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Reduced inflammation in genetically hypertensive rat airways is associated with reduced tachykinin NK_1 receptor numbers

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Abstract

The airways of the genetically hypertensive rat (GH) are hyperinnervated by substance P-containing sensory nerves and exhibit reduced inflammatory responsiveness to substance P and to capsaicin. The present study measured tracheal inflammation to resiniferatoxin (1.0 μg/kg i.v.), a capsaicin analogue, which lacks the hypotensive action of capsaicin itself, alone or after the neuronal nitric oxide synthase inhibitor 1-(2-trifluoromethylphenyl)imidazole (TRIM) (50 mg/kg i.p.). The inflammatory response to resiniferatoxin alone was 50% lower in untreated GH than in control rats, a similar strain difference to that seen previously with capsaicin. Pre-treatment with TRIM had no effect on inflammation in either strain. Binding kinetics of the tachykinin NK_1 receptor antagonist [^{3}H](*S*)-1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl}-4-phenyl-l-azoniabicyclo[2,2,2,]octane chloride ([^{3}H]SR140333)-(0.125-16.0 nM) showed 50% reduction of B_{max} in GH versus control tracheae (74 ± 13 cf.165 ± 26 fmol/mg protein). Our results indicate that the reduced neurogenic inflammatory responsiveness in GH rats can be attributed entirely to reduced tachykinin NK_1 receptor numbers.

Keywords: Tachykinin NK_1 receptor; Substance P; Inflammation; Hypertension genetic; GH (genetically hypertensive) rat; Nitric oxide (NO)

1. Introduction

Substance P released from polymodal sensory nerve endings is a major mediator of the normal inflammatory response and contributes to both vasodilation and increased venular permeability (Lembeck and Donnerer, 1981; Kenins et al., 1984; Chahl, 1991). Sensory axons of this class are widely distributed throughout the body, including the airways (Maggi and Meli, 1988). It has been shown that asthmatics have increased numbers of intrapulmonary substance P-containing axons and exhibit increased levels of substance P in broncho-alveolar lavage, relative to non-asthmatic control subjects (Ollerenshaw et al., 1991). As well, lavage levels of substance P are elevated further by allergen challenge (Nieber et al., 1992; Khwaja and Rogers, 1996). These findings, together with others, support a crucial role for neurogenic substance P in the airway oedema that characterizes acute asthma (Barnes and Belvisi, 1997). It is therefore of some importance to understand the factors that regulate this pathway.

The genetically hypertensive inbred strain of rat (GH) possesses elevated numbers of substance P-containing sensory neurons, relative to a genetically related normotensive control strain; and GH airways show hyper-innervation by substance P-containing axons (Bakhle and Bell, 1994). Paradoxically, however, a recent study of neurogenic airways oedema demonstrated that inflammatory responses to both substance P and to sensory nerve activation by the selective neurotoxin capsaicin are substantially reduced in the GH although responsiveness to an unrelated inflammatory mediator, serotonin, is normal (Bakhle, et al., 1999).

We have now examined the basis for the difference between airways inflammatory responsiveness to substance P in GH and in control rats. We reasoned that one or more of three mediator steps could be involved. First, both substance P and capsaicin cause generalised vasodilation, with capsaicin in addition producing profound bradycardia and hypotension due to activation of the Bezold–Jarisch reflex (Donnerer and Lembeck, 1982). This effect is more

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pronounced in GH than in control rats (Bakhle et al., 1999), so it could reduce venular hydrostatic pressure and consequently reduce oedema formation. Second, as the inflammatory effect of substance P appears to be mediated in part via nitric oxide (Hughes et al., 1990; Kajekar et al., 1995) and production of nitric oxide is decreased in the GH strain (Winquist et al., 1984; Ledingham and Laverty, 1997), the transduction of this step of the inflammatory pathway may be inhibited in the GH strain. Finally, the number or affinity of receptors for substance P might be decreased in GH tissues. The present paper reports the results of experiments designed to differentiate between these possibilities, using a capsaicin analogue that is devoid of hypotensive circulatory actions, a nitric oxide synthase (NOS) inhibitor to block the production of nitric oxide in vivo and radioligand-binding techniques to quantify the kinetics of tachykinin NK_1 receptor behaviour.

