Neurogenic plasma leakage in mouse airways

^{*,1,2}Peter Baluk, ^{1,2}Gavin Thurston, ^{1,2}Thomas J. Murphy, ^{3,4}Nigel W. Bunnett & ^{1,2}Donald M. McDonald

¹Cardiovascular Research Institute, University of California, San Francisco, California 94143-0130, U.S.A.; ²Department of Anatomy, University of California, San Francisco, California 94143-0130, U.S.A.; and ³Department of Surgery, University of California, San Francisco, California 94143-0130, U.S.A.; ⁴Department of Physiology, University of California, San Francisco, California 94143-0130, U.S.A.

1 This study sought to determine whether neurogenic inflammation occurs in the airways by examining the effects of capsaicin or substance P on microvascular plasma leakage in the trachea and lungs of male pathogen-free C57BL/6 mice.

2 Single bolus intravenous injections of capsaicin (0.5 and 1 $\mu\text{mol kg}^{-1}$, i.v.) or substance P (1, 10 and 37 nmol kg^{-1} , i.v.) failed to induce significant leakage in the trachea, assessed as extravasation of Evans blue dye, but did induce leakage in the urinary bladder and skin.

3 Pretreatment with captopril (2.5 mg kg^{-1} , i.v.), a selective inhibitor of angiotensin converting enzyme (ACE), either alone or in combination with phosphoramidon (2.5 mg kg^{-1} , i.v.), a selective inhibitor of neutral endopeptidase (NEP), increased baseline leakage of Evans blue in the absence of any exogenous inflammatory mediator. The increase was reversed by the bradykinin B_2 receptor antagonist Hoe 140 (0.1 mg kg^{-1} , i.v.).

4 After pretreatment with phosphoramidon and captopril, capsaicin increased the Evans blue leakage above the baseline in the trachea, but not in the lung. This increase was reversed by the tachykinin (NK_1) receptor antagonist SR 140333 (0.7 mg kg^{-1} , i.v.), but not by the NK_2 receptor antagonist SR 48968 (1 mg kg^{-1} , i.v.).

5 Experiments using Monastral blue pigment as a tracer localized the leakage to postcapillary venules in the trachea and intrapulmonary bronchi, although the labelled vessels were less numerous in mice than in comparably treated rats. Blood vessels of the pulmonary circulation were not labelled.

6 We conclude that neurogenic inflammation can occur in airways of pathogen-free mice, but only after the inhibition of enzymes that normally degrade inflammatory peptides. Neurogenic inflammation does not involve the pulmonary microvasculature.

Keywords: Plasma leakage; endothelial cells; vascular permeability; substance P; capsaicin; Hoe 140; SR 140333; SR 48968; phosphoramidon; captopril

Abbreviations: ACE, angiotensin converting enzyme; NEP, neutral endopeptidase

Introduction

Capsaicin induces the release of substance P and other neurotransmitters from sensory nerves, causing plasma leakage *via* the activation of tachykinin NK_1 receptors on endothelial cells (Abelli *et al.*, 1991a; Bowden *et al.*, 1994; Jancsó *et al.*, 1967). The neurogenic plasma leakage, part of the phenomenon known as neurogenic inflammation (Geppetti & Holzer, 1996; McDonald, 1997), has been well characterized in rat and guinea-pig airways, but it is not clear whether such leakage occurs in airways of mice, and if so, under what circumstances. Published studies of mouse airways report apparently contradictory results; some investigators have failed to observe substance P-induced plasma leakage in mouse airways (Buckley & Nijkamp, 1994), whereas others have reported it (Figini *et al.*, 1997). In human airways, there is as yet no direct evidence for neurogenic inflammation (McDonald, 1997), and even high doses of capsaicin fail to induce plasma leakage (Greiff *et al.*, 1995).

