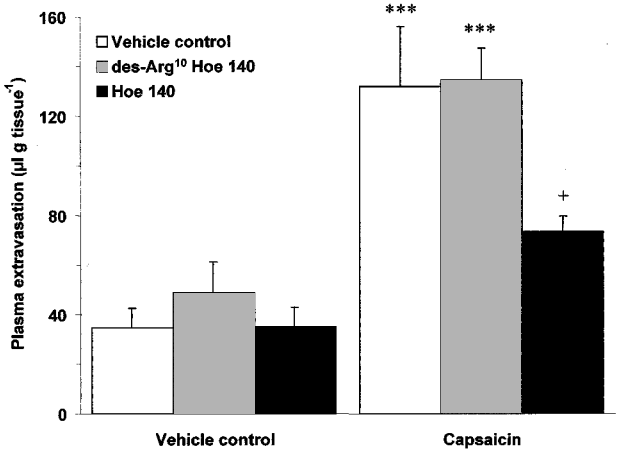

Figure 5 Effect of kinin antagonists on capsaicin ear oedema. Plasma extravasation in the ears of wildtype mice pretreated (−5 min; i.v) with vehicle control, des-Arg^{10} Hoe 140 (1 μmol kg^{-1}) or Hoe 140 (100 nmol kg^{-1}) at 30 min following topical application of capsaicin (200 μg ear^{-1}). All data are presented as mean± s.e.mean. ***$P<0.001$ vs topical vehicle control in the same animal, +$P<0.05$ vs animals treated with topical capsaicin and i.v. vehicle control; $n=5$.

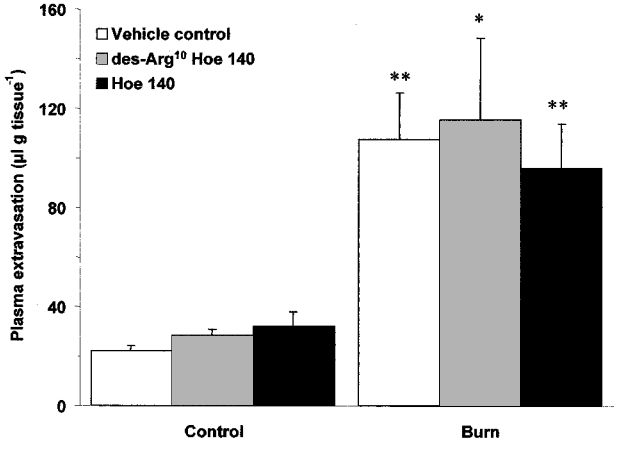

Figure 6 Effect of kinin antagonists on thermic oedema in NK_1 knockout mice. Plasma extravasation in the ears of NK_1 knockout mice pretreated (−5 min; i.v) with vehicle control, des-Arg^{10} Hoe 140 (1 μmol kg^{-1}) or Hoe 140 (100 nmol kg^{-1}) at 30 min post-burn (55°C). All data are presented as mean±s.e.mean. *$P<0.05$ vs control in the same animal, **$P<0.01$ vs control in the same animal; $n=5$.

burn and occurs in a manner progressive with time (Arturson & Jakobsson, 1985; Haegerstrand *et al*., 1987). In keeping with these studies, we have described a murine model of cutaneous thermal injury that has been applied to the study of tachykinins and kinins involved in mediating acute burn oedema. Initially, temperature-response curves were obtained. It was observed that plasma extravasation in the ears of mice was dependent on the temperature of exposure and that a threshold of 55°C caused a significant increase in plasma leakage compared to controls in this model. The degree of plasma extravasation following a 55°C burn was also seen to be dependent on time post-burn. It should be noted that plasma extravasation did not change in the contralateral, control ears at any of the time points or temperatures investigated, indicating that the injury induced was localized and did not lead to the release of circulating factors that may have affected the vascular permeability of sites remote to the burn.

