

RESEARCH PAPER

Epithelium-dependent modulation of responsiveness of airways from caveolin-1 knockout mice is mediated through cyclooxygenase-2 and 5-lipoxygenase

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BACKGROUND AND PURPOSE

Acute silencing of caveolin-1 (Cav-1) modulates receptor-mediated contraction of airway smooth muscle. Moreover, COX-2 and 5-lipoxygenase (5-LO)-derived prostaglandin and leukotriene biosynthesis can influence smooth muscle reactivity. COX-2 half-life can be prolonged through association with Cav-1. We suggested that lack of Cav-1 modulated levels of COX-2 which in turn modulated tracheal contraction, when arachidonic acid signalling was disturbed by inhibition of COX-2.

EXPERIMENTAL APPROACH

Using tracheal rings from Cav-1 knockout (KO) and wild-type mice (B6129SF2/J), we measured isometric contractions to methacholine and used PCR, immunoblotting and immunohistology to monitor expression of relevant proteins.

KEY RESULTS

Tracheal rings from Cav-1 KO and wild-type mice exhibited similar responses, but the COX-2 inhibitor, indomethacin, increased responses of tracheal rings from Cav-1 KO mice to methacholine. The phospholipase A₂ inhibitor, eicosatetraynoic acid, which inhibits formation of both COX-2 and 5-LO metabolites, had no effect on wild-type or Cav-1 KO tissues. Indomethacin-mediated hyperreactivity was ablated by the LTD₄ receptor antagonist (montelukast) and 5-LO inhibitor (zileuton). The potentiating effect of indomethacin on Cav-1 KO responses to methacholine was blocked by epithelial denudation. Immunoprecipitation showed that COX-2 binds Cav-1 in wild-type lungs. Immunoblotting and qPCR revealed elevated levels of COX-2 and 5-LO protein, but not COX-1, in Cav-1 KO tracheas, a feature that was prevented by removal of the epithelium.

CONCLUSION AND IMPLICATIONS

The indomethacin-induced hypercontractility observed in Cav-1 KO tracheas was linked to increased expression of COX-2 and 5-LO, which probably enhanced arachidonic acid shunting and generation of pro-contractile leukotrienes when COX-2 was inhibited.

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Keywords

caveolin-1; contraction; 5-lipoxygenase; airway smooth muscle; PGE₂

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Abbreviations

5-LO, 5-lipoxygenase; ASM, airway smooth muscle; Cav-1, caveolin-1

Introduction

Caveolae are flask-shaped invaginations of the plasma membrane found in variable numbers in different cell types, being most prominent in vascular endothelial cells, adipocytes, fibroblasts, epithelial cells and smooth muscle cells. They segregate receptors and signalling intermediates and form a micro-environment where local kinases and phosphatases can modify downstream signalling events and cell responses (Okamoto *et al.*, 1998; Simons and Toomre, 2000; Stan, 2005). Caveolin-1 (Cav-1), the primary structural protein of caveolae, plays a key role in orchestrating activation of pathways that underpin cell proliferation, migration and contraction (Cohen *et al.*, 2004; Halayko and Stelmack, 2005). In smooth muscle, caveolae are enriched in Ca^{2+} -handling and binding proteins, and trimeric G-proteins (Li *et al.*, 1995; de Weerd and Leeb-Lundberg, 1997; Darby *et al.*, 2000; Gosens *et al.*, 2007a) which is of functional significance, as acute disruption of plasma membrane caveolae suppresses GPCR-mediated contraction in airway smooth muscle (ASM) (Gosens *et al.*, 2007b; Prakash *et al.*, 2007; Sharma *et al.*, 2010). ASM contraction *in situ* is regulated both by intrinsic cellular pathways and by mediators released from neighbouring cells, such as the airway epithelium. Thus, there is an intrinsic role for caveolins in regulating isolated ASM cell contraction. However, a systematic assessment of mechanisms that integrate contraction of intact multicellular airways from Cav-1 knockout (KO) & wild type mice is still lacking.