Recently, a capsaicin receptor has been cloned and localized in sensory neurons in dorsal root ganglia of rats and mice (Caterina *et al.*, 1997), but it is not clear if it is present on the vagal sensory neurons that innervate the airways. In mouse

organs such as urinary bladder, skin, and parts of the gastrointestinal tract innervated by capsaicin-sensitive nerves, plasma leakage can occur in response to substance P, either given directly or released by capsaicin, or in response to bradykinin (Figini *et al.*, 1997; Inoue *et al.*, 1993; Maggi *et al.*, 1987a). Capsaicin-sensitive, substance P-containing nerves may have a role in mucosal exudation in mouse airways associated with a delayed hypersensitivity reaction (Buckley & Nijkamp, 1994) and in immune complex activation of the lung (Bozic *et al.*, 1996). However, it is not clear which anatomical categories of vessels become leaky, and to our knowledge, there are no reports of the effect of capsaicin on plasma leakage in mouse airways. Furthermore, it has been suggested that in addition to the major role played by NK_1 tachykinin receptors in neurogenic plasma leakage in the airways of some species, NK_2 receptors can also have a minor role (Tousignant *et al.*, 1993). However, there are no data on this point in mouse airways.

Our main goal was to determine whether neurogenic inflammation occurs in mouse airways. Another aim was to determine which airway blood vessels are involved, and whether there is any leakage in the lung. Lastly, we sought to examine the possible role of NK_1 and NK_2 receptors in neurogenic plasma leakage in the mouse trachea. Our strategy was to quantify the plasma leakage induced by substance P or

*Author for correspondence; E-mail: pbaluk@itsa.ucsf.edu

capsaicin using Evans blue dye as a tracer. Because tachykinins can be rapidly degraded by neutral endopeptidase (NEP) and angiotensin converting enzyme (ACE), we inhibited these enzymes, using phosphoramidon and captopril respectively. Because airway infections can change the activity of these enzymes (Borson *et al.*, 1989), increase the expression of tachykinin receptors (Baluk *et al.*, 1997), and potentiate plasma leakage (McDonald *et al.*, 1991), all studies used specific pathogen-free male C57BL/6 mice, a strain in which airway responses have been studied in 'knockout' mice (Bozic *et al.*, 1996; Figini *et al.*, 1997). Leaky vessels were identified histologically using Monastral blue as a marker of plasma leakage, and the labelled vessels in mice were compared to corresponding vessels in rats. To examine the relative contribution of NK₁ and NK₂ tachykinin and B₂ bradykinin receptors in the leakage we used the selective receptor antagonists SR 140333 and SR 48968 and Hoe 140.

Methods

Animals

Pathogen-free male C57BL/6 mice (20–25 g in weight) from Charles River Laboratories (Hollister, CA, U.S.A.) were maintained in a barrier facility to prevent respiratory tract infections. The mice were anaesthetized with ketamine (100 mg kg⁻¹, i.m.) and xylazine (5 mg kg⁻¹, i.m.), and their body temperature was maintained by a heating pad. In experiments done for comparison, pathogen-free male F344 rats (200–250 g) from Simonsen Laboratories (Gilroy, CA, U.S.A.) were treated in an identical fashion to the mice.

Experimental protocol

Mice were pretreated with vehicle (0.9% NaCl) or with phosphoramidon (2.5 mg kg⁻¹, i.v.), captopril (2.5 mg kg⁻¹, i.v.), or both. Five min later Evans blue dye (30 mg kg⁻¹, i.v.) or Monastral blue pigment (30 mg kg⁻¹, i.v.) was injected and immediately thereafter substance P (1, 10 and 37 nmol kg⁻¹, i.v.) or capsaicin (0.5 or 1 µmol kg⁻¹, i.v.) was injected over 20 s (to minimize apnea). A stock solution of 5 µmol ml⁻¹ capsaicin was prepared in a 1:1:8 mixture of ethanol, Tween-80 and 0.9% NaCl and diluted further for use with 0.9% NaCl; other drugs were dissolved in 0.9% NaCl. Baseline leakage of