2. Materials and methods

2.1. Animals

The rats used were age-matched adult males (12–20 weeks) bred in the Trinity College Bioresources Unit from stock obtained from the Wellcome Medical Research Institute, University of Otago (New Zealand) and consisting of GH and control animals from the colony that produced the original GH strain (Phelan, 1968). The integrity of each line was confirmed by establishing that the systolic arterial blood pressures of adults of each generation, as measured by tail-cuff plethysmography or by direct arterial cannulation, were consistently < 145 mm Hg in control animals and > 190 mm Hg in GH animals. All experimental protocols were approved in advance by the local ethics committee.

2.2. Microvascular permeability studies

Drugs used were Evans blue, 6,7-deepoxy-6,7-dihydro-5-deoxy-21-dephenyl-21-(phenylmethyl)-daphnetoxin (20-4-hydroxy-3-methoxybenzeneacetate, resiniferatoxin) and 1-(2-trifluoromethylphenyl)imidazole (TRIM). TRIM was supplied by Lancaster Synthesis (UK); the other drugs were supplied by Sigma-Aldrich. Evans blue was dissolved at 30 mg/ml in normal saline containing heparin 300 IU/ml and passed before use successively through a 5.0 μm and a 0.2 μm Millipore filter. Resiniferatoxin was dissolved in ethanol–Tween 80 (1:1; v/v) to give a stock solution of 0.1 mg/ml and was subsequently diluted with saline to 1 μg/ml. The vehicle used as a control was the same ethanol–Tween mixture diluted 100-fold with saline. TRIM was dissolved in normal saline at a concentration of 4 mg/ml.

Rats were anaesthetized with sodium pentobarbitone (60 mg/kg i.p.), placed on a stainless steel operating plate maintained at 35°C and allowed to breathe spontaneously. A polyethylene cannula (PE50) was passed down one internal jugular vein to the region of the right atrium for injection of drugs. In three animals, a second cannula was inserted into the right femoral artery for measurement of mean arterial blood pressure, via a Statham P23AC pressure transducer.

Evans blue (30 mg/kg) was injected via the jugular vein catheter, followed 90 s later by resiniferatoxin (1 μg/kg), or an equal volume of vehicle. After a further 5 min, the chest was opened and the rat was perfused transcardially with 120 ml isotonic saline at a pressure of 100 cm water. The trachea was then dissected free, blotted dry and weighed and the Evans blue content was extracted in 1.5 ml formamide (Sigma) overnight. The optical density of each extract was measured at 630 nm in a microplate reader and the Evans blue concentration was calculated from a standard curve of Evans blue in formamide, prepared and determined on the same day. Extravasation was expressed as ng dye/(mg wet weight tissue).

In a second series of rats of each strain, injection of Evans blue was preceded 30 min earlier by intraperitoneal administration of TRIM (50 mg/kg) or an equal volume of normal saline and followed by intravenous injection of resiniferatoxin (1 μg/kg) or an equal volume of its vehicle.

2.3. Ligand-binding experiments

Rats were overdosed with sodium pentobarbitone (180 mg/kg i.p.). Tracheae or C6 spinal cords were suspended in 2 ml of ice-cold 50 mM Tris–HCl buffer (pH 7.4 at 4°C) containing 0.32 M sucrose, 4 μg/ml leupeptin, 4 μg/ml chymostatin, 4 μg/ml bacitracin, and 20 μM phenylmethylsulphonyl fluoride and homogenised with a PowerGen 125 homogenizer at 5000 rpm in 10 s bursts. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C to remove debris, the supernatant was centrifuged at $15\,000 \times g$ for 30 min at 4°C and the resulting pellet was resuspended in binding buffer (50 mM Tris hydrochloride buffer (pH 7.4) containing 10 μM phosphoramidon, 4 μg/ml leupeptin, 4 μg/ml chymostatin, 40 μg/ml bacitracin, 3 mM manganese chloride and 0.2% w/v bovine serum albumin), frozen and stored at −80°C. Protein concentrations were determined using the Bradford assay from a bovine serum albumin standard curve.