Arachidonic acid metabolites play an important role in cellular physiology, and their aberrant biosynthesis via COX-2 or 5-lipoxygenase (5-LO) pathways has been linked with allergic and inflammatory diseases (Wenzel, 1997; Wasserman, 1988; Peters-Golden and Henderson, 2007). The increased contractile function of ASM, as seen in chronic airway diseases, can be regulated by the airway epithelium which is a rich source of lipid mediators that regulate ASM tone and contractility (Barnes *et al.*, 1988; Holtzman, 1992; Raeburn and Webber, 1994; Chung and Barnes, 1999). Epithelium-associated changes in COX-2 activity and levels of endogenous PGE_2 modulated changes in airway reactivity and bronchial inflammation in experimental models of asthma (Gavett *et al.*, 1999; Peebles *et al.*, 2002; Torres *et al.*, 2008). Recent research also suggests a role for Cav-1 in chronic lung diseases such as asthma, chronic obstructive pulmonary disease and pulmonary fibrosis (Gosens *et al.*, 2010; Xia *et al.*, 2010; Ryter *et al.*, 2011). Moreover, allergen-naïve caveolin-1 knockout (Cav-1 KO) mice exhibit an altered lung phenotype that is likely to affect airway physiology (Murata *et al.*, 2007). Interestingly, although reduced Cav-1 expression in whole lung has been demonstrated in allergen-challenged mice (Le Saux *et al.*, 2008), the opposite effect has recently been reported for ASM upon allergen challenge of guinea pigs (Gosens *et al.*, 2010). The COX-2 enzyme is co-localized with Cav-1 in caveolae, and the binding of

COX-2 with Cav-1 targets the former for degradation via endoplasmic reticulum-associated mechanisms (Liou *et al.*, 2001; Chen *et al.*, 2010a). These studies indicate a novel function for Cav-1 in regulating both COX-2 expression and activity.

Therefore, we used Cav-1 KO mice to test the hypothesis that the lack of Cav-1 would modulate COX-2 abundance and that this modulation would affect control of tracheal contractility when signalling through the arachidonic acid cascade was disrupted by selective inhibition of the cyclooxygenase pathway. We found that responses to methacholine of tracheal preparations from Cav-1 KO mice were highly sensitive to pharmacological manipulation of COX but this was not observed in rings from wild-type mice. Notably, the effects of COX inhibition could be reversed using 5-LO pathway inhibitors, indicating shunting of arachidonic acid towards pro-contractile 5-LO metabolites in Cav-1 null airways. We also found that removal of the epithelium modified COX-2 sensitivity in Cav-1 KO preparations, and that COX-2 and 5-LO abundance was markedly increased in the airway epithelium of Cav-1 KO mice. These data suggest that, despite an increase in both COX-2 and 5-LO proteins in Cav-1 KO airways, normal functional balance was maintained because intrinsic airway contractility was not affected. However, due to the elevated activity of both pathways, when COX-2 was inhibited there was an enhanced generation of pro-contractile arachidonic acid metabolites via 5-LO, and these products maintained hypercontractility of Cav-1-deficient airways through montelukast-sensitive LT receptors.

Methods

Isolated tracheal ring preparation and epithelium removal

All animal care and experimental protocols were approved by the University of Manitoba Animal Care Committee. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). All animals were maintained on a 12 h dark and light cycle and were fed with regular laboratory chow while in-house at the university facility. Cav-1 KO Cav1^{tm1Mis/J} and, as genetic controls, B6129SF2/J (wild-type) mice were used in this study. All mice were female, aged between 8 and 12 weeks (total = 41 Cav-1 KO mice and 41 wild-type) and were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were killed with an overdose of pentobarbital (90 mg·kg⁻¹) before tracheal isolation. For this, the chest cavity contents were removed en masse and placed in Krebs-Henseleit bicarbonate solution (K-H) of the following composition (in mM): 118 NaCl, 23.5 NaCO₃, 4.69 KCl, 1.18 KH₂PO₄, 1.00 MgCl₂, 2.50 CaCl₂ and 5.55 dextrose. The K-H solution was gassed with 95% O₂-5% CO₂ to maintain a pH between 7.3 and 7.5. Tracheal isola-

tions were carried out in cold K-H solution (4°C) by pinning the apex of the heart and the voice box of trachea to a dissecting dish and removing extraneous tissue. Lungs were removed and frozen for protein and RNA analysis (see below).

