Rosiglitazone elicits \textit{in vitro} relaxation in airways and precision cut lung slices from a mouse model of chronic allergic airways disease

1,2\textsuperscript{*}Chantal Donovan, 3\textsuperscript{*}Simon R Bailey, 2Jenny Tran, 2Gertrudd Haitsma, 2Zaridatul A Ibrahim, 2Simon R Foster, 4Mimi L K Tang, 1,2,4\textsuperscript{+}Simon G Royce, 1,2\textsuperscript{+}Jane E Bourke

1\textsuperscript{Biomedicine Discovery Institute and Department of Pharmacology, Monash University, Clayton, Australia}
2\textsuperscript{Lung Health Research Centre, Department of Pharmacology and Therapeutics, University of Melbourne, Parkville, Australia}
3\textsuperscript{Faculty of Veterinary Science, University of Melbourne, Parkville, Australia}
4\textsuperscript{Department of Allergy and Immunology, Murdoch Children’s Research Institute, Royal Children’s Hospital, Melbourne, Australia}

\textsuperscript{*}joint first authors
\textsuperscript{+}joint senior authors

Author for correspondence:
Dr Jane E Bourke,
Biomedicine Discovery Institute and Department of Pharmacology,
Monash University,
Clayton, Victoria, 3800
Australia

Email: jane.bourke@monash.edu

Running title: Bronchodilator actions of rosiglitazone in mouse airways

Copyright © 2015 by the American Physiological Society.
25 Authorship contribution statement
26 CD, SRB, JT, GH, ZAI, SRF, SGR, JEB made substantial contributions to conception, design, acquisition, analysis and/or interpretation of data of the work; MLKT made substantial contributions to design of the work; CD, SRB, JT, GH, ZAI, SRF, JEB drafted the work; CD, SRB, JT, GH, ZAI, SRF, MLKT, SGR, JEB revised it critically for important intellectual content, gave final approval of the version to be published and agreed to be accountable for all aspects of the work.
Abstract

Rosiglitazone (RGZ), a Peroxisome Proliferator Activated Receptor γ (PPARγ) ligand, is a novel dilator of small airways in mouse precision cut lung slices (PCLS). In this study, relaxation to RGZ and β-adrenoceptor agonists were compared in trachea from naïve mice and guinea pigs, and trachea and PCLS from a mouse model of chronic allergic airways disease (AAD). Airways were pre-contracted with methacholine before addition of PPARγ ligands (RGZ, ciglitazone (CGZ) or 15-deoxy-PGJ₂) or β-adrenoceptor agonists (isoprenaline, salbutamol). Effects of T0070907 and GW9662 (PPARγ antagonists) or epithelial removal on relaxation were assessed. Changes in force of trachea and lumen area in PCLS were measured using preparations from saline-challenged mice and mice sensitised (days 0, 14) and challenged with ovalbumin (3 times/week, 6 weeks). RGZ and CGZ elicited complete relaxation with greater efficacy than β-adrenoceptor agonists in mouse airways but not guinea pig trachea, while 15-deoxy-PGJ₂ did not mediate bronchodilation. Relaxation to RGZ was not prevented by T0070907 or GW9662, or by epithelial removal. RGZ-induced relaxation was preserved in trachea and increased in PCLS after ovalbumin-challenge. Although RGZ was less potent than β-adrenoceptor agonists, its effects were additive with SALB and ISO, and only RGZ maintained potency and full efficacy in maximally contracted airways or after allergen challenge. Acute PPARγ-independent, epithelial-independent airway relaxation to RGZ is resistant to functional antagonism and maintained in both trachea and PCLS from a model of chronic AAD. These novel efficacious actions of RGZ support its therapeutic potential in asthma when responsiveness to β-adrenoceptor agonists is limited.
Keywords: Allergic airways disease, bronchodilation, lung slice, peroxisome proliferator activated receptor γ, rosiglitazone

Abbreviations: allergic airways disease, AAD; airway hyperresponsiveness, AHR; airway smooth muscle, ASM; ciglitazone, CGZ; cyclo-oxygenase, COX; 15-deoxy-Δ^{12,14}-prostaglandin J₂, 15-deoxy-PGJ₂; Depolarising Potassium Solution, DKS; dimethyl sulfoxide, DMSO; Dulbecco’s Modified Eagle’s Medium, DMEM; methacholine, MCh; nitric oxide synthase, NOS; ovalbumin, OVA; precision cut lung slices, PCLS; Peroxisome Proliferator Activated Receptor γ, PPARγ; prostaglandin E₂, PGE₂; rosiglitazone, RGZ; saline, SAL; salbutamol, SALB.
Introduction

There is increasing evidence that rosiglitazone (RGZ) and other agonists of peroxisome proliferator activated receptor γ (PPARγ) may offer therapeutic benefit in asthma. Numerous studies suggest that PPARγ ligands can inhibit allergen-induced inflammation and the development of airway remodelling and airway hyperresponsiveness (AHR) in vivo (reviewed in (8)), as well as exert direct dilator effects on airway smooth muscle in vitro (3, 12).