For saturation experiments, aliquots of approximately 50 μg of tracheal or 200 μg of spinal cord membrane preparation were incubated in triplicate with a range of concentrations (0.125–16.0 nM) of the NK_1 receptor antagonist [^{3}H](*S*)-1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2,2,2,]octane chloride ([^{3}H]SR140333; Amersham, UK; specific activity 26.0 Ci/mmol) for 1 h at 25°C in a final volume of 200 μl of binding buffer. Non-specific

binding was defined as the binding in the presence of substance P (10 μM). The reaction was terminated by rapid filtration under reduced pressure over Whatman GF/B glass fibre filters that had been presoaked for 1 h at 4°C in 50 mM Tris–HCl buffer (pH 7.4) containing 0.05% bovine serum albumin. The filters were washed with 5 × 1 ml of ice-cold 50 mM Tris–HCl buffer and left to dry for 2 h at 20°C. Each filter was counted in 3-ml scintillation fluid (Cocktail-T) in a liquid scintillation counter (Packard 1500).

Binding parameters for the saturation studies of the tachykinin NK_1 receptors (K_d — the equilibrium dissociation constant, and B_{max} — the maximum number of binding sites) were determined by regression analysis of data using the computer programme InStat (GraphPad Software).

2.4. Statistical analysis

Results were expressed as mean ± S.E.M. and differences between means were assessed by Welch's test (extravasation) or by a two-tailed, unpaired Student's *t*-test (ligand binding), using the Instat programme. In all cases, a P value of < 0.05 was taken to denote significance of difference.

3. Results

3.1. Microvascular permeability responses

Tracheae from animals that had received only vehicle after Evans blue showed minimal extravasated dye. The extent of this baseline extravasation was similar for both control and GH strains (control 31 ± 6 ng dye/(mg wet weight), $n = 5$; GH, 25 ± 2 ng dye/(mg wet weight), $n = 6$; $P > 0.27$) (Fig. 1) and these values were also similar to the comparable vehicle data reported in a previous study, using the same strains of rat (Bakhle et al., 1999).

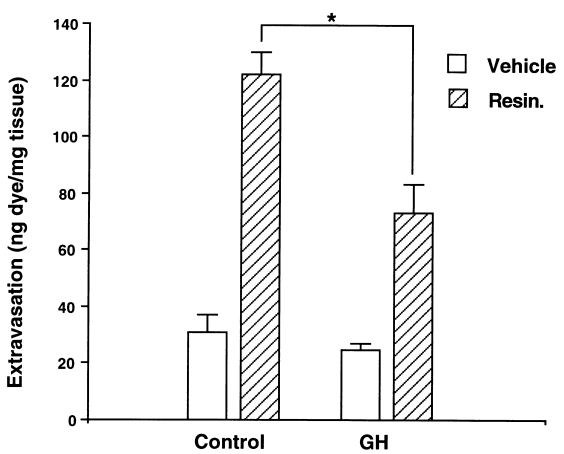

Fig. 1. Increased microvascular permeability in the tracheae of control and GH rats following systemic administration of resiniferatoxin (1 μg/kg i.v.). In both strains, dye extravasation was markedly increased by resiniferatoxin (hatched columns) over that induced by vehicle alone (open columns); however, the effect of resiniferatoxin in GH was only around one-half that in control animals ($^*P < 0.01$). Values shown indicate means ± S.E.M., vehicle $n = 5$ for control, 6 for GH, resiniferatoxin $n = 5$ for control, 8 for GH.