Each isolated trachea was cut into four segments with each segment containing three to four cartilage rings. Tracheal ring preparations were mounted between two pins: – one pin firmly fixed and the other attached to an isometric force transducer in one chamber of a Danish Myo Technology (Aarhus, Denmark) organ bath system. The paries membranaceus of the tracheal ring preparation (containing the smooth muscle layer) was placed between the two support pins. Tissue preparations were maintained in gassed K-H solution at 37°C and pH 7.3–7.5, for all subsequent studies.

For some studies, epithelium-denuded tracheal preparations were used. To remove the epithelium, tracheal segments were threaded onto silk surgical threads (Ethicon P4888C size 5); then the rings were rolled 3 revolutions on a paper towel soaked with K-H solution (Ndukwu *et al.*, 1994). Randomly selected tracheal preparations were saved for histological evaluation of epithelial removal whilst others were used for immunohistochemical staining of Cav-1, 5-LO and COX-2.

Smooth muscle equilibration

To establish resting tension, reference length and stable baseline, tracheal rings were equilibrated for 90–120 min with intermittent (~20 min) instillation of 63 mM KCl-substituted K-H solution (usually three exposures) in order to contract the tissues isometrically in a receptor-independent manner. Reference length of preparations was established by stretching rings after each KCl stimulation to achieve a resting tension of 0.6 mN. The isometric force developed for each smooth muscle preparation in response to the third exposure to the KCl-substituted K-H solution was used as the reference force for subsequent contractions elicited with methacholine.

Methacholine concentration-response studies

After equilibration, tracheal rings were incubated for 30 min with either dimethyl sulfoxide (vehicle control; 1 $\mu\text{L}\cdot\text{mL}^{-1}$), indomethacin (non-selective COX inhibitor; 3 μM), eicosatetraynoic acid (ETYA; phospholipase A_2 (PLA $_2$) inhibitor, 10 μM) alone, or in combination with either montelukast (LTC $_4$ and LTD $_4$ receptor antagonist; 10 μM) or zileuton (5-LO inhibitor; 10 μM). Tracheal rings from Cav-1 KO and wild-type mice were randomly assigned to treatment groups. After incubation with inhibitors, concentration-response studies with methacholine (1.0 nM–1.0 mM) were performed.

Preparation of lung and tracheal tissue lysates from Cav-1 KO and wild-type mice

After the final administration of methacholine in myography studies, rings were washed with K-H solution and stored in protein lysis buffer at –80°C (buffer composition: 40 mM Tris, 150 mM NaCl, 1% IgepalCA-630, 1% deoxycholic acid, 1 mM NaF, 5 mM β -glycerophosphate, 1 mM Na $_3$ VO $_4$, 10 $\mu\text{g}\cdot\text{mL}^{-1}$ aprotinin, 10 $\mu\text{g}\cdot\text{mL}^{-1}$ leupeptin, 7 $\mu\text{g}\cdot\text{mL}^{-1}$ pepstatin A, 1 mM PMSE, pH 8.0). Entire lungs were cut into small fragments in ice cold K-H solution. For protein isolation, tissues were stored in protein lysis buffer at –80°C. To prepare homogenates, samples were thawed, homogenized using a Polytron,

and supernatant collected after centrifugation (10 000 $\times$ g, 5 min) and stored at –20°C for subsequent protein assay and immunoblot analyses.

Protein blot analysis

Protein content in samples was determined using the Bio-Rad protein (Bio-Rad, Hercules, CA, USA). Immunoblotting was performed using standard techniques described previously (Halayko *et al.*, 1999; Sharma *et al.*, 2008). Briefly, samples were size separated under reducing conditions using SDS-polyacrylamide gels, and thereafter transferred to nitrocellulose membranes. After blocking membranes using skim milk in Tris-buffered saline (TBS) with Tween-20 (0.2%), membranes were incubated with primary antibodies diluted in TBS/1% skim milk/Tween-20 overnight, then with HRP-conjugated secondary antibodies prior to visualization on film using enhanced chemiluminescence reagents (Amersham, Buckinghamshire, UK). β -actin was used to correct for equal loading of all samples.