PPARγ expression is increased in bronchial biopsies from patients with asthma, including in the epithelial and smooth muscle (ASM) layers (2). In vitro, human ASM cytokine secretion and proliferation have been shown to be suppressed by the PPARγ agonists, RGZ and ciglitazone (CGZ) (10, 27, 30, 34, 37). However, only some of these anti-inflammatory and anti-remodelling activities were inhibited by the selective PPARγ antagonist GW9662, suggesting both PPARγ-dependent and -independent mechanisms (30, 34).

The effects of chronic treatment with PPARγ ligands have also been examined in mouse models of allergic airway disease (AAD), where sensitisation and nebulisation with ovalbumin (OVA) causes airway inflammation, airway wall remodelling and AHR to methacholine (MCh). In BALB/c mice, treatment with either RGZ or CGZ inhibited OVA-induced airway inflammation and fibrosis as well as the development of AHR (14, 32, 35), with the effects of the synthetic PPARγ ligands mimicked by AdPPARγ (15, 17) and abrogated by GW9662 (14, 15, 17, 35). Although RGZ also reduced the development of AHR in OVA-challenged C57BL/6 mice, the increase in BAL inflammatory cells in this mouse strain was not affected, suggesting that RGZ may also modulate AHR by a mechanism that is independent of inhibition of inflammatory cell
recruitment to the airway (33).

In addition to these inhibitory effects in models of chronic AAD, RGZ has been shown to elicit acute relaxation of mouse tracheal segments and intrapulmonary airways in precision cut lung slices (PCLS) pre-contracted with muscarinic agonists \textit{in vitro} (3, 12). In PCLS, RGZ elicited complete relaxation under conditions of impaired $\beta$-adrenoceptor agonist responsiveness, opposing contraction by inhibiting calcium oscillations and sensitivity (3). However, these novel direct dilator actions have yet to be confirmed when large and small airway reactivity to contractile agonists is altered by allergen challenge.

The aim of the current study was to further characterise dilator responses to RGZ relative to $\beta$-adrenoceptor agonists in isolated trachea from mice and guinea pigs, and to demonstrate its efficacy in both large and small airways from a mouse model of chronic AAD where \textit{in vitro} responsiveness to MCh is altered (6). Here, we confirmed that RGZ elicits tracheal bronchodilation, albeit at lower potency than either salbutamol (SALB) or isoprenaline (ISO). However, in contrast to these $\beta$-adrenoceptor agonists, both the potency and complete relaxation to RGZ were maintained in maximally contracted trachea. Despite increased \textit{in vitro} responsiveness to MCh in trachea after allergen challenge, RGZ-mediated bronchodilation was similar in trachea from saline- and OVA-challenged mice. Of particular interest, relaxation to RGZ was greater in small airways in PCLS from OVA-challenged mice relative to saline controls, whereas only partial $\beta$-adrenoceptor-mediated relaxation was observed. These results suggest that PPAR$\gamma$ agonists including RGZ may offer significant therapeutic advantages in asthma by exerting control over pathways that are not susceptible to inhibition by current treatments.
Methods

Chronic AAD model

For the chronic AAD model, female BALB/c mice were administered grade V chicken egg OVA (10 μg OVA per 0.4 mg aluminum potassium sulfate adjuvant (alum) in 0.5 mL saline i.p.) or equivalent volumes of adjuvant on days 0 and 14 followed by nebulised OVA (2.5 % w v⁻¹ saline) or nebulised saline three times per week between days 21 – 63 as previously described (20).

Myograph and organ bath experiments

Experimental details are as previously described (6). Mice were killed by a slow-fill asphyxiation method (80% CO₂ in 20% O₂) or by cardiac puncture under anaesthesia with ketamine (200 μg/g) and xylazine (10 μg/g). Guinea pigs were concussed by a blow to the head prior to exsanguination. Tracheae were removed, immersed in Krebs-Henseleit buffer (59 mM NaCl, 2.3 mM KCl, 0.69 mM MgSO₄, 7H₂O, 2.5 mM CaCl₂.6H₂O, 0.6 mM KH₂PO₄, 10 mM EDTA, 25 mM NaHCO₃ and 6 mM glucose, containing 3 μM indomethacin for guinea pig tissues only) and dissected into 2-4 mm segments. In some experiments, the tracheal epithelium was denuded by gently rubbing a cocktail stick inside the lumen. Epithelial removal was confirmed histologically and by a reduced dilator response to Substance P as previously described (1).

