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Steroids augment relengthening of contracted airway smooth muscle: potential additional mechanism of benefit in asthma

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ABSTRACT: **Breathing (especially deep breathing) antagonises development and persistence of airflow obstruction during bronchoconstrictor stimulation. Force fluctuations imposed on contracted airway smooth muscle (ASM) *in vitro* result in its relengthening, a phenomenon called force fluctuation-induced relengthening (FFIR). Because breathing imposes similar force fluctuations on contracted ASM within intact lungs, FFIR represents a likely mechanism by which breathing antagonises bronchoconstriction. While this bronchoprotective effect appears to be impaired in asthma, corticosteroid treatment can restore the ability of deep breaths to reverse artificially induced bronchoconstriction in asthmatic subjects. It has previously been demonstrated that FFIR is physiologically regulated through the p38 mitogen-activated protein kinase (MAPK) signalling pathway. While the beneficial effects of corticosteroids have been attributed to suppression of airway inflammation, the current authors hypothesised that alternatively they might exert their action directly on ASM by augmenting FFIR as a result of inhibiting p38 MAPK signalling.**

This possibility was tested in the present study by measuring relengthening in contracted canine tracheal smooth muscle (TSM) strips.

The results indicate that dexamethasone treatment significantly augmented FFIR of contracted canine TSM. Canine tracheal ASM cells treated with dexamethasone demonstrated increased MAPK phosphatase-1 expression and decreased p38 MAPK activity, as reflected in reduced phosphorylation of the p38 MAPK downstream target, heat shock protein 27.

These results suggest that corticosteroids may exert part of their therapeutic effect through direct action on airway smooth muscle, by decreasing p38 mitogen-activated protein kinase activity and thus increasing force fluctuation-induced relengthening.

KEYWORDS: **Asthma, bronchoconstriction, bronchoprotection, deep breaths, steroids, tidal breathing**

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Tidal breathing, especially with larger tidal volumes, antagonises the development and persistence of airflow obstruction during bronchoconstrictor stimulation in normal animals and humans [1–6]. However, this bronchoprotective effect is impaired in individuals suffering from asthma [5, 7, 8]. Corticosteroids have long been a mainstay in asthma therapy. These agents reduce airway constrictor hyperresponsiveness in mice with experimental allergen-induced airway inflammation [9, 10] and improve or restore the bronchodilatory effect of deep inspiration that is typically impaired in asthmatic patients [11–13]. The mechanisms by which breathing confers this beneficial effect in normal individuals and how corticosteroids restore this effect in asthmatic individuals are not clear. The current authors propose a potential mechanism by which breathing antagonises bronchoconstriction and suggest a novel hypothesis of how corticosteroids may restore this effect.

It is known that superimposing load fluctuations (that mimic those generated by breathing) upon isotonically contracted tracheal smooth muscle (TSM) strips causes them to relengthen [14–16], a phenomenon termed force fluctuation-induced relengthening (FFIR). It has been proposed that FFIR may be one mechanism by which breathing antagonises bronchoconstriction. Importantly, FFIR can be physiologically regulated, since pharmacological inhibition of actin polymerisation [17] or p38

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mitogen-activated protein kinase (MAPK) signalling [14] augments FFIR *in vitro*.