Immunoprecipitation

Immunoprecipitation was carried out as previously described (Sharma *et al.*, 2010). Protein-G-conjugated sepharose beads (GE Healthcare, Uppsala, Sweden) were mixed with tissue lysates and incubated overnight with Cav-1 antibody at 4°C. Beads were washed with TBS/0.1% Tween 20 and PBS, then stored at –80°C until used for protein blot analysis for COX-2 protein.

RNA isolation and real-time RT-PCR analysis

Lungs were cut into small fragments in ice cold K-H solution then stored in RNAlater* buffer at –80°C. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Mississauga, ON, Canada). After assessing RNA concentration and purity (Chirgwin *et al.*, 1979), RNA (1 μg) was reverse transcribed using the Quantitect Reverse Transcription Kit (Qiagen). Real-time PCR was carried out with the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using primer pairs for COX-2, 5-LO, Cav-1 and 18S RNA [calibrator gene for qPCR using previously published method (Sharma *et al.*, 2008)]. The primers are described in Table 1. The reaction conditions used are described previously (Sharma *et al.*, 2008). Product specificity was determined by dissociation curve analysis. Relative quantitation of gene expression was performed using the 7500 Sequence Detection software v.1.4 (Applied Biosystems).

Histology and immunohistochemistry

Paraffin sections (4 μm thickness) were prepared using a Shandon Finesse E Microtome Sectioner (Fisher Scientific, Ottawa, ON, Canada). After mounting on slides, sections were deparaffinized and rehydrated in xylene and graded ethanol. Heat-induced epitope retrieval was performed by placing slides in a pre-warmed steamer (60–90°C) with citrate buffer (0.1 M citric acid, 0.1 M sodium citrate, pH 6.0) for 30 min. After washing with 1X PBS, samples were incubated in blocking solution (3% BSA/PBS) for 30 min at room temperature, then washed and incubated in 3% H $_2$ O $_2$ /PBS for 10 min, and last in Avidin and Biotin blocking solution (Vector Inc, Burlington, ON, Canada) for 15 min. Thereafter,

Table 1

Primers used for RT-PCR analyses

Gene	Forward primer	Reverse primer
COX-2 (PES-2)	TTGCTGTACAAGCAGTGGCAAAGG	ACAGGAGAGAAGGAAATGGCTGA
5-LO	CCATTGCCATCCAGCTCAACCAAA	TGTCTGAGGTGTTTGGTATCGCCA
Cav-1	CTCCGAGGGACATCTCTACAC	CAGCAACATCCGCATCAGCAC
GAPDH	AGCAATGCCTCCTGCACCACCAAC	AGACTGTGGATGGCCCCTCCGG
18s RNA	CGCCGCTAGAGGTGAAATTC	TTGGCAAATGCTTTCGCTC

samples were incubated in diluted primary antibodies (4°C overnight). When mouse primary antibodies were used, sections were then incubated with unconjugated AffiniPure Fab Fragment goat anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA, USA) for 1 h. Sections were then incubated with diluted biotinylated secondary antibody for 1 h, washed, and finally in ABC solution (Vector Inc) for 30 min. To develop slides, 3,3-diaminobenzidine substrate was added to the sections and incubated for 2–5 min, then washed in distilled water before nuclei were counter-stained with Mayer's haematoxylin and sections were mounted with Permount (Thermo Fisher Scientific, Ottawa, ON, Canada). As a negative control, primary antibodies were omitted.

Data analysis

Data are shown as means $\pm$ SEM, unless otherwise stated. Student's unpaired *t*-test or one-way ANOVA was used where appropriate. When ANOVA revealed a difference among groups, Tukey's multiple range test was used to determine significant differences between groups; *P*-values < 0.05 were considered significant.