Current evidence suggests that the neuropeptide, substance P is released from sensory neurones and acts on endothelial NK_1 receptors to mediate plasma extravasation following thermal injury. Early studies showed that post-burn oedema formation was suppressed in rats pretreated with capsaicin to cause selective degeneration of sensory C-fibres (Saria, 1984; Yonehara *et al*., 1987) and a number of reports show that substance P release is increased in both experimental (Jonsson *et al*., 1986; Yonehara *et al*., 1987; Hu *et al*., 1996) and clinical thermal injury (Onuoha & Alpar, 2001). In addition, selective NK_1 receptor antagonists have been shown to be of benefit in reducing post-burn oedema in the rat (Siney & Brain, 1996; Lofgren *et al*., 1999). The initial aim of this study was to confirm the importance of NK_1 receptors in mediating thermic oedema *via* the use of NK_1 receptor knockout mice. Our results show that in comparison to the very large and rapid plasma extravasation seen in wildtype mice following scald injury, there was a marked depression of this parameter in NK_1 knockout mice at 10 and 30 min post-burn. However, at the later time point of 60 min, the degree of plasma extravasation in the two populations was comparable, confirming that the NK_1 receptor plays an essential role in mediating the early, but not the late phase of plasma extravasation following thermal injury. To validate these findings, the selective NK_1 receptor antagonist, SR140333 was administered to our model in a dosing regime identical to that described by Cao *et al*. (2000) where a similar reduction in the inflammatory response was seen in wildtype mice treated with SR140333 and NK_1 receptor knockouts following an intradermal injection of carrageenan. In this study, SR140333 completely ablated thermic oedema in wildtype animals at 10 min post-burn. These results are directly comparable to those of Siney & Brain (1996) who reported that SR140333 blocked thermal skin oedema in the rat at 5–35 min, but not at 65–95 min post-burn.

The kinins are formed from kininogen precursors by a group of serine proteases collectively known as the kallikreins (Burch *et al*., 1990). The biologically active peptide bradykinin and its metabolite, des-Arg^9 bradykinin are generated *via* activation of this system. In inflammation, bradykinin acts primarily through constitutive B_2 receptors to cause vasodilation and increased vascular permeability, while des-Arg^9 bradykinin is thought to have a greater selectivity for B_1 receptors (Hall, 1992). The kinins have been implicated in mediating post-burn plasma extravasation. Although the role of the B_2 receptor in mediating thermic oedema is well established (Wirth *et al*., 1992; Nwariaku *et al*., 1996), the relative contribution of the B_1 receptor is not known. As such, the second aim of this study was to investigate the effect of B_1 and B_2 receptor antagonists in a murine model of thermal injury. The B_1 antagonist, des-Arg^{10} Hoe 140 administered at a dose reported to selectively inhibit allergen-induced bronchial hyperresponsiveness in the rat (Huang *et al*., 1999) was seen to significantly reduce oedema formation in wildtype mice at 10 and 30 min post-burn, but not at 60 min. A second B_1 antagonist, des-Arg^9 Leu^8

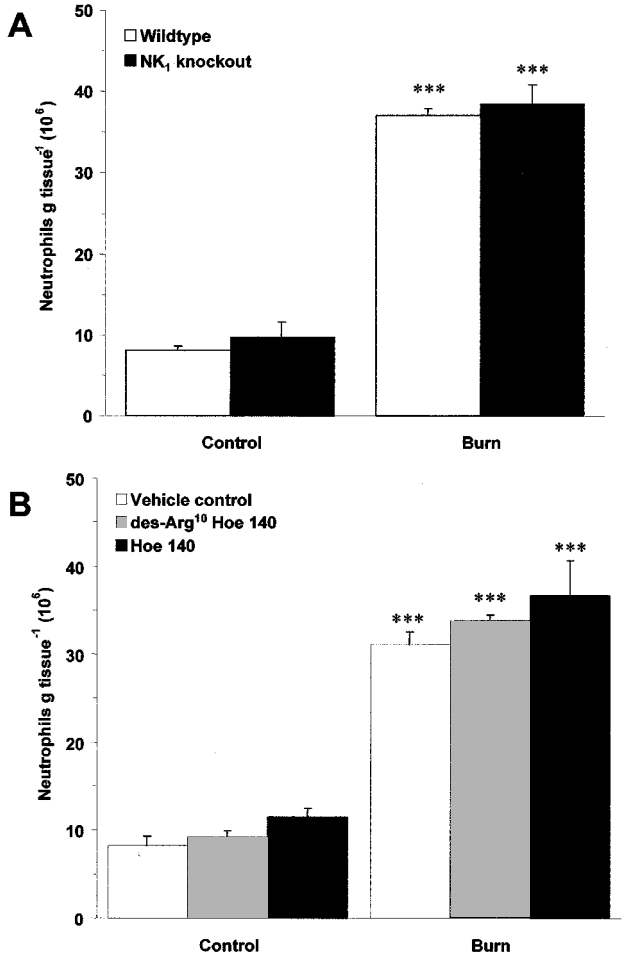

Figure 7 Effect of NK_1 deletion and kinin antagonists on thermic neutrophil accumulation. (A) Neutrophil accumulation, as assessed by MPO assay in the ears of wildtype and NK_1 knockout mice at 4 h post-burn (55°C). All data are presented as mean ± s.e.mean. ***$P<0.001$ vs wildtype control; $n=11$. (B) Neutrophil accumulation in the ears of wildtype mice pretreated (−5 min; i.v) with vehicle control, des-Arg^{10} Hoe 140 (1 μmol kg^{-1}) or Hoe 140 (100 nmol kg^{-1}) at 4 h post-burn (55°C). ***$P<0.001$ vs control in the same animal; $n=5$.