Airway segments were mounted under static conditions in temperature-controlled myograph baths (mouse, Danish MyoTechnology; Aarhus, Denmark) or standard organ baths (guinea pig) containing Krebs-Henseleit buffer (pH 7.4, 37°C, aerated with 95% O₂ / 5% CO₂) for continuous recording of changes in isometric
tension ($\Delta$mN) using Power Lab and Chart software (ADI Instruments). An optimal resting tension was determined based on the maximum response to Depolarising Potassium Solution (DKS: containing 123.7 mM K$^+$), prior to generation of a full concentration-response curve to MCh. Tissues were then re-contracted to 20%, 75% or 100% of the maximal MCh response, followed by cumulative addition of PPAR$\gamma$ agonists (RGZ, CGZ, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15-deoxy PGJ$_2$)), $\beta$-adrenoceptor agonists (SALB, ISO) or SALB in the presence of 10 $\mu$M RGZ (after 75% MCh maximal response only). After maximally effective dilator responses to SALB or ISO were established, the effects of subsequent addition of 100 $\mu$M RGZ were also tested (after 100% MCh maximal response only). The effects of PPAR$\gamma$ antagonists (T0070907, 10 $\mu$M or GW9662, 10 $\mu$M, added 15 min prior to MCh), a nitric oxide synthase (NOS) inhibitor (nitro-L-arginine (NOLA), 10 $\mu$M, 10 min) or a non-selective cyclo-oxygenase (COX) inhibitor (indomethacin, 10 $\mu$M, 15 min) on RGZ-induced relaxation were assessed. The effect of epithelial removal on RGZ-induced relaxation was also determined. Comparisons of dilator responses to RGZ and ISO were made between tracheae from saline- and OVA-challenged mice.

**Precision cut lung slice experiments**

PCLS were prepared as previously described (3, 6, 9). Mice were killed with an overdose of sodium pentobarbitone (0.45 ml, 60 mg ml$^{-1}$ i.p.). Tracheae were cannulated and the lungs inflated with warm ultra pure low melting point agarose (~1.2 ml of 2% w/v$^{-1}$ in Hank’s Balanced Salt Solution supplemented with 40 mM HEPES (sHBSS), Invitrogen). A small bolus of air was then used to push the agarose into alveolar spaces. After the agarose solidified (20 min, 4°C), a single lobe was mounted
in cold HBSS in a vibratome (VT 1000S, Leica Microsystems) to cut 150 μm slices commencing at the lung periphery. Slices were cultured overnight in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 1% penicillin-streptomycin solution (37°C, 5% CO₂).

Individual slices were then placed in a custom-made perfusion chamber between 2 cover glasses (~100 μL volume) and covered in fine wire mesh (Small Parts Inc.) with a small hole cut over a single airway (150 – 400 μm diameter). Viability was confirmed by observation of an intact epithelial layer displaying ciliary activity under phase contrast microscopy using 10 x objective lens, zoom adaptor, reducing lens and camera (Nikon Eclipse Ti-U; Pulnix CCD camera model TM-62EX). Digital images (744 x 572 pixels) recorded in time lapse (0.5 Hz) using image acquisition software (Video Savant; IO industries, Inc.) were converted to TIFF files and analysed using PC software NIH/Scion (Scion Corporation). Using an appropriate grey scale threshold to distinguish between the airway lumen and surrounding tissue, the lumen area in each image could be calculated by pixel summation.

Comparisons of constrictor and dilator responses were made in perfused PCLS from saline- and OVA-challenged mice. In matched PCLS from the same mice, full concentration-response curve to MCh were generated or airways were pre-contracted with MCh (100 nM) followed by cumulative addition of RGZ or SALB, delivered at a constant rate under gravity.

**Drugs and reagents**

acetyl-β-methacholine chloride (MCh), indomethacin, ISO, NOLA, penicillin-streptomycin solution, salbutamol hemisulphate salt (SALB), CGZ, 15-deoxy-PGJ₂,
GW9662, PGZ, RGZ and T0070907 from Cayman (USA); ultra pure low melting point agarose, 10 X HBSS, 1M HEPES buffer and DMEM from Gibco (Invitrogen, Australia).

Animal welfare and Ethics statements

Tissues were obtained from male and female BALB/c mice (Animal Resources Centre, Western Australia) and male guinea pigs (Biological Research Facility, University of Melbourne). All experiments were conducted with approval from Ethics Committees of the University of Melbourne (approval #070127, #0808107), and Murdoch Children’s Research Institute (approval #A597). All experimental procedures used were as humane as possible.

Balb/C mice and guinea pigs were housed in Biological Research Facility at the University of Melbourne; additional Balb/C mice were housed at the Murdoch Children’s Research Institute. All animals were housed on a 12:12 h day:night cycle and provided with food and water ad libitum.

For tracheal preparations, mice were killed by a slow-fill asphyxiation method (80% CO₂ in 20% O₂) or by cardiac puncture under anaesthesia with ketamine (200 μg/g) and xylazine (10 μg/g). For lung slice preparations, mice were killed by an overdose of sodium pentobarbitone (0.45 ml, 60 mg ml⁻¹ i.p.).