In general, the beneficial effects of corticosteroids have been attributed to their anti-inflammatory actions, as pro-inflammatory cytokines can modulate airway smooth muscle (ASM) contractile and relaxant function. HAKONARSON *et al.* [18] have shown that interleukin (IL)-1β and tumour necrosis factor (TNF)-α increase ASM contractility to acetylcholine (ACh) and impair ASM relaxation with isoproterenol, changes prevented when ASM is pre-treated with dexamethasone. However, dexamethasone also increases relaxation with KCl [19] and isoproterenol [20] in contracted TSM even in the absence of a pro-inflammatory environment, suggesting that corticosteroids might exert direct effects on ASM contraction independent of their anti-inflammatory effect. However, since these studies were conducted on rabbit tracheal and bronchial ring segments with intact epithelium, corticosteroids may have exerted their effect indirectly by acting on the epithelium or mucosa rather than on the smooth muscle itself. Corticosteroids have also been reported to induce expression of MAPK phosphatase (MKP)-1, which dephosphorylates and inactivates p38 MAPK [21], as well as other MAP kinases [22, 23]. Since pharmacological inhibition of p38 MAPK enhances FFIR [14], the current authors reasoned that corticosteroid treatment might augment FFIR as well. Indeed, in the present study, dexamethasone treatment was found to increase FFIR of contracted canine trachealis strips (epithelium removed) *in vitro*, and such treatment increased MKP-1 expression in cultured canine tracheal myocytes. The latter was accompanied by decreased p38 MAPK activity, as reflected in diminished phosphorylation of heat shock protein (HSP)27, a well-established downstream target of p38 MAPK [24]. Together, these results suggest that FFIR contributes to the bronchoprotective effect of breathing and that corticosteroid treatment may restore this effect in asthma by augmenting FFIR *via* reduction of p38 MAPK activation.

METHODS

Assessment of FFIR of ACh-contracted canine TSM strips

In accordance with Institutional Animal Care and Use Committee approved protocols, random source dogs were anaesthetised and killed by overdose with pentobarbital sodium (30 $mg \cdot kg^{-1}$ *i.v.*). Tracheas were excised and rinsed several times in Krebs-Henseleit (K-H) solution (115 mM NaCl, 25 mM $NaCO_3$, 1.38 mM KH_2PO_4, 2.51 mM KCl, 2.46 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 11.2 mM dextrose). K-H was gassed with 95% O_2/5% CO_2 to maintain a pH between 7.3 and 7.5. Some tissues were stored for up to 4 days at 4°C prior to study, without apparent effect on results. All studies were conducted at 37°C in K-H solution. As described previously [17], parallel-fibred bundles of canine TSM (CTSM) were dissected free of all overlying connective tissue and epithelium and fastened at either end in aluminium foil clips (Laser Services Inc., Westford, MA, USA). The strips were then placed in a horizontal dip-tray style of organ bath and connected to a 300B lever arm/force transducer (Aurora Scientific, Aurora, Canada); the 300B lever arm measures both force output and length changes. All force and length changes of the TSM strips were monitored using Powerlab Chart software (ADInstruments, Colorado Springs, CO, USA).

As described previously [17], after equilibration, reference length (L_{ref}) of the tissues measured between 3.5 and 6.0 mm and maximal response (F_{max}) to 100 μM ACh was determined. L_{ref} and F_{max} in response to ACh were then used as base parameters for force oscillation contraction sequences. Muscles were allowed to relax by re-perfusing with K-H alone. Tissues were re-exposed to 100 μM ACh 20 min after force reached relaxed baseline, and allowed to shorten isotonically against an afterload of 32% F_{max} for 20 min and then without delay and during continued ACh exposure, force oscillations were superimposed (frequency 0.2 Hz, amplitude ±16% F_{max}) for 20 min; thereafter, TSM strips were allowed to relax by switching to ACh-free K-H solution. All length changes were noted. Next, tissues were incubated for ~2 h in K-H solution containing 4 μM dexamethasone or vehicle control. This concentration was chosen based on studies performed on a separate cohort of TSM strips that showed it to have minimal effects on isometric force (data not shown). After this equilibration period, the entire isotonic contraction sequence was repeated in the continued presence of dexamethasone or vehicle; length changes during contractions before and after inhibitor treatment were expressed as percentage of L_{ref}. FFIR was calculated as the extent of relengthening from the end of the isotonic shortening period until the end of the oscillation period. Differences between the first and second isotonic/force oscillation sequence (*i.e.* before and after dexamethasone or vehicle) were expressed as ΔFFIR.