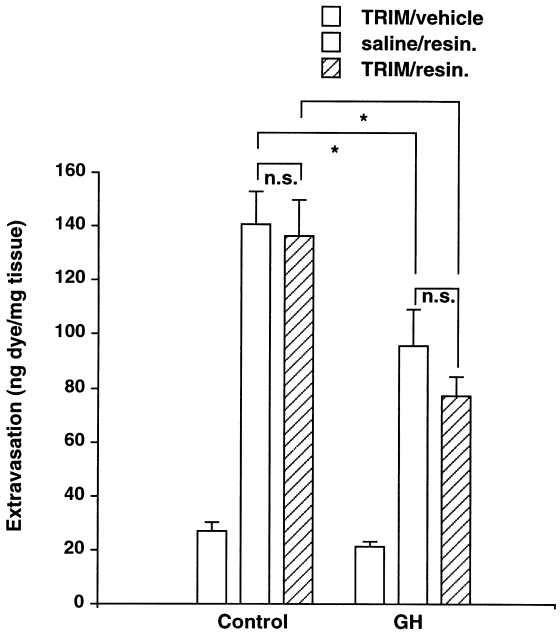

Fig. 2. Increased microvascular permeability in the tracheae of control and GH rats following systemic administration of resiniferatoxin (1 μg/kg) after pretreatment with saline (stippled columns) or TRIM (50 mg/kg, hatched columns). Baseline values where resiniferatoxin was substituted by vehicle following TRIM are shown by the open columns. Responses to resiniferatoxin were significantly less in tracheae of GH than of control animals in both TRIM-and saline-pretreated groups ($^*P < 0.02$). However, the effect of resiniferatoxin was not altered significantly (n.s.) in either strain by pretreatment with TRIM. Values shown indicate means ± S.E.M., TRIM/vehicle $n = 4$ for each strain; TRIM/resiniferatoxin and saline/resiniferatoxin $n = 5$ for each strain.

The 1 μg/kg dose of resiniferatoxin used was chosen on the basis of preliminary studies on control animals, because this dosage caused a substantial and reproducible degree of tracheal extravasation above vehicle levels, comparable to that produced in our previous study by 75 μg/kg capsaicin (Bakhle et al., 1999), where the mean value for extravasated Evans blue was 136 ng dye/(mg wet weight). The mean value for extravasated Evans blue obtained with resiniferatoxin in control rats was 122 ng dye/(mg wet weight) ($n = 5$). By contrast, extravasation was markedly less ($P < 0.01$) in tracheae of GH animals ($n = 8$), with a mean value of only 73 ng dye/(mg wet weight) (Fig. 1). In three control animals examined, resiniferatoxin produced rapid elevation of mean blood pressure, as reported previously by Szolcsanyi et al. (1990). This response reached a maximum of 40–50 mm Hg and then declined towards resting levels over the ensuing 4–5 min. A qualitatively similar pressor response was seen in

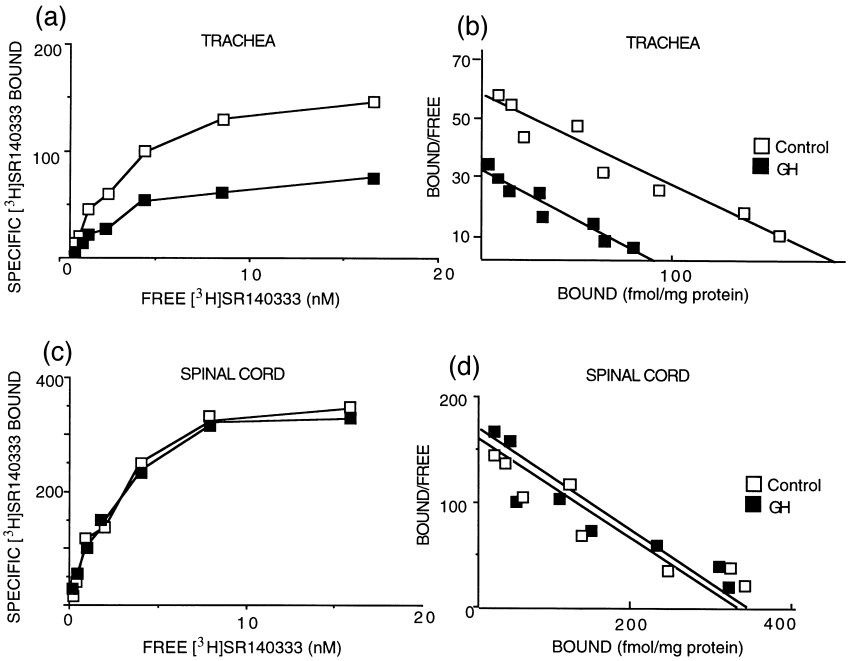

Fig. 3. Representative saturation curves (a,c) and Scatchard plots (b,d) of [^{3}H]SR140333 binding to membranes isolated from tracheae (a,b) and spinal cords (c,d) of an control (open squares) and a GH (filled squares) rat. Membranes were incubated with a range of concentrations of [^{3}H]SR140333 for 1 h at 25°C and substance P (10 μM) was used to determine non-specific binding. Similar results were obtained from eight separate animals of each strain.

three GH animals, but the maximum rise in pressure did not exceed 20 mm Hg in any of these.