Materials

Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG were obtained from Sigma, and primary antibodies were obtained from the following sources: caveolin-1 (BD Transduction Labs (Franklin Lakes, NJ, USA) and Santa Cruz Biotechnology, Santa Cruz, CA, USA), 5-LO (Santa Cruz Biotechnology), COX-2 (Calbiochem, EMD Millipore, Darmstadt, Germany). Montelukast was generously provided by Merck Frosst Canada Inc., (Kirkland Lake, PQ, Canada) and zileuton was supplied by R&D Systems, Inc., (Minneapolis, MN). Indomethacin, methacholine and ETYA were from Sigma-Aldrich, (St Louis, MO) and pentobarbital from Ceva Santé Animale (Libourne, France). All other chemicals used were of analytical grade. Receptor nomenclature follows Alexander *et al.*, (2011).

Results

Inhibiting COX induces hyperreactivity selectively in tracheas from Cav-1 KO mice

In the absence of indomethacin, tracheal rings from Cav-1 KO mice and the wild-type controls responded similarly metha-

choline (Figure 1A). However, in the presence of indomethacin (3 μ M), tracheal rings from Cav-1 KO mice developed hyperreactivity to methacholine whereas responses of tracheal rings from wild-type mice were unaffected (Figure 1A). In contrast to the selective effects of indomethacin on Cav-1 KO tissues, treatment with either the cysteinyl leukotriene (CysLT₁) receptor antagonist montelukast (10 μ M) or the 5-LO inhibitor zileuton (10 μ M) was without effect on the responses of tracheal rings to methacholine, regardless of the mouse strain (Figure 1B,C). To establish the role of arachidonic acid signalling as a determinant of tracheal responses, we treated preparations with the phospholipase A₂ inhibitor ETYA (10 μ M) which prevents the liberation of arachidonic acid and thereby the formation of both COX and 5-LO metabolites. ETYA was without effect on basal tone or responses to methacholine in tissues from either mouse strain (data not shown), suggesting that the functional balance of COX and 5-LO metabolites produced was not changed in Cav-1 KO mice. Thus, the effects of indomethacin on Cav-1 KO tissue could be due to a disruption of the intrinsic balance between the COX and 5-LO pathways of the arachidonic acid cascade. To assess this possibility, in Cav1 KO tracheal rings treated with indomethacin, we also added montelukast (10 μ M) or zileuton (10 μ M). Both co-treatments reversed indomethacin-induced hyperresponsiveness (Figure 1D). These data suggest that COX inhibition in Cav-1 KO, but not in wild-type tissues, may have shunted a functionally significant fraction of arachidonic acid through the 5-LO pathway to promote contractile responses, or blocked production of COX-derived prostanoids that control contraction.

Indomethacin-induced methacholine hyperreactivity in Cav-1 KO airways is epithelium dependent

As arachidonic acid signalling is prominent in epithelial cells, tracheal rings from Cav-1 KO and wild-type mice were denuded of epithelium (Figure 2A) and the effects on responses to methacholine in the absence and presence of indomethacin measured (Figure 2B). Responses to methacholine in the absence of epithelium were indistinguishable between strains and were not different from epithelium-intact preparations. In contrast to our studies with epithelium-intact preparations, the addition of indomethacin had no effect on responses to methacholine of Cav-1 KO preparations, after removal of epithelium. These data suggest that hyperreactivity unmasked by COX inhibition in Cav-1