Data analysis and statistical procedures

All data are expressed as mean ± SEM for either contraction (ΔmN or % initial lumen area) or relaxation (% relaxation of MCh pre-contraction), with n representing the numbers of individual tissues from different mice. Concentration-response curves were fitted using a single-site curve-fitting model for estimation of EC₅₀ values and
maximum responses (GraphPad Prism 5 software). Since a maximum plateau response for RGZ was not achieved, pEC$_{50}$ values were estimated from relaxation curves and experimental responses to 100 μM RGZ are presented. Unpaired t-tests, paired t-tests with Bonferroni post hoc tests were used to analyse data where appropriate. P<0.05 was considered significant.

Results

RGZ causes relaxation of isolated mouse and guinea pig tracheal segments pre-contracted with MCh

Dilator responses to RGZ were compared with the β-adrenoceptor agonists SALB and ISO (Figure 1). A typical trace from an isolated mouse tracheal segment demonstrates bronchodilator responses to RGZ at concentrations above 1 μM (Figure 1A). After contraction to ~75% of the maximum response to MCh, cumulative addition of RGZ caused concentration-dependent and complete relaxation. Responses to either SALB or ISO were more rapid than RGZ, with plateau responses to each drug addition occurring within 2 min for the β-adrenoceptor agonists (not shown) versus 10 min for RGZ (Figure 1A). When measured after similar MCh pre-contraction, (Figure 1B) the potency of RGZ in mouse tracheal segments was lower than either β-adrenoceptor agonist. However, only RGZ was able to elicit complete relaxation (Figure 1C; Table 1).

In guinea pig tracheal segments, both β-adrenoceptor agonists were also more potent than RGZ, with all dilators fully reversing the matched MCh-induced contraction in this species (Figure 1D,E).
Relaxation to RGZ, but not β-adrenoceptor agonists, is maintained with increasing contraction of mouse tracheal segments

To assess the influence of the level of pre-contraction on dilator potency and efficacy, responses to RGZ (Figure 2A) were compared with SALB and ISO (Figures 2B, C) in mouse tracheal segments pre-contracted to low (20% MCh maximum), sub-maximal (75% MCh maximum) or high (100% MCh maximum) tone. Representative pre-contractions to MCh are shown (Figure 2D, prior to ISO).

RGZ maintained both its potency and capacity to cause near complete relaxation even in maximally contracted tissues (Figure 2A, Table 1). With increasing tone, both the potency and partial relaxation for SALB were decreased (Figure 2B, Table 1). Although ISO potency was not significantly reduced, the complete relaxation to ISO at low tone was reduced by > 80% in tissues contracted to high tone (Figure 2C, D, Table 1).

Relaxation to RGZ is additive with β-adrenoceptor agonists in mouse tracheal segments

To determine if RGZ had additive effects with β-adrenoceptor agonists, trachea were pre-contracted with MCh to 75% maximum, prior to assessment of SALB-mediated relaxation in the absence or presence of RGZ (Figure 3A). After a submaximal relaxation to RGZ was established (% relaxation to 10 μM RGZ alone: 31.4±9.5%), increasing concentrations of SALB elicited further relaxation, but the maximal response to combined treatment was still incomplete relaxation (SALB+RGZ 63.0±13.7%) (Figure 3A).

To determine if responses to RGZ were maintained after β-adrenoceptor-mediated relaxation had failed to overcome functional antagonism, dilator responses
were measured in airways maximally contracted with MCh (Figure 3B). Consistent with
earlier findings (Figure 2), the β-adrenoceptor agonists alone were relatively
ineffective in maximally contracted airways (% relaxation: 100 μM SALB 8±5%; 30 μM
ISO 26±7, n=4). However, subsequent addition of 100 μM RGZ elicited marked further
relaxation (RGZ + SALB 71±5%; RGZ + ISO 110±3%).

RGZ- and CGZ-induced relaxation is independent of PPARγ activation

To explore potential PPARγ dependence, the effects of RGZ were compared with two
other PPARγ ligands, the structurally-related CGZ and the putative endogenous agonist
15-deoxy-PGJ₂ (Figure 4A). In mouse tracheal segments pre-contracted with MCh to
similar levels, CGZ and RGZ elicited relaxation at similar potency and efficacy while 15-
deoxy-PGJ₂ had no effect (Figure 4A).

The effect of pre-incubation with either of the PPARγ antagonists T0070907
or GW9662, at concentrations previously shown to inhibit PPARγ activation (16, 18) on
relaxation to RGZ and CGZ was then tested. Both the potency and maximal agonist
effect of these dilators were maintained, with both causing near-complete relaxation
(Figure 4B-D).

RGZ-induced relaxation is epithelium-independent

The potential role of epithelial-derived factors in the dilator response to RGZ was
assessed. The level of MCh-induced pre-contraction was similar in matched tracheal
segments, irrespective of pre-incubation with inhibitors or the absence of an intact
epithelium (data not shown). RGZ-induced relaxation was maintained in the presence
of the COX inhibitor indomethacin and the NOS inhibitor NOLA (Figure 5A, B). RGZ also
still elicited complete relaxation, with no loss of potency, in preparations where epithelium-denudation was confirmed by a significant reduction in relaxation to the epithelium-dependent bronchodilator substance P (Figure 5C, D).