bradykinin, used at a dose previously shown to attenuate carrageenan induced neutrophil accumulation in Sv129 + C57BL/6 mice (Cao *et al*., 2000), also inhibited plasma extravasation at 30 min post-burn indicating a role for B_1 receptor. To confirm that the doses chosen to antagonize the B_1 receptor were acting in a selective manner, des-Arg^{10} Hoe 140 was co-administered with a dose of the B_1 agonist, des-Arg^9 bradykinin previously reported to exacerbate adjuvant induced arthritis in the rat (Rupniak *et al*., 1997). des-Arg^9 bradykinin prevented the inhibition of plasma extravasation seen with des-Arg^{10} Hoe 140 at 30 min post-burn. Taken together, the results suggest that kinin B_1 receptors mediate at least the early phase of thermic oedema in Sv129 + C57BL/6 mice. The effect of the B_2 antagonist, Hoe 140 in this model was also examined. A dose of Hoe 140 previously reported to prevent carrageenan induced neutrophil accumulation in Sv129 + C57BL/6 mice (Cao *et al*., 2000) suppressed the early phase of oedema formation, but not the later phase, indicating that as expected, the B_2 receptor mediates post-burn plasma extravasation.

These findings were of interest for a number of reasons. Firstly, to our knowledge this is the first time that kinin B_1 receptors have been demonstrated to have an active role in the burn wound. Secondly, the rapid activation of B_1 receptors seen in this model (e.g. 10 min post-injury) would indicate that they are present in the murine microvasculature in a constitutive form. This is interesting because B_1 receptors are generally not considered to be constitutively expressed in naïve tissues and are commonly reported to be upregulated in experimental animals following sub-acute exposure to proinflammatory cytokines (Campos *et al*., 1999) and bacterial endotoxins (Campos *et al*., 1996). However, there are a number of exceptions to this supposition. Functional studies indicate that constitutive B_1 receptors are present in the rat (Boschcov *et al*., 1984; Calixto & Medeiros, 1992; Campos & Calixto, 1994; Wotherspoon & Winter, 2000) and more recently it has been suggested that they mediate experimental pleurisy (Vianna & Calixto, 1998) and neuronal activity (Maas *et al*., 1995; Pesquero *et al*., 2000) in the mouse. In keeping with these studies, we surmise that constitutive B_1 receptors are activated by endogenous ligands to mediate the early stages of plasma extravasation following thermal injury. Nonetheless, it should be noted that the B_1 receptor antagonists des-Arg^{10} Hoe 140 and des-Arg^9 Leu^8 bradykinin had no effect on plasma extravasation at 60 min post-burn in our model. This is perplexing, since even in models where constitutive B_1 receptors are present, upregulation is still thought to occur following exposure to pro-inflammatory stimuli (Pesquero *et al*., 2000) and as such, it could be assumed that the B_1 component of response would increase with time, especially as the B_1 receptor is not prone to desensitization (Austin *et al*., 1997). However, it is well established that thermal injury results in the activation of many inflammatory pathways (Arturson, 1996) and at the later time point, it is conceivable that any B_1 receptor mediated effects could be masked by the involvement of other mediators.

It was observed that the pattern and time course of plasma extravasation following thermal injury was very similar in wildtype mice treated with kinin antagonists and NK_1 receptor knockout mice. To further investigate a link between these receptor systems, kinin receptor antagonists were administered to NK_1 receptor knockout mice prior to the induction of thermal injury. Neither B_1 or B_2 receptor blockade had any additional inhibitory effect on oedema formation at 30 min post-burn suggesting that the kinins interact with NK_1 related pathways in this model. In accordance with this concept, Yonehara *et al*. (1995) reported that plasma extravasation and substance P release was suppressed in burn wounds in kininogen-deficient rats. Furthermore, in other models of experimental inflammation, it has been reported that inducible B_1 (Ferreira *et al*., 2000) and constitutive B_2 receptors (Ricciardolo *et al*., 1994; Lindstrom & Andersson, 1997; Schuligoi *et al*., 1998) mediate tachykinin release from afferent sensory neurons. Taken together, we conclude that a similar interaction may occur in thermally injured tissues. In an effort to further understand this mechanism, the effect of the kinin antagonists on capsaicin induced ear oedema was examined in Sv129 + C57BL/6 wildtype mice. It was observed that the