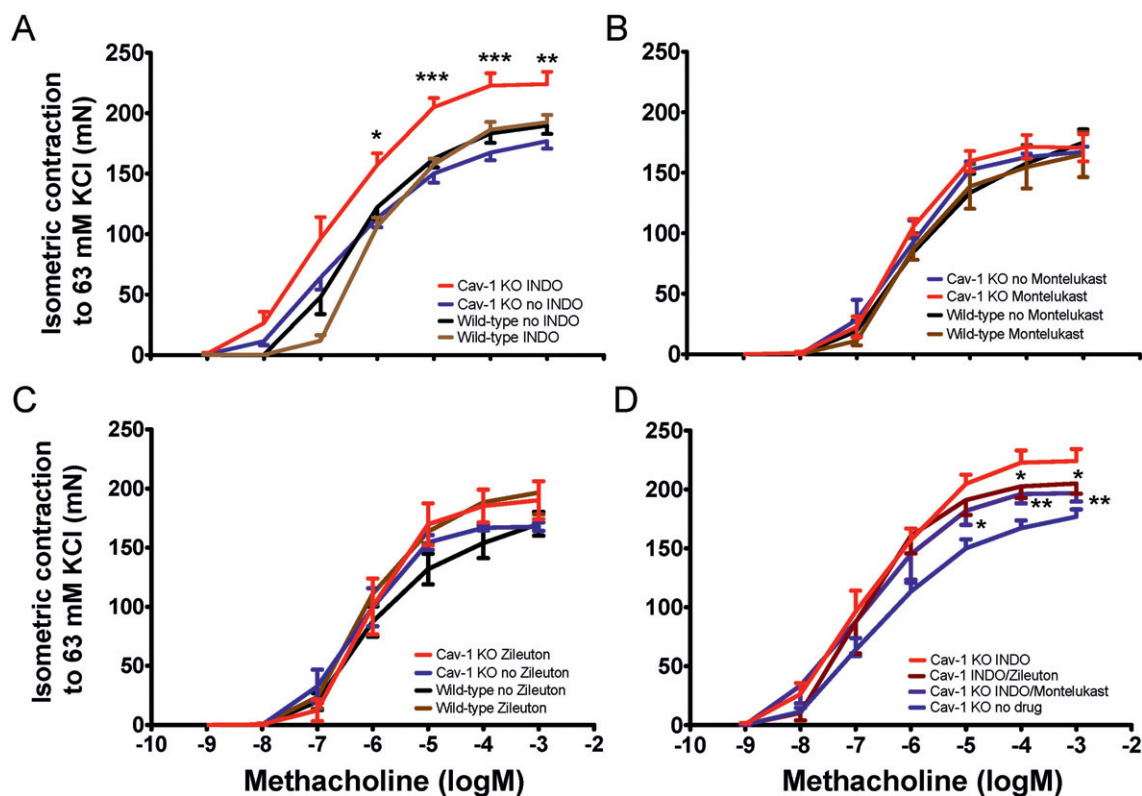


Figure 1

MCh-induced isometric force produced by Cav-1 KO and wild-type tracheal rings in the presence of epithelium. Trachea from Cav-1 KO and wild-type mice was isolated and sliced to obtain four equal-sized segments containing three to four cartilage rings. Tissues were equilibrated for 90–120 min with intermittent (~20 min) instillation of 63 mM KCl-substituted K-H solution to obtain a stable resting tension at ~0.6 mN. Isometric force was measured with increasing cumulative concentrations of methacholine (10^{-9} – 10^{-3} M). (A) Some tissues were incubated with indomethacin (INDO); ($n = 12$ rings from 6 Cav-1 KO and $n = 9$ rings from 5 wild-type mice) while others received DMSO (vehicle) alone ($n = 21$ rings from 13 Cav-1 KO and $n = 31$ rings from 17 wild-type mice). With indomethacin treatment, there were larger responses in Cav-1 KO preparations, compared with indomethacin-free Cav-1 KO tissues, to methacholine between 10^{-6} and 10^{-3} M. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$: one-way ANOVA. (B) Tracheal rings were incubated for 30 min with montelukast (10 μ M) alone before performing methacholine concentration-response studies. No significant differences were seen between Cav-1 KO and wild-type groups ($n = 8$ rings from 4 mice; one-way ANOVA). (C) Tracheal rings were incubated for 30 min with zileuton (10 μ M) alone before performing methacholine concentration-response studies. No significant differences were seen between Cav-1 KO and wild-type groups ($n = 7$ rings from 4 mice; one-way ANOVA). (D). Tracheal rings were incubated for 30 min with indomethacin in the presence or absence of montelukast or zileuton (10 μ M) before performing methacholine concentration-response studies. Compared with indomethacin-treated Cav-1 KO rings, montelukast ($n = 8$ rings from 8 mice, $*P < 0.05$, one-way ANOVA) and zileuton ($n = 4$ rings from 4 mice, $***P < 0.01$, one-way ANOVA) both significantly reversed indomethacin-induced responses in Cav-1 KO mice.