Assessment of MKP-1 expression and HSP27 phosphorylation in cultured CTSM cells

Since it was previously found that the inhibition of p38 MAPK augmented FFIR [14] and that corticosteroids induce the expression of dual-specific phosphatases, especially MKP-1 [21–23], which in turn dephosphorylates and inactivates p38 MAPK [21], MKP-1 expression and HSP27 phosphorylation (a downstream target of p38 MAPK) were measured in CTSM cells. Airway myocytes were dissociated from adult canine trachealis and cultured using previously described methods [25, 26]. Briefly, TSM cells were enzymatically digested from dissected trachealis using 10 $U \cdot mL^{-1}$ elastase, 600 $U \cdot mL^{-1}$ collagenase and 2 $U \cdot mL^{-1}$ Nagarse protease. Myocytes were grown on uncoated plastic culture plates in Dulbecco's modified Eagle medium/F12 supplemented with 10% foetal bovine serum, 50 $U \cdot mL^{-1}$ penicillin, 50 $\mu g \cdot mL^{-1}$ streptomycin and 50 $\mu g \cdot mL^{-1}$ gentamicin. Low passage (1–3) myocytes from five different primary cell lines were treated with 4 μM dexamethasone (or in media alone as untreated control) for 1 or 2 h. Protein lysates from treated and untreated myocytes were collected using CelLytic lysis/extraction buffer (Sigma-Aldrich Co., St Louis, MO, USA). Following the manufacturer's protocol, the cells were washed with PBS, lysed for 15 min with 0.5 mL lysis buffer supplemented with Complete protease inhibitor cocktail mix (Roche, Basel, Switzerland), then centrifuged to pellet the cellular debris. The protein containing supernatant was then used for western blots.

Proteins from dexamethasone- and vehicle-treated CTSM cells were extracted as described previously [17, 27]. All lanes in all gels were loaded with equal volumes and concentrations of total protein extracts. Denatured proteins were separated by SDS-polyacrylamide gel electrophoresis (NuPage 4–12% gels; Invitrogen, Carlsbad, CA, USA), transferred to Immobilin-P