Evans blue was measured in mice injected with 0.9 NaCl (1 ml kg⁻¹, i.v.). Some groups of mice were pretreated with the antagonists Hoe 140 (D-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-bradykinin; 0.1 mg kg⁻¹, i.v.) selective for bradykinin B₂ receptors (Wirth *et al.*, 1991), or with SR 140333 ((S)-1-[2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenyl)acetyl] piperidin-3-yl]ethyl]-4-phenyl-1-azoniabicyclo [2,2,2]octane chloride; 0.7 mg kg⁻¹, i.v.) selective for tachykinin NK₁ receptors (Emonds-Alt *et al.*, 1993), or with SR 48968 ((S)-N-methyl-[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)-butyl] benzamide; 1 mg kg⁻¹, i.v.) selective for NK₂ tachykinin receptors (Emonds-Alt *et al.*, 1992; Maggi *et al.*, 1993), administered 5 min before the phosphoramidon and/or captopril or before vehicle only. Five minutes after the inflammatory stimulus the chest was opened, both atria were cut, and the systemic circulation was perfused with a fixative containing paraformaldehyde (McDonald *et al.*, 1991) for 2 min at a pressure of 120–140 mmHg via an 18 gauge needle inserted into the left ventricle. In addition, the pulmonary circulation was flushed for 1 min at a pressure of 20 mmHg. Tissues (trachea, 5 mm thick sections of left lung containing the hilum and main bronchus, dorsal skin of hind foot, and urinary bladder) were dissected, gently blotted, and weighed. Evans blue dye was extracted from the tissues using formamide, measured spectrophotometrically and expressed as ng dye mg⁻¹ wet weight of tissue (Saria & Lundberg, 1983). Tracheas and urinary bladders used for identifying leaky vessels labelled by extravasated Monastral blue were prepared as whole mounts (McDonald *et al.*, 1991), and 20 µm thick cryostat sections were cut of the lung.

Materials used

We used the following reagents: capsaicin, captopril, formamide, Monastral blue, phosphoramidon, phosphate buffered saline, Tween 80 (Sigma Chemical Company, St. Louis, MO, U.S.A.), Evans blue (EM Sciences, Cherry Hill, NJ, U.S.A.), ketamine (Parke-Davis, Morris Plains, NJ, U.S.A.), substance P (Peninsula Laboratories, Belmont, CA, U.S.A.), and xylazine (Butler, Columbus, OH, U.S.A.). The selective NK₁ and NK₂ receptor antagonists SR 140333 and SR 48968, respectively, were gifts from Dr Xavier Emonds-Alt, Sanofi Recherche, Montpellier, France, and Hoe 140, a selective bradykinin B₂ receptor antagonist, was a gift from Dr Klaus Wirth, Hoechst, Frankfurt, Germany.

Table 1 Microvascular leakage of Evans blue in urinary bladder and skin of C57BL/6 mice

Treatment	Urinary bladder (ng dye mg ⁻¹ tissue)	Skin (ng dye mg ⁻¹ tissue)
Baseline	2.43 ± 0.22	3.85 ± 0.68
Substance P 1 nmol kg ⁻¹	5.88 ± 1.01	7.64 ± 4.03
Substance P 10 nmol kg ⁻¹	33.01 ± 7.91*	11.71 ± 2.26*
Substance P 37 nmol kg ⁻¹	21.66 ± 5.05*	10.40 ± 2.34*
Capsaicin 0.5 µmol kg ⁻¹	5.98 ± 1.61	4.98 ± 0.34
Capsaicin 1.0 µmol kg ⁻¹	20.28 ± 9.29*	4.50 ± 0.51
Phosphoramidon	13.30 ± 4.39*	5.30 ± 1.02
Captopril	20.68 ± 3.56*	7.02 ± 1.02
Phosphoramidon + captopril	31.55 ± 11.32*	7.98 ± 1.84
Hoe 140	2.19 ± 0.12	4.73 ± 0.83
SR 140333	2.66 ± 0.43	2.34 ± 0.53
Hoe 140 + phosphoramidon + captopril	3.70 ± 0.44*	7.03 ± 1.35*
Phosphoramidon + captopril + capsaicin 0.5 µmol kg ⁻¹	45.98 ± 5.91*	9.70 ± 2.27*
SR 140333 + phosphoramidon + captopril + capsaicin 0.5 µmol kg ⁻¹	18.98 ± 7.11*	7.00 ± 1.26

Microvascular leakage in urinary bladder and skin of anaesthetized pathogen-free C57BL/6 mice expressed as ng Evans blue dye mg⁻¹ wet weight of tissue. Values are means ± s.e.mean; *n* = 6–8 for all groups, except for substance P 1 nmol kg⁻¹ (*n* = 5). *Significantly different from baseline group (*P* < 0.05).