Contraction of isolated trachea and PCLS to methacholine are differentially altered in a model of chronic AAD

To confirm our previously reported effects of allergen challenge on contractile responses (6), concentration-response curves to MCh were prepared in tracheal segments and PCLS from saline- and OVA-challenged mice. Trachea from OVA-challenged mice were hyperresponsive to MCh (Figure 6A), with a greater than 50% increase in the maximum force of contraction (P<0.05 versus SAL, unpaired t-test). In PCLS following OVA challenge, MCh potency was decreased approximately 3-fold (Figure 6B) (P<0.05 versus SAL, unpaired t-test), with no significant change in the maximum reduction in airway area.

RGZ-induced relaxation of isolated mouse trachea is maintained in a model of chronic AAD

Comparisons of RGZ- and ISO-induced relaxation were made in trachea from saline- and OVA-challenged mice. Tissues were pre-contracted to 75% of the maximal MCh response in same preparation (Figure 6A), such that precontraction was relatively higher after allergen challenge, prior to addition of RGZ (Figure 7A, B). RGZ was equally effective at causing complete relaxation with no loss of potency in trachea after chronic exposure to OVA (Figure 7B, Table 2). ISO-mediated relaxation was also
maintained under these conditions, but failed to fully overcome the pre-contraction to MCh in tracheal segments from either saline- or OVA-challenged mice (Figure 7C, D).

RGZ potency is increased in small airways in PCLS from a mouse model of chronic AAD

Dilator responses to RGZ and SALB were compared in perfused PCLS from saline- and OVA-challenged mice, measuring changes in airway lumen area. Reduced MCh potency was established in PCLS from the same mice after OVA challenge (Figure 6B). The average submaximal precontraction with MCh before RGZ was also reduced, but the reduction in airway area in response to this single MCh concentration was variable between PCLS in both groups (Figure 8A).

RGZ induced complete relaxation in small airways from both saline- and OVA-challenged mice, at higher potency than trachea in both groups (Figure 8B, Table 2). As shown in traces from PCLS from saline- and OVA-challenged mice matched for precontraction, and also in combined results where MCh contraction was relatively lower, RGZ showed ~3-fold higher potency in PCLS after chronic OVA challenge (Figure 8B,C, Table 2). In contrast, SALB failed to fully reverse the MCh-induced contraction in either group (Figure 8E,F), while subsequent perfusion with 100 μM RGZ in the same preparations caused complete relaxation (Figure 8F).

Discussion and Conclusions

While inhaled β-adrenoceptor agonists remain the gold standard treatment for relief of asthma symptoms, their efficacy may be limited during a severe asthma attack and reduced by receptor desensitization associated with frequent use (11, 24, 25). There
has been increasing recent interest in identifying novel bronchodilators that target alternative mechanisms to overcome some of these limitations.

We have previously demonstrated that RGZ mediates relaxation of small airways in perfused mouse PCLS under conditions of reduced β-adrenoceptor responsiveness, inhibiting MCh-induced calcium oscillations and sensitivity (3, 7). In the current study, we have explored dilator responses to RGZ in both trachea and small airways, and confirmed its in vitro efficacy after chronic allergen challenge. In summary, we showed that tracheal relaxation to RGZ was not mediated by PPARγ, was independent of epithelial-derived relaxing factors, and unlike β-adrenoceptor agonists, was maintained with increasing contraction. Critically, RGZ overcame the hyperresponsiveness to MCh that persisted in vitro in tracheal segments from a mouse model of chronic AAD. Of note, responses to RGZ, but not SALB, were increased in small airways in PCLS from this model. These combined findings demonstrate acute PPARγ-independent dilator responses to RGZ in inflamed airways that are superior to β-adrenoceptor agonists. These actions are in addition to its previously reported inhibitory effects on chronic allergen-induced inflammation, airway wall remodelling and AHR (reviewed by (8)).

In establishing the therapeutic potential of novel dilator agents, it is critical to characterize their in vitro efficacy relative to existing therapy and to define their underlying mechanisms of action. Our previous studies have described bronchodilator responses to RGZ in small airways in situ in perfused PCLS, opposing contraction to MCh, serotonin and endothelin-1, with greater efficacy than SALB (3, 7). More recently, we have shown that RGZ and the bitter taste receptor agonist chloroquine, but not SALB or ISO, maintained their potency and efficacy following β-adrenoceptor
desensitization. However, only pre-treatment with RGZ, but not chloroquine or the β-adrenoceptor agonists, also inhibited the development of MCh-induced contraction and reduced MCh potency in PCLS (7).

We have now extended these studies to confirm an isolated report of dilator actions of RGZ in mouse trachea (12). We demonstrate that RGZ elicits full relaxation in both mouse and guinea pig tracheal segments, albeit at lower potency than the β-adrenoceptor agonists. In mouse trachea where β₁-adrenoceptors are known to play a major role in mediating relaxation (13), increasing the level of MCh pre-contraction markedly compromised the partial relaxation to both SALB and ISO. Consistent with our previous findings in PCLS (3), RGZ was able to maintain potency and elicit marked relaxation in maximally contracted trachea, overcoming the functional antagonism shown to limit β-adrenoceptor responsiveness (19).