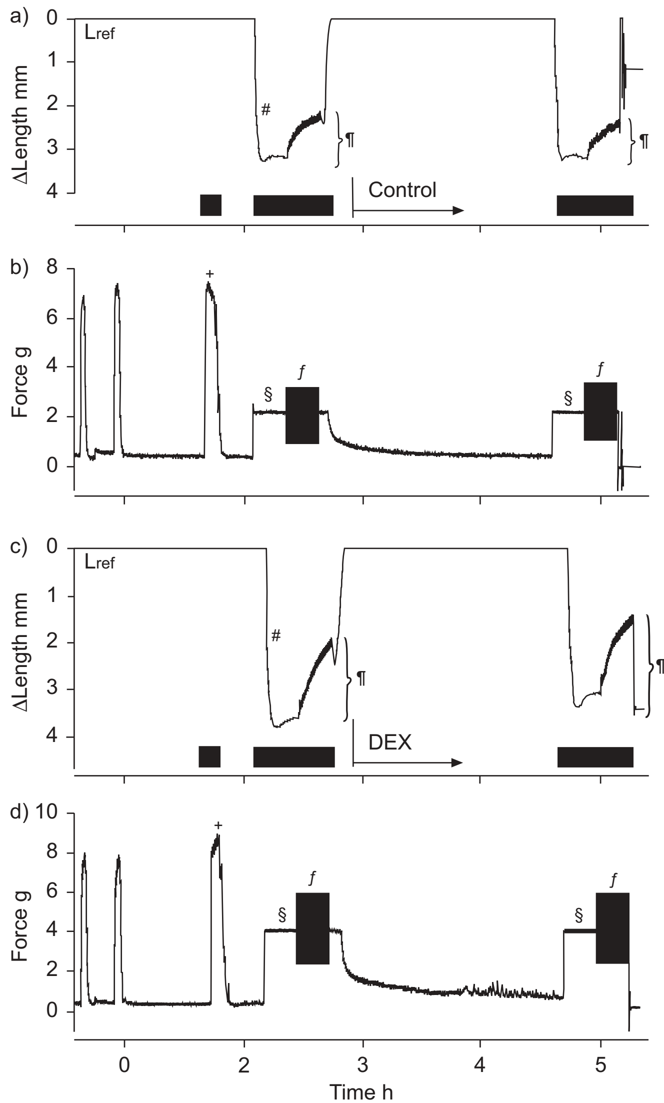

FIGURE 1. Experimental protocol to assess drug effect on force fluctuation-induced relengthening (FFIR). Changes (Δ) in length (a and c) and force (b and d) of tracheal smooth muscle (TSM) strips were measured. TSM strips were attached to a force/length transducer and equilibrated allowing for determination of reference length (L_{ref}) and isometric maximal force (F_{max}; $^{+}$) upon exposure to 100 μM acetylcholine (Ach; ■ in a and c). For the next exposure to ACh, TSM strips were contracted against a load equal to 32% of established F_{max} (§), thus producing isotonic shortening ($^{\#}$). At 20 min into the isotonic contraction, sinusoidal force fluctuations (0.2 Hz to simulate tidal breathing) were superimposed on 32% F_{max} that were of amplitude $\pm$16% F_{max} (^{f}). FFIR (¶) was noted at the end of 20 min. ACh was again washed out and baseline force and L_{ref} re-established. TSM was then incubated for ~2 h in vehicle (Control; a and b) or 4 μM dexamethasone (DEX; c and d). Differences in FFIR post- *versus* pre-treatment were compared.

polyvinylidene difluoride membranes (Millipore Corporation, Medford, MA, USA), and probed for phosphorylated and non-phosphorylated HSP27, MKP-1 and β-actin. Phosphorylated and nonphosphorylated HSP27 were detected on the same gels using Pierce SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA). Membranes initially probed for phosphorylated HSP27 were stripped for 30 min at 50°C (0.76% Tris base, 2% SDS, 0.7% β-mercaptoethanol, pH 6.7) and re-probed for total HSP27. Blot intensities (volumes) were calculated using a BioRad S710 densitometer and software (Bio-Rad Laboratories Inc., Hercules, CA, USA). The ratios of phosphorylated to total HSP27 and MKP-1 to β-actin expression were assessed relative to data derived for vehicle-treated cells, on the same western blot. All primary antibodies were raised in rabbits, except for β-actin (which was raised in mice), and were from the following sources: HSP27 was a gift from W.T. Gerthoffer (Dept of Biochemistry, University of South Alabama, Mobile, AL, USA); phosphorylated HSP27 was from Stressgen Bioreagents (Assay Designs Inc., Ann Arbor, MI, USA); anti-MKP-1 was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); and anti-β-actin was from Sigma-Aldrich Co.

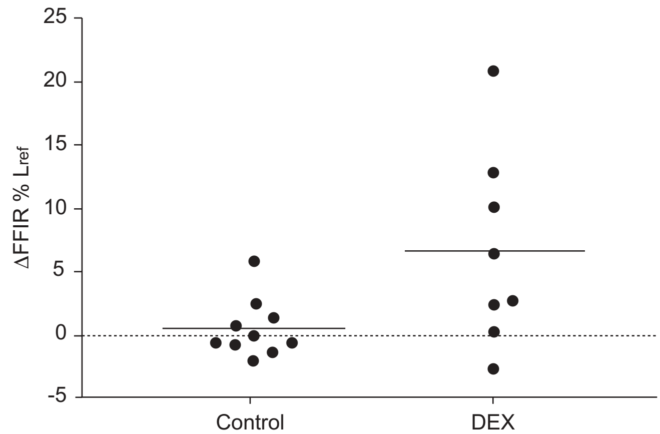

FIGURE 2. Cumulative data for force fluctuation-induced relengthening (FFIR) in control and dexamethasone (DEX)-treated canine tracheal smooth muscle strips. DEX-treated strips demonstrated a greater increase in FFIR (ΔFFIR), expressed as % of reference length (L_{ref}), than control strips when comparing FFIR post-treatment with pre-treatment.