KO tissues was likely to have been the result of the absence of Cav-1 from epithelial cells, and not a change in contractile capacity of the ASM *per se*. Consistent with this conclusion, we found that KCl-induced isometric force generated by epithelium-intact or epithelium-denuded isolated tracheal rings from either strain showed no difference in maximal response to 63 mM KCl (Figure 2C). Moreover, there was no strain-dependent difference in the baseline tone of epithelium-intact or epithelium-denuded tracheal rings (Figure 2D).

Increased COX-2 and 5-LO expression in Cav-1 KO airways and lungs

We estimated the abundance of COX-2, 5-LO and COX-1 in isolated tracheas of Cav-1 KO and wild-type mice by immunoblotting. Significantly higher levels of COX-2 and 5-LO were evident in protein lysates of whole tracheas from Cav-1

KO mice compared with those from wild type mice (Figure 3A,B), however, there was no difference in COX-1 abundance (Figure 3C). We also assessed COX-2, 5-LO and COX-1 abundance in lysates from tracheas that had been denuded of epithelium. In wild-type mouse tissues, epithelial removal had little effect on COX-2 or 5-LO abundance. However, in epithelium-denuded Cav-1 KO tracheas, we found a significantly reduced abundance of both proteins that was similar to that of epithelium-denuded or epithelium-intact wild-type tissues (Figure 3A,B). COX-1 abundance was unchanged in the epithelium-denuded tracheal rings in both strains (Figure 3C).

In order to determine whether the differences in COX-2 and 5-LO expression that we measured in Cav-1 KO tracheas were also evident in the lungs, we performed immunoblotting using lung lysates and found that COX-2 and 5-LO protein levels to be significantly higher compared with