B_2 (Hoe 140), but not the B_1 antagonist (des-Arg^{10} Hoe 140) attenuated plasma extravasation induced by capsaicin. A conceivable explanation for this is that the B_1 and B_2 receptors are found in different locations within the microvasculature. It is possible, that in murine skin, B_1 receptors are located prejunctionally on the sensory neurones since their blockade does not affect oedema induced by capsaicin, but it does prevent oedema induced by endogenous kinins (e.g. generated following thermal injury), which we suggest interact with sensory neurones to cause substance P release. In contrast, it would appear that a population of B_2 receptors are located postjunctionally on the post-capillary venule endothelial cells since their blockade can prevent sensory neuropeptide mediated plasma extravasation induced by capsaicin. Alternatively, the results may simply reflect the fact that the burn wound is an environment highly favourable to the production of the endogenous B_1 receptor agonist, des-Arg^9 bradykinin *via* the action of carboxypeptidase N on bradykinin, whereas other degradation pathways (e.g. plasma proteases) maybe of greater importance in removing bradykinin from dermal tissues acutely exposed to capsaicin. In addition, the differential effects of des-Arg^{10} Hoe 140 and Hoe 140 in the capsaicin ear oedema model would indicate that the doses chosen were selective to the kinin B_1 and B_2 receptors respectively.

In a separate series of experiments, the effects of NK_1 receptor deletion and kinin antagonists on neutrophil accumulation following thermal injury were examined. The relative importance of the NK_1 receptor in mediating neutrophil accumulation remains to be determined. Bozic *et al*. (1996) reported that neutrophil accumulation in the lung was suppressed in NK_1 knockout mice following immune complex challenge and exogenous substance P has been shown to cause neutrophil accumulation in a variety of animal models (Perretti *et al*., 1993; Baluk *et al*., 1995; Walsh *et al*., 1995). Furthermore, in the murine air pouch model, neutrophil accumulation stimulated by IL-1β is attenuated by both NK_1 antagonists (Ahluwalia *et al*., 1998). However, activation of NK_1 receptors does not induce neutrophil accumulation in naïve rat (Pinter *et al*., 1999) or mouse (Cao *et al*., 2000) skin. In agreement with these findings, it was observed that post-burn neutrophil accumulation in NK_1 receptor knockout mice was akin to that seen in wildtype mice at both 30 min and 4 h post-burn. As such, it would appear that the NK_1 receptor does not play a critical role in mediating neutrophil accumulation following cutaneous thermal injury. Similarly, neither the B_1 antagonist, des-Arg^{10} Hoe 140 or the B_2 antagonist, Hoe 140 had any effect on neutrophil accumulation in this model indicating that like the NK_1 receptor, the kinin receptors are not critical for neutrophil infiltration into the burn wound. This result was in direct contrast to the findings reported in other models of experimental inflammation where it has been shown that both the B_1 (Bozic *et al*., 1996; Ahluwalia & Perretti, 1996; Cao *et al*., 2000; McLean *et al*., 2000) and the B_2 receptor (Cao *et al*., 2000) can mediate leukocyte accumulation. However, as previously discussed, thermal injury initiates the activation of an array of inflammatory pathways and can lead to the production of cytokines (Ono *et al*., 1995), complement (Oldham *et al*., 1988) and leukotrienes (Dobke *et al*., 1987), all of which are potent neutrophil chemoattractants and/or activators. Thus, it is possible that kinin mediated neutrophil accumulation is masked by the involvement of other mediators in this model.

In conclusion, a murine model of thermal injury has been described and applied to the investigation of tachykinin and kinin involvement in the burn wound. We have demonstrated that tachykinin NK_1 receptors and the kinin B_1 and B_2 receptors play an essential role in mediating the early, but not the delayed phase of plasma extravasation following thermal injury. This is the first time that functional B_1 receptors have been shown to play a role in the burn wound and we propose that the kinin receptors are able to modulate the activity of tachykinins by stimulating their release from sensory neurones under these circumstances. Furthermore, we have shown that constitutive B_1 receptors are present in the murine microvasculature and suggest that des-Arg^9 bradykinin is generated in the burn wound. In addition, the possibility remains that the kinin B_1 and B_2 receptors are expressed in anatomically distinct regions of the murine microvasculature, the former being present on sensory neurones and the latter on the endothelium of post-capillary venules. If such mechanisms exist in man, it is possible that combinational therapy of tachykinin and kinin antagonists may be of therapeutic value in the treatment of accidental burns and other types of dermal trauma.

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