Data analysis

All data were expressed as mean ± SE. Results from the control and dexamethasone-treated groups were compared with unpaired t-tests. Significant differences were defined when $p<0.05$.

RESULTS

FFIR in CTSM strips

Superimposition of force fluctuations upon a steady load against which ACh-stimulated CTSM strips had shortened caused them to relengthen (fig. 1). After dexamethasone treatment, CTSM strips demonstrated significantly increased FFIR, whereas no change in FFIR was observed in vehicle-treated CTSM strips; the increase in FFIR (ΔFFIR) was significantly larger in dexamethasone-treated trachealis strips than in control strips (6.6±2.70% *versus* 0.52±0.72% change, respectively; $p=0.029$; fig. 2). Isotonic shortening post-treatment was not different in dexamethasone- and vehicle-treated tissues (94.65±2.59 *versus* 96.32±4.44% of initial shortening, respectively; $p=0.737$; fig. 3), so this parameter could not account for the differences observed in ΔFFIR in dexamethasone-treated TSM.

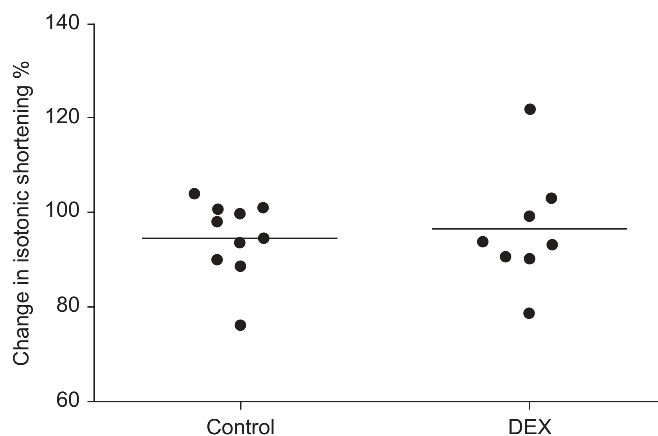

FIGURE 3. Cumulative data for isotonic shortening of control and dexamethasone (DEX)-treated canine tracheal smooth muscle strips. There was no significant difference in the change in isotonic (no oscillations) shortening after treatment, between control and DEX-treated tissues.

CTSM cell culture experiments

CTSM cells incubated in 4 μM dexamethasone demonstrated significantly increased MKP-1 expression within 1 h compared with control cells (1.69±0.23 *versus* 1.00, respectively; $p=0.040$; fig. 4a and c). Although at 1 h there was no difference in HSP27 phosphorylation between dexamethasone-treated cells and control cells, by 2 h, cells treated with dexamethasone demonstrated a significant decrease in HSP27 phosphorylation, compared with control cells (0.54±0.10 *versus* 1.00, respectively; $p=0.041$; fig. 4b and d).

DISCUSSION

The present study demonstrated enhancement of FFIR of ACh-contracted TSM strips by treatment with corticosteroids, a class of drugs that also augments the ability of deep breaths to reverse bronchoconstriction in people with asthma. Application of large force-fluctuations resulted in significant relengthening of isotonically shortened smooth muscle strips, and dexamethasone treatment further enhanced this effect. Therefore, it seems possible that corticosteroids might help restore the bronchodilatory effect of deep inspiration in asthmatic patients [11–13] in part by enhancing FFIR in their ASM.