Dilator responses mediated through β₂-adrenoceptors in guinea pig trachea may be of greater relevance to human airways (23, 36). The higher potency and efficacy of SALB in tracheal segments from guinea pigs relative to mice is consistent with this, while the finding of marked relaxation to RGZ at similar potency in both species is suggestive of potential benefit independent of β-adrenoceptor density.

We then explored the potential contribution of PPARγ activation to RGZ-mediated tracheal relaxation. We observed that the rates of relaxation to both RGZ and CGZ, another thiazolidinedione PPARγ agonist, were relatively rapid compared to the time likely to be required for genomic effects via PPARγ. In addition, although both RGZ and CGZ mediated relaxation, a structurally unrelated PPARγ ligand 15-deoxy-PGJ₂, failed to elicit bronchodilation. Critically, relaxation to both RGZ and CGZ were maintained in the presence of two structurally distinct and selective PPARγ
antagonists, T0070907 and GW9662 (16, 18). These combined findings suggest that relaxation to both RGZ and CGZ in mouse tracheal segments is independent of PPARγ activation, as we have reported for RGZ in PCLS (3).

We have previously explored mechanisms underlying relaxation to RGZ in PCLS, utilising the unique capacity of this approach to assess regulation of calcium signalling within ASM by dilator drugs. In this setting, RGZ inhibited the amplitude and frequency of MCh-induced calcium oscillations and reduced MCh-induced calcium sensitivity of ASM cells when relaxing intrapulmonary airways in PCLS (3). However, the possible contribution of RGZ-mediated release of epithelial-derived relaxing factors to relaxation could not be assessed in perfused PCLS, as this approach precludes the accumulation of factors such as prostaglandin E2 (PGE2) or NO. In the current study, the relatively slow reversal of an established contraction by RGZ in isolated tracheal segments in a static organ bath was suggestive of a mechanism whereby accumulation of epithelial-derived relaxing factors might play a role. Previously, RGZ-mediated relaxation of mouse tracheal segments was associated with increased PGE2 levels, and attributed to RGZ-mediated inhibition of PGE2 breakdown rather than its increased synthesis (12). However, we did not find any evidence to support this epithelial-dependent mechanism, since relaxation to RGZ was maintained in the presence of the COX inhibitor indomethacin, and also with the NOS inhibitor NOLA and following epithelial denudation.

Further assessment of the relative dilator efficacy of RGZ and β-adrenoceptor agonists was conducted when contractile reactivity was altered by allergen challenge. Tracheal preparations and PCLS were prepared from an established model of chronic AAD characterised by *in vivo* inflammation, epithelial remodeling, airway fibrosis and
AHR (20). We and others have previously reported that in vivo hyperresponsiveness to MCh is maintained in isolated trachea from OVA-challenged mice and rats when measured as a greater increase in force (6, 22). In contrast, we have recently shown that MCh is less potent in small airways in after OVA-challenge when measured in situ as a change in area in PCLS (6). In this latter setting, we proposed that MCh-induced narrowing in PCLS was opposed by altered interactions of remodelled airways with parenchymal tissue after allergen challenge.

Despite the increased tracheal contraction to MCh after allergen challenge, RGZ elicited complete relaxation, with no loss of potency. This was consistent with its ability to overcome functional antagonism in maximally contracted trachea from naïve mice. Previous studies have shown reduced β-adrenoceptor-mediated relaxation in rat and guinea pig trachea after allergen challenge (4, 5, 21). However, we did not find evidence to support this, with partial relaxation to ISO maintained, although the level of pre-contraction to MCh was not significantly increased in this subset of OVA-challenged mice.

An increase in small airway sensitivity to RGZ was observed in PCLS from OVA-challenged mice relative to saline-challenged mice. The increased potency of RGZ was evident in traces from single PCLS matched for precontraction from the two groups, as well as group data in which the average contraction to MCh was relatively reduced in PCLS from OVA-challenged mice. These findings were unexpected since RGZ potency in either trachea or PCLS (3) from naïve mice was similar irrespective of the level of pre-contraction. Relaxation to RGZ may have been increased because the airway narrowing to MCh had been limited by OVA-induced remodelling and altered airway-parenchymal interactions within PCLS. Alternatively, OVA challenge may have had a
selective effect on an as-yet unidentified mechanism underlying RGZ-mediated relaxation in small airways. A possible explanation for the failure to see a similar increase in sensitivity to SALB may be that the reduced functional antagonism (dependent on the level of MCh pre-contraction) was negated by an allergen-induced decrease in β-adrenoceptor density in the peripheral lung, although this was not tested here. Nevertheless, our current and previous findings have now shown in both trachea and PCLS from naïve, saline- and OVA-challenged mice that RGZ potency is independent of the level of pre-contraction, with consistently greater efficacy than β-adrenoceptor agonists (3).