The baseline extravasation in the absence of resiniferatoxin was not significantly different after pretreatment with TRIM to that seen in untreated animals, for either strain (control 27 ± 3 ng dye/(mg wet weight); GH 21 ± 2 ng dye/(mg wet weight), $n = 4$ for each strain)(Fig. 2). Furthermore, the extent of extravasation in response to resiniferatoxin was not altered appreciably from that seen in non-pretreated animals by pretreatment with either TRIM or saline, in either strain ($n = 5$ in each group)(Fig. 2).

3.2. Ligand binding

The B_{max} and K_d values for [^{3}H]SR140333 binding in trachea and cervical spinal cord were estimated by Scatchard analysis and results of a typical experiment are illustrated in Fig. 3. In tracheae of control rats, B_{max} was 165 ± 26 fmol/mg protein and K_d was 0.30 ± 0.05 nM ($n = 8$). In tracheae of GH rats, B_{max} was reduced by approximately 50% ($P < 0.01$), to 74 ± 13 fmol/mg protein ($n = 8$). However, the K_d in GH tracheae was identical to that in control animals (0.31 ± 0.08 nM).

By contrast with the tracheal data, no strain differences were seen in the binding properties of [^{3}H]SR140333 in the spinal cord. The B_{max} values were 350 ± 120 and 302 ± 88 fmol/mg protein for control and GH, respectively, while the K_d values were 0.45 ± 0.05 and 0.49 ± 0.12 nM ($n = 4$ each strain).

4. Discussion

Resiniferatoxin is a vanilloid that acts similarly to capsaicin at the terminals of polymodal sensory axons, releasing intraneuronal substance P and inducing local inflammation (Szallasi and Blumberg, 1989; Szolcsanyi et al., 1990). In the present experiments, we found that extravasation in response to resiniferatoxin was substantially less in airways of the GH hypertensive rats than in those of its closely related normotensive control strain, confirming our previous observation with capsaicin (Bakhle et al., 1999) that the GH has reduced capacity for neurogenic airways inflammation. Resiniferatoxin has been shown to be up to several orders of magnitude more potent than capsaicin (Szallasi and Blumberg, 1989; Szolcsanyi et al., 1990) and this was confirmed by our finding that similar levels of Evans blue extravasation were induced by 1 μg/kg resiniferotixin as required administration of around 100 times this dose of capsaicin.

In some respects, however, actions of these two vanilloids differ. As well as causing generalised neurogenic inflammation, systemic capsaicin causes profound apnoea, bradycardia and hypotension, due to activation of the Bezold–Jarisch reflex (Donnerer and Lembeck, 1982).

Resiniferatoxin, by contrast, is virtually devoid of this reflex effect (Szolcsanyi et al., 1990). In our previous study, we observed that systemic capsaicin produced a far larger fall in blood pressure in GH than in control animals, although depressor responses to acetylcholine and to substance P were similar between the strains (Bakhle et al., 1999). In view of this, we reasoned that the attenuated inflammatory reaction of the GH to capsaicin could be secondary to the effect of this agent on intravenular hydrostatic pressure. The present experiments, however, demonstrate that the strain difference in inflammatory responsiveness is as pronounced with resiniferatoxin as with capsaicin. Thus, the difference cannot be attributed to a more pronounced effect of capsaicin on intravenular pressure in GH than in control animals. The pressor response to resiniferatoxin suggests a vasoconstrictor action of this agent, which could also reduce hydrostatic pressures in the microcirculation. However, the magnitude of the pressor response was, if anything, less in GH than in control animals. We conclude, therefore, that the strain difference in neurogenic inflammation is not secondary to a circulatory artefact but is related to the mediation of the inflammatory response itself.