Dexamethasone might conceivably affect smooth muscle FFIR by a number of mechanisms. Corticosteroids are potent anti-inflammatory agents. Dexamethasone decreases bronchoconstriction in sensitised animals [9, 10] and enhances the bronchodilatory effect of deep inspiration in asthmatic individuals [11–13]. In the present study, it seems less likely that dexamethasone influenced FFIR through an anti-inflammatory action, as the TSM strips used were obtained from nonsensitised, healthy dogs. However, length oscillations applied to bovine smooth muscle strips have been found to induce the expression of IL-6 and IL-8 genes, and this

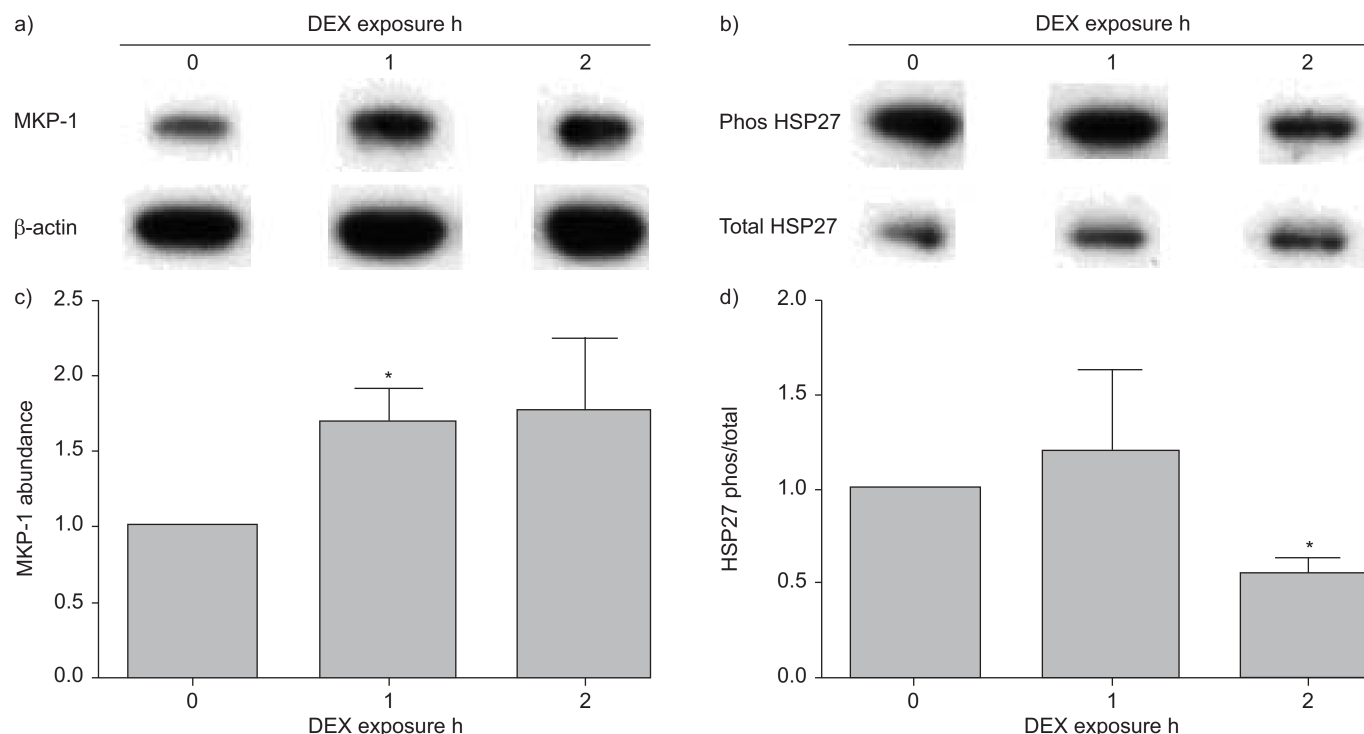

FIGURE 4. Mitogen-activated protein kinase phosphatase (MKP)-1 (a and c) and heat shock protein (HSP)27 (b and d) expression in dexamethasone (DEX)-treated canine tracheal smooth muscle (TSM) cells, shown as western blots (a and b) and quantitatively (c and d). After 1 h of incubation in 4 μM DEX, TSM cells demonstrated a significant increase in MKP-1 expression (relative to β-actin) compared with control (0 h DEX) cells. Although HSP27 phosphorylation (phos; relative to total HSP27) was unchanged at 1 h incubation, by 2 h there was significantly less HSP27 phosphorylation in cells incubated in DEX compared with control (0 h DEX) cells. Molecular weights: HSP27, total and phosphorylated ~27 kD; MKP-1 and β-actin ~40 kD. *: $p<0.05$.