The implications of these findings for the treatment of asthma should be considered. An exploratory clinical trial for RGZ has been conducted in smokers with asthma, a difficult-to-treat group that show resistance to current therapies (31). In this cohort, treatment with RGZ (8 mg, once daily, oral) over 4 weeks produced improvements in lung function compared with inhaled beclometasone dipropionate, despite the absence of detectable anti-inflammatory actions. These authors suggested that the improvements in forced expiratory flow values with RGZ treatment may reflect reduced small airway obstruction. More recently, treatment with RGZ (4 mg, twice daily for 28 days, oral) was associated with a modest (15%) reduction in the late asthmatic reaction in the allergen challenge model of asthma (28). Of note, 12 week treatment with progressively increasing oral doses of RGZ (2 mg for 4 weeks, then 4mg for 4 weeks, then 8 mg for 4 weeks) decreased responsiveness to MCh (2.5 fold increase in PC_{20}), albeit without effects on exhaled NO or FEV1 (29). However, these findings have yet to be extended to assess responses to acute inhalation of PPARγ agonists. This route of administration and/or identification of more potent agents
eliciting airway relaxation by the same mechanisms as RGZ, would minimize the reported adverse cardiovascular effects that have limited the systemic use of RGZ in diabetes (26) and would achieve the higher local concentrations that may be required to exert direct effects on airway contraction.

In conclusion, this study provides additional evidence of dilator efficacy of RGZ under conditions of impaired or limited β-adrenoceptor responsiveness. RGZ was able to oppose increases in MCh-induced contraction and OVA-induced AHR in trachea, and showed increased potency in small airways after allergen challenge. The use of RGZ may provide a novel therapeutic approach targeting both large airways and small airways in the distal lung to improve clinical outcomes in patients with severe asthma whose symptoms are poorly controlled with β2-adrenoceptor agonists.

Grants

This work was supported by the National Health and Medical Research Council [Grant 509239, 1041575].

Disclosures

None to declare


**Figure Legends**

**Figure 1**  RGZ causes greater relaxation than salbutamol in isolated trachea from mice and guinea pigs.

(A) A representative trace from a mouse trachea shows a full concentration-response curve to methacholine (MCh) and re-contraction to ~75% maximum prior to cumulative addition of RGZ. Pre-contraction responses to MCh (ΔmN) and subsequent relaxation to isoprenaline (ISO), salbutamol (SALB) or RGZ are shown for (B,C) mouse and (D,E) guinea pig trachea. Relaxation responses (mean ± SEM) are calculated as % relaxation of the submaximal MCh contraction for n=3-6 tracheal segments from different animals.

**Figure 2**  Relaxation to rosiglitazone, but not β-adrenoceptor agonists, is maintained in maximally contracted mouse trachea.

Mouse tracheal segments were pre-contracted to 20%, 75% or 100% of the maximal methacholine (MCh) response for the same tissue prior to cumulative addition of (A) rosiglitazone (RGZ), (B) salbutamol (SALB) or (C) isoprenaline (ISO). Relaxation responses (mean ± SEM) are expressed as % relaxation of the corresponding MCh contraction (ΔmN), shown for (D) pre-ISO. Similar pre-contractions were obtained prior to addition of RGZ or SALB (not shown). Data were obtained from n=3-6 tracheal segments from different mice. *P < 0.05 versus maximum MCh contraction, paired t-test.

**Figure 3**  Relaxation to rosiglitazone is additive with salbutamol, and maintained when relaxation to β-adrenoceptor agonist efficacy is limited in maximally contracted mouse trachea.

Mouse tracheal segments were pre-contracted to (A) 75% of the maximal methacholine (MCh) response for the same tissue prior to cumulative addition of salbutamol (SALB) in the absence or presence of 10 μM rosiglitazone (RGZ) or (B) 100% of the maximal MCh response prior to maximally effective concentrations of SALB or isoprenaline (ISO), followed by 100 μM RGZ. Relaxation responses (mean ± SEM) are expressed as % relaxation of the corresponding MCh contraction (ΔmN). Data were obtained from n=3-5 tracheal segments from different mice.

**Figure 4**  Relaxation to rosiglitazone and ciglitazone is not inhibited by PPARγ antagonists T0070907 or GW9662.

Mouse tracheal segments were pre-contracted to 75% of the maximal methacholine (MCh) response for the same tissue prior to cumulative addition of (A) rosiglitazone (RGZ), ciglitazone (CGZ) or 15-deoxy-Δ12,14-prostaglandin J2 (15-deoxy PGJ2) or (B)(C)(D) RGZ or CGZ in the absence or presence of the PPARγ antagonists T0070907 (10 μM) or GW9662 (10 μM). Relaxation responses (mean ± SEM) are expressed as % relaxation of the corresponding MCh contraction (ΔmN) for n=4-10 tracheal segments from different mice.
Figure 5  Relaxation to rosiglitazone is not inhibited by indomethacin, nitro-L-arginine or epithelial removal.