Statistics

Values are expressed as means $\pm$ s.e.mean. Differences between groups were determined by analysis of variance followed by the Dunn-Bonferroni *t*-test and were considered significant when $P < 0.05$.

Figure 1 Comparison of plasma leakage in (A) trachea and (B) urinary bladder of pathogen-free C57BL/6 mice 5 min after intravenous injection of substance P or capsaicin. Baseline values measured in mice after vehicle alone. Values are means $\pm$ s.e.mean $n = 5-8$ mice per group. * $P < 0.05$ compared to baseline group.

Results

In the absence of any stimulus, baseline leakage of Evans blue in the trachea, urinary bladder and other organs (Figure 1 and Table 1) was modest. In the absence of pretreatment, substance P (1, 10 and 37 nmol kg⁻¹, i.v.) or capsaicin (0.5 and 1 μmol kg⁻¹, i.v.) did not increase Evans blue leakage significantly above baseline in the trachea (Figure 1A). No blood vessels in the trachea were labelled with Monastral blue after substance P or capsaicin alone. Intravenous injection of capsaicin at doses up to 1 μmol kg⁻¹ caused a transient (<1 min) apnea.

In the same mice, substance P (10 and 37 nmol kg⁻¹) significantly increased Evans blue leakage in the urinary bladder and skin, although the response to 37 nmol kg⁻¹ was less than to 10 nmol kg⁻¹ (Figure 1B and Table 1). On average, the leakage in the urinary bladder increased 2 and 8 fold after the 0.5 and 1 μmol kg⁻¹ i.v. doses of capsaicin (Table 1).

Pretreatment with Hoe 140 significantly reduced baseline leakage in the trachea, but not in the urinary bladder or skin (Figure 2 & Table 1). Pretreatment with SR 140333 did not influence baseline in any organ (Figure 2 & Table 1). After pretreatment with captopril alone, but without capsaicin or substance P, there was a significant increase in the Evans blue leakage in the trachea (Figure 2). Captopril plus phosphoramidon had a slightly larger effect, but phosphoramidon alone had no effect (Figure 2). The leakage induced by captopril plus phosphoramidon was blocked by pretreatment with Hoe 140 (Figure 2). Similar results were found in the urinary bladder (Table 1).

Capsaicin produced a further dose-dependent increase in Evans blue leakage in the trachea of mice pretreated with captopril plus phosphoramidon (Figure 3). This increase was prevented by pretreatment with SR 140333, but not by SR

Figure 2 Increased plasma leakage in tracheas of pathogen-free C57BL/6 mice pretreated with phosphoramidon plus captopril 5 min before Evans blue and vehicle. Leakage is not increased when mice are pretreated with the bradykinin B₂ receptor antagonist Hoe 140 10 min before Evans blue. Pretreatment with Hoe 140 alone, but not SR 140333 alone, reduces baseline leakage. Values are means $\pm$ s.e.mean; $n = 6-8$ mice per group. * $P < 0.05$ compared to baseline group.

48968 (Figure 3 and Table 1). However, neither SR 140333 nor SR 48968 blocked the component of the leakage produced by captopril plus phosphoramidon pretreatment. Similar results were found in the urinary bladder and skin (Table 1).

In the lung, baseline Evans blue values were higher than in the trachea and other tissues, but none of the treatments significantly increased the values above this baseline (Figure 4).

Monastral blue labelling showed that in the trachea the leaky vessels after captopril plus phosphoramidon pretreatment, followed by capsaicin were postcapillary venules and collecting venules (Figure 5A). The labelled venules in the mouse trachea were less branched and less abundant than in comparably treated rats (Figure 5B). After phosphoramidon and captopril pretreatment alone a few postcapillary venules were also labelled. The distribution pattern was similar to that observed after pretreatment and subsequent capsaicin injection, but the number of labelled vessels and intensity of labelling was much less. Monastral blue labelled venules extended to the extrapulmonary bronchi. Corresponding Monastral blue labelled venules were also found in the urinary bladder and skin. In cryostat sections of mouse lung, some postcapillary venules around the larger intrapulmonary bronchi with smooth muscle were labelled (Figure 5C), but smaller bronchioles and the pulmonary vasculature were not (Figure 5D). No labelled vessels were found in alveolar walls.