expression is reduced when the frequency of oscillations is reduced [28]. Other studies using cultured cells and mechanical stretch/strain have shown similar results [29, 30]. In cultured human ASM cells, corticosteroids reduce TNF-α-induced IL-6 release from cultured ASM cells by upregulation of MKP-1 [31]. These data raise the possibility that the reduction of cytokine release from ASM itself by corticosteroids may be involved in their enhancement of FFIR.

Dexamethasone has multiple effects on smooth muscle function [32], which include reducing intracellular calcium [33], uncoupling of H1 histamine receptors [34] and reducing muscarinic receptor expression [35]. Together, these effects could act in concert to reduce smooth muscle contractile activation in response to a variety of stimuli. However, in the present study, dexamethasone-treated TSM demonstrated similar isotonic shortening to control tissues upon ACh exposure, and the 4 μM concentration of dexamethasone was chosen because it did not significantly affect isometric force in a separate cohort of TSM strips, suggesting that smooth muscle contractile activation was likely not impaired by dexamethasone. Glucocorticoids can also enhance smooth muscle relaxation by increasing adenylate cyclase activity [36], reducing β_2 receptor desensitisation [37], increasing the number of β_2 receptors [38] and increasing Na^+/K^+ pump activity [19]. The latter effect, in particular, may be relevant because it occurs in <1 h. Corticosteroids can also reduce smooth muscle proliferation [39–41], although one might not expect change in cell number to be relevant within the short time-course of the current experiments.

Numerous studies have demonstrated that corticosteroids increase the expression of dual-specific phosphatases, especially MKP-1 [21–23], and by doing so decrease p38 MAPK activity, which is dephosphorylated and inactivated by MKP-1 [21]. It has previously been demonstrated that when p38 MAPK activity is inhibited pharmacologically with SB203580, FFIR of smooth muscle is enhanced [14]. In the present study, CTSM cells incubated with dexamethasone demonstrated increased MKP-1 expression within 1 h and decreased HSP27 phosphorylation by 2 h. HSP27 is a well-established downstream phosphorylation target of p38 MAPK signalling [42, 43]; thus, the current data strongly suggest that dexamethasone treatment suppresses p38 MAPK activity. HSP27 is also an actin-capping protein that, when phosphorylated, promotes actin polymerisation [43–45]. Inhibition of HSP27 phosphorylation could be expected to decrease actin polymerisation; it was previously demonstrated that inhibition of actin polymerisation with latrunculin B increases FFIR [17, 46]. Thus, the current authors propose that dexamethasone may enhance FFIR of contracted TSM by inducing MKP-1 expression, which in turn reduces p38 MAPK activation and HSP27 phosphorylation.

It is noteworthy that the effect of dexamethasone on the ability of contracted airway smooth muscle to maintain shortening was revealed through a loading protocol that simulates physiological conditions. This effect would otherwise not have been apparent, had only isotonic shortening or isometric force been measured. Thus, most traditional studies of muscle contractility have not considered the physiological pathways and mechanisms that are evoked here.

In conclusion, corticosteroids enhance force fluctuation-induced relengthening in contracted airway smooth muscle and inhibit the p38 mitogen-activated protein kinase pathway. Previous studies have demonstrated the importance of deep breaths in reversing bronchoconstriction and that this phenomenon is impaired in asthma but restored by corticosteroid treatment. The present study suggests that force fluctuation-induced relengthening is a mechanism by which deep inspirations protect against bronchoconstriction and that corticosteroids may restore this effect that is impaired in asthma, through inhibition of the p38 mitogen-activated protein kinase pathway and augmentation of force fluctuation-induced relengthening. These results suggest that novel therapies that enhance force fluctuation-induced relengthening, perhaps by targeting p38 mitogen-activated protein kinase, may have a beneficial effect in asthma.

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