Inflammatory responses to substance P or to sensory nerve stimulation, manifested as increased venular permeability and oedema, are decreased by inhibition of endogenous NOS (Hughes et al., 1990; Kajekar et al., 1995). Recently, neurogenic inflammation induced by electrical stimulation of the saphenous nerve in rats was shown to be decreased by treatment with a NOS inhibitor (TRIM) that is selective for the neuronal isoform of the enzyme (nNOS) (Towler et al., 1998). For the GH strain, there is already evidence for decreased endothelial-dependent vasodilation, relative to its control strain, both in isolated vessels (Winquist et al., 1984) and in intact animals (Ledingham and Laverty, 1997). Thus, this strain might possess a generally decreased capacity for nitric oxide production. To assess whether such a deficit could underlie the decreased neurogenic inflammatory response in GH, we compared extravasation induced by resiniferatoxin alone and after pretreatment with TRIM at the dose known to inhibit oedema induced by saphenous nerve stimulation in vivo (Towler et al., 1998). We found that TRIM did not attenuate extravasation in either control or GH tissues.

The lack of effect of nNOS inhibition in the GH demonstrated that the diminished response to resiniferatoxin was clearly not due to an abnormal release of nitric oxide in this strain. However, the failure of TRIM to modulate responses to resiniferatoxin in the control strain was unexpected. Although electrical stimulation and the vanilloid toxins both induce release of substance P from sensory neurones, the molecular mechanisms involved are different (Maggi, 1995). It is possible therefore that activation of the vanilloid receptor does not lead to the activation of nNOS in the same way as an axonally conducted depolarization of the nerve terminal.

The third putative mechanism that we had considered as a basis for this reduced response was some alteration in receptor processing of the substance P signal. This was investigated by quantifying the binding characteristics of [^{3}H]SR140333, a selective antagonist for the tachykinin NK_1 receptor that transduces the inflammatory response to substance P (Emonds-Alt et al., 1993). This antagonist has been shown previously to abolish entirely the extravasation induced by systemic capsaicin in our model (Bakhle et al., 1999). We found that the number of tracheal binding sites for [^{3}H]SR140333 was reduced by approximately 50% in tissues from GH rats compared to those from the control strain, while receptor affinity for the ligand was constant between the two groups of animals. The magnitude of reduction in binding sites was strikingly similar to the magnitude of reduction in inflammatory response, suggesting strongly that the lowered inflammatory capacity is due entirely to the change in receptor numbers.

The GH is known to possess elevated numbers of substance P-containing spinal sensory neurons, due to defective programmed cell death in early postnatal life, and this is associated with hyperinnervation by substance P-containing axons of a number of peripheral tissues, including the airways (Gurusinghe and Bell, 1989; Messina and Bell, 1991; Bakhle and Bell, 1994). Functional loss of receptors from the cell surface is a known feature of substance P/tachykinin NK_1 receptor interactions, resulting from internalization of the receptors following their activation (Garland et al., 1994; Quartara and Maggi, 1997). Since the cells of GH tissues are likely to be exposed chronically to elevated amounts of neurogenic substance P, it is tempting to speculate that this tachyphylactic process is the basis for the altered receptor population.

Interestingly, we found that there was no strain difference in numbers of tachykinin NK_1 receptor binding sites in an area of spinal cord that receives sensory inputs from the trachea. Spatial distribution of substance P-containing terminals in the dorsal spinal laminae correlates well with the density of tachykinin NK_1 receptors (McLeod et al., 1998), supporting the view that neurotransmission from these inputs to the spinal interneurons is likely to be mediated via this receptor type. The lack of strain difference in receptor numbers therefore suggests that tonic release of substance P from peripheral axon terminals may occur independently of anterograde sensory activation of the same axons.

In summary, we have demonstrated that the tracheae of GH rats show a reduced inflammatory response to resiniferatoxin, as well as to capsaicin. The inflammation is not modulated by endogenous nitric oxide derived from nNOS in either the GH or the control, normotensive strain. Thus, the decreased inflammatory response cannot be attributed to a defect in local nitric oxide generation. Ligand binding studies show that the tachykinin NK_1 receptors in GH airways have normal affinity for substance P; by

contrast, they are substantially reduced in numbers and this seems likely to be the cause of the reduced inflammatory response. There may be a causal relationship with the known increased innervation density of the airways by substance P-containing axons in the GH strain.

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