Discussion

The main finding of this study was that neurogenic plasma leakage could be induced in C57BL/6 mouse airways, but only

after the inhibition of the enzymes that degrade tachykinins. The leaky vessels in the trachea and bronchi were postcapillary venules supplied by the systemic circulation. Neurogenic plasma leakage appeared to be mediated by NK₁ receptors. Blood vessels of the pulmonary circulation did not become leaky. In the trachea, leaky blood vessels were less abundant, and simpler in their branching pattern in mice than in rats.

Injection of even quite high doses of substance P and capsaicin failed to induce plasma leakage in airways of normal C57BL/6 mice. These inflammatory stimuli were sufficient to induce leakage in other organs supplied by sensory nerves, indicating that sufficient amounts of tachykinins were available to induce leakage in these organs. The highest dose of substance P used is more than that required to induce maximal leakage in the trachea of rats and guinea-pigs (Brokaw & White, 1994; Rogers *et al.*, 1988), and this dose produces severe hypotension (Maggi *et al.*, 1987b). We did not investigate higher doses of capsaicin because of the risk of fatal apnea.

According to the original description, the characteristics of neurogenic inflammation are plasma leakage and vasodilatation (Jancsó *et al.*, 1967). In recent years, however, the term neurogenic inflammation has been used somewhat more broadly and vaguely, and has come to include other responses to activation of sensory nerves, including smooth muscle contraction, hypersecretion, mast cell degranulation, and activation of immune cells (Geppetti & Holzer, 1996). In the present report, we adhere to the original definition for clarity and for consistency with our earlier publications (McDonald, 1997).

Neurogenic inflammation apparently does not occur in species such as hamsters and rabbits that have a higher

Figure 3 Capsaicin-induced leakage in tracheas of pathogen-free C57BL/6 mice pretreated with captopril alone or with phosphoramidon plus captopril given 5 min before Evans blue injection. The increase in leakage induced by capsaicin is prevented by pretreatment with the NK₁ receptor antagonist SR 140333 10 min in advance. Values are means $\pm$ s.e.mean; $n = 6-8$ mice per group. * $P < 0.05$ compared to baseline group. † $P < 0.05$ compared to phosphoramidon + captopril pretreated group.

Figure 4 Plasma leakage in the middle portion of left lung containing the main bronchus of pathogen-free C57BL/6 mice. None of the treatments induced leakage significantly different from the baseline group. Values are means $\pm$ s.e.mean; $n = 5-8$ mice per group.

Figure 5 Photomicrographs of Monastral blue-labelled venules in whole mount preparations of tracheas from pathogen-free (A) C57BL/6 mice and (B) F344 rats, and in cryostat sections of mouse lung (C, D) after pretreatment with phosphoramidon plus captopril and challenge with capsaicin $0.5 \mu\text{mol kg}^{-1}$ (i.v.). (A) Labelled postcapillary venules (arrow) are sparse in mouse trachea (c, cartilage), but are more abundant (arrows) and more branched in rat trachea (B). (C) Mouse main stem intrapulmonary bronchus photographed by Nomarski differential interference microscopy (e, epithelium; sm, smooth muscle; arrow, Monastral blue-labelled postcapillary venule in bronchial adventitia). (D) Vessels around the epithelium (e) of a smaller bronchiole and vessels in the wall of an alveolus (al) are unlabelled. Scale bar: $200 \mu\text{m}$ for (A, B) and $50 \mu\text{m}$ for (C, D).

tolerance to capsaicin than mice, rats, and guinea-pigs (Glinsukon *et al.*, 1980; McDonald, 1997). It is still not clear whether neurogenic inflammation really occurs in human airways (Baluk & McDonald, 1998; Barnes, 1996; McDonald, 1997). Although human airways undoubtedly contain sensory nerves, few of them contain substance P (Hislop *et al.*, 1990; Luts *et al.*, 1993). On the other hand, administration of substance P onto the nasal mucosa causes an increased amount of albumin in nasal secretions, and this can be interpreted as a manifestation of plasma leakage (Braunstein *et al.*, 1991). However, there is no evidence that capsaicin can induce plasma leakage in human nasal mucosa (Greiff *et al.*, 1995). In this respect, the situation in mouse airways, where it is rather difficult to induce neurogenic inflammation, is more similar to human airways than to rat or guinea-pig airways, where it is easy to induce leakage.