Mouse tracheal segments were pre-contracted to 75% of the maximal methacholine (MCh) response for the same tissue prior to cumulative addition of RGZ. Responses were compared in the absence or presence of (A) indomethacin (10 μM) or (B) nitro-L-arginine (NOLA, 10 μM) or (C) between epithelial-intact and epithelial-denuded tracheal segments in which (D) dilator responses to Substance P (1 μM) were also assessed. Relaxation responses (mean ± SEM) are expressed as % relaxation of the corresponding MCh contraction (ΔmN) for n=4-8 tracheal segments from different mice. **P < 0.01 versus control, paired t-test.

Figure 6  Contraction to MCh is increased in mouse trachea but decreased in PCLS following OVA challenge.

Concentration-response curves to MCh were prepared in (A) tracheal segments from saline (SAL)- and OVA-challenged mice (n=11, 13), expressed as change in force (ΔmN) and (B) PCLS from SAL- and OVA-challenged mice (n=4, 5), expressed as % initial airway area. Contraction responses are mean ± SEM.

Figure 7  Rosiglitazone, but not isoprenaline, elicits complete relaxation in trachea from a mouse model of chronic AAD

Mouse tracheal segments from saline- and OVA-challenged mice were pre-contracted to 75% of the maximal methacholine (MCh) response for the same tissue (A) (C) prior to cumulative addition of (B) RGZ or (D) ISO. Relaxation responses (mean ± SEM) are expressed as % relaxation of the corresponding MCh contraction (ΔmN) for n=3-8 tracheal segments from different mice. **P < 0.05 versus saline, unpaired t-test.

Figure 8  Rosiglitazone, but not salbutamol, elicits complete relaxation at higher potency in small airways from a mouse model of chronic AAD.

Small airways in PCLS from saline- and OVA-challenged mice were pre-contracted with (A)(D) methacholine (MCh, 100 nM) prior to cumulative addition of (B) RGZ or (E) SALB. Representative traces obtained in slices from saline- and OVA-challenged mice are shown for (C) RGZ alone and (F) SALB followed by 100 μM RGZ. Relaxation responses (mean ± SEM) are expressed as % relaxation of the corresponding MCh contraction (% reduction in airway lumen area) for n=4-6 PCLS from different mice. *P < 0.05 versus saline, unpaired t-test.
**Table 1** Comparison of relaxation responses to rosiglitazone, isoprenaline and salbutamol in mouse trachea.

<table>
<thead>
<tr>
<th>Dilator</th>
<th>n</th>
<th>MCh (% max contraction)</th>
<th>pEC$_{50}$</th>
<th>Maximal agonist effect (% relaxation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGZ</td>
<td>4</td>
<td>4.4 ± 0.1</td>
<td>93.8 ± 8.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.5 ± 0.1</td>
<td>123.8 ± 7.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.2 ± 0.0</td>
<td>80.2 ± 5.0</td>
<td></td>
</tr>
<tr>
<td>ISO</td>
<td>6</td>
<td>7.7 ± 0.2</td>
<td>110.4 ± 4.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7.4 ± 0.2</td>
<td>53.6 ± 2.3**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.3 ± 0.2</td>
<td>33.1 ± 1.9**</td>
<td></td>
</tr>
<tr>
<td>SALB</td>
<td>4</td>
<td>6.2 ± 0.2</td>
<td>77.4 ± 4.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.8 ± 0.2</td>
<td>36.1 ± 2.3**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5.2 ± 0.2*</td>
<td>15.0 ± 1.6**</td>
<td></td>
</tr>
</tbody>
</table>

Average pEC$_{50}$ and maximal agonist effect values obtained from fitted concentration-response curves from each tissue were compared by unpaired t-tests, as appropriate. Values are mean ± SEM. *P < 0.05, **P < 0.01 versus 20% for same dilator.
Table 2 Comparison of relaxation responses to rosiglitazone in mouse trachea and in small airways in lung slices from a mouse model of chronic allergic airways disease.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Group</th>
<th>n</th>
<th>(pE_{C50})</th>
<th>Maximal agonist effect (% relaxation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trachea</td>
<td>saline</td>
<td>6</td>
<td>4.4±0.1</td>
<td>103.7±6.7</td>
</tr>
<tr>
<td></td>
<td>OVA</td>
<td>7</td>
<td>4.4±0.02</td>
<td>100.4±4.4</td>
</tr>
<tr>
<td>Small airway</td>
<td>saline</td>
<td>6</td>
<td>5.5±0.1*</td>
<td>103.0±6.0</td>
</tr>
<tr>
<td></td>
<td>OVA</td>
<td>7</td>
<td>6.3±0.3*+</td>
<td>109.1±4.4</td>
</tr>
</tbody>
</table>

Average \(pE_{C50}\) and maximal agonist effect values obtained from fitted concentration-response curves from each tissue were compared by unpaired t-tests, as appropriate. Values are mean ± SEM. *P < 0.05 versus same treatment in trachea, †P < 0.05 versus saline treatment in small airways.