In agreement with previous studies in rats (Brokaw & White, 1994), we found that inhibition of neutral endopeptidase and angiotensin converting enzyme by phosphoramidon and captopril can elicit leakage even in the absence of capsaicin or substance P, suggesting that endogenous mediators are involved. Mice also resemble guinea-pigs and rats (Brokaw & White, 1994; Lötvall *et al.*, 1991) in that inhibition of neutral endopeptidase alone by phosphoramidon is insufficient to increase leakage above baseline levels in the airways, although it can do so in the urinary bladder (Lu *et al.*, 1997). A likely candidate for the endogenous inflammatory mediator that increases leakage in airways after captopril plus phosphor-

amidon is bradykinin. Evidence for this possibility is that bradykinin is efficiently degraded by angiotensin converting enzyme (Welches *et al.*, 1993) and can induce dose-dependent leakage in the mouse trachea (Figini *et al.*, 1997). The increased leakage was prevented by the bradykinin B_2 receptor antagonist Hoe 140, and pretreatment with Hoe 140 alone actually reduced baseline leakage.

It is unclear why different investigators have found different amounts of substance P-induced plasma leakage in mouse airways. Differences in routes of administration, measurement techniques, and the strains of mice used (Balb/c, Swiss-Morini, and C57BL/6) may contribute (Buckley & Nijkamp, 1994; Figini *et al.*, 1997, present study). Another possibility is the presence of undetected respiratory tract infections. In rat airways, *Mycoplasma pulmonis* and Sendai virus infections can potentiate the effects of substance P (McDonald *et al.*, 1991; Piedimonte *et al.*, 1990). A reduction in neutral endopeptidase activity may be involved (Borson *et al.*, 1989). *M. pulmonis* infections can also increase expression of NK_1 receptors on endothelial cells (Baluk *et al.*, 1997). Mice with *M. pulmonis* infection appear to resemble rats in this respect and their airway blood vessels become more sensitive to substance P, so that substance P (3.7 nmol kg^{-1} , i.v.; lower than the doses used here) induced significant leakage in infected mice, but not in pathogen-free controls (Thurston *et al.*, 1998). Therefore, only specific pathogen-free mice were used in the present study.

We examined whether NK_1 or NK_2 receptors mediate the leakage in mouse airways by blocking the capsaicin-induced

increase in leakage with the selective receptor antagonists SR 140333 and SR 48968. We found that the NK₁ receptor antagonist blocked the response but the NK₂ antagonist did not, and we conclude that neurogenic plasma leakage in the mouse trachea is mediated by NK₁ receptors, with no contribution from NK₂ receptors detectable by our technique. Similar findings have been reported for neurogenic inflammation in mouse skin (Inoue *et al.*, 1997), and rat airways and urinary bladder (Abelli *et al.*, 1991b; Ahluwalia *et al.*, 1994).

Leaky vessels were less numerous in mice than in rats. In addition to the smaller sizes of airways and volumes of tissue, substance P-containing nerves may be sparser. The trachea of mice resembles bronchioles of larger species and sensory nerve fibres have been reported to be sparse in the mucosa and absent in the epithelium (Pack *et al.*, 1984). In contrast, substance P-containing nerve fibres are abundant in the epithelium of guinea-pig and rat trachea (Baluk *et al.*, 1992; Lundberg *et al.*, 1984). In mouse lungs substance P-containing

nerve fibres supply the airways, but not the intrapulmonary blood vessels (Verastegui *et al.*, 1997). Finally, in mice, as in rats and guinea-pigs, only postcapillary venules of the tracheal and bronchial circulation become leaky, and vessels of the pulmonary circulation appear unresponsive to tachykinins or bradykinin.

We conclude that neurogenic inflammation can occur in mouse airways after the inhibition of enzymes that normally degrade inflammatory peptides, but does not involve the pulmonary microvasculature.

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