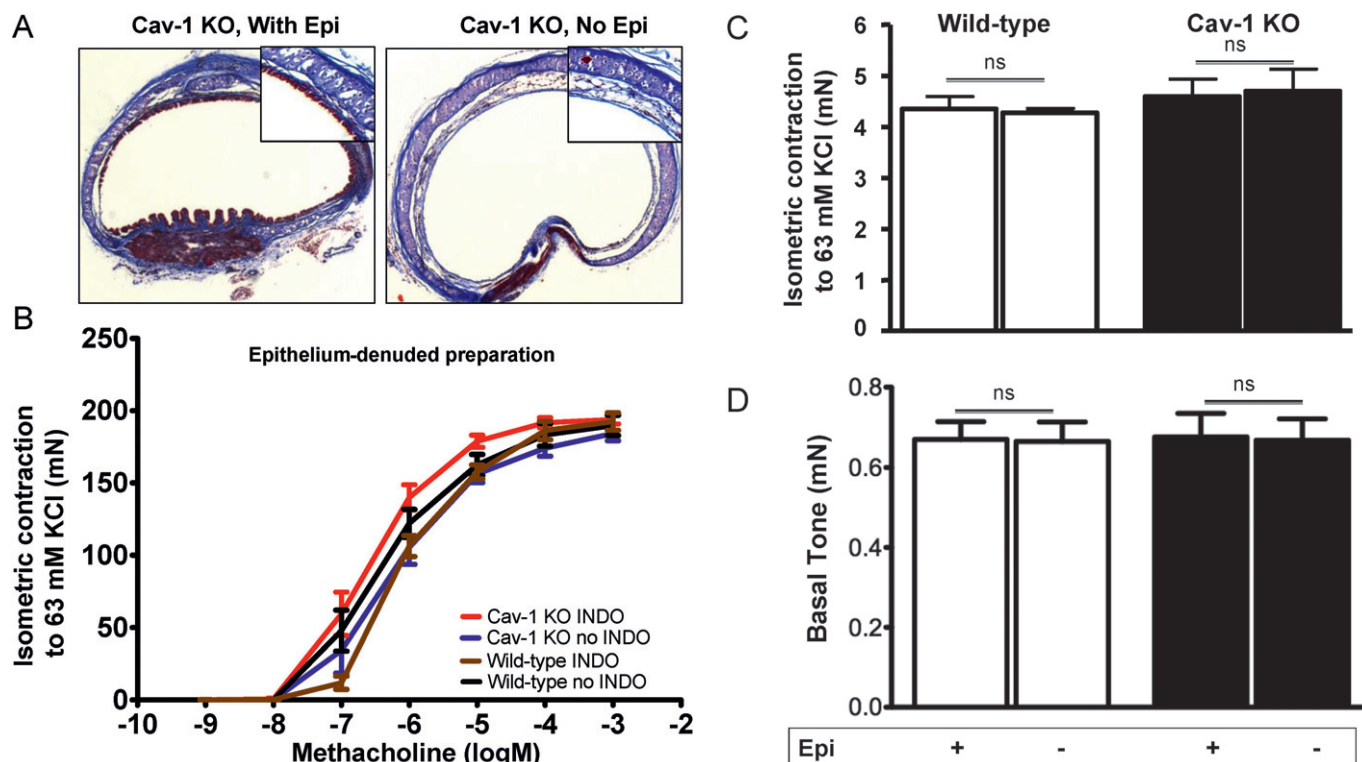


Figure 2

Epithelium denudation and KCl-induced isometric force in tracheal rings from Cav-1 KO and wild-type mice. The epithelium of excised tracheal segments was removed by threading with surgical silk then rolling the rings for 3 revolutions on a wetted paper towel. (A) Masson's trichrome staining of randomly selected tracheal preparations showing result of epithelial (Epi) denudation. Tracheal specimens from Cav-1 KO mice are shown. (B) Isometric methacholine concentration-response studies were performed using epithelium-denuded tracheal segments from Cav-1 KO (9 rings from 6 mice) and wild-type (9–12 rings from 5–7 animals) mice. After equilibration, rings were incubated for 30 min with either vehicle or indomethacin (INDO), then isometric force was measured with increasing concentrations of methacholine (10^{-9} – 10^{-3} M). Results shown are the mean $\pm$ SEM, indomethacin had minimal effects on responsiveness to methacholine (one-way ANOVA, $P > 0.05$). (C) Receptor-independent force generating capacity of tracheal rings with (+) and without (–) epithelium (Epi) from wild-type and Cav-1 KO mice was assessed based on isometric force measured after 63 mM KCl substituted with K-H solution treatment. For each group, two to three tracheal rings from at least three to four mice were studied. There were no significant differences between the groups (one-way ANOVA, $P > 0.05$). (D) Histogram comparing basal tone in tracheal rings from Cav-1 KO and wild-type mice with (+) and without (–) epithelium. For each group, five to seven tracheal rings from at least three to four mice were studied. There were no significant differences between the groups (one-way ANOVA, $P > 0.05$). ns, not significant.

wild-type tissue (Figure 4A,B). As Cav-1 is linked to regulation of COX-2 transcription in cancer cell lines (Rodriguez *et al.*, 2009), we also measured COX-2 and 5-LO mRNA using qPCR. Indeed, COX-2 mRNA levels were increased by 70% and there was a threefold increase in 5-LO mRNA levels in Cav-1 KO lungs compared with wild-type tissue (Figure 4C). There is evidence for COX-2 and Cav-1 binding (Liou *et al.*, 2001; Perrone *et al.*, 2007) which is linked to COX-2 half-life (Rodriguez *et al.*, 2009; Chen *et al.*, 2010a) and so, using wild-type mouse lungs, we also performed immunoprecipitation of Cav-1 with subsequent immunoblotting for COX-2 (Figure 4D). This analysis confirmed that COX-2 did associate with Cav-1 in wild-type mouse lung cells.

These data suggested that elevated COX-2 and 5-LO abundance in the respiratory system of Cav-1 deficient mice may be principally associated with accumulation in the airway epithelia, hence we next assessed the distribution of COX-2 and 5-LO by immunohistochemistry, in resident airway cells of tracheas from Cav-1 KO and wild-type mice (Figure 5). This analysis demonstrated the epithelium to be the primary com-

partment in which COX-2 and 5-LO were expressed, although low levels of 5-LO and COX-2 staining were also evident in smooth muscle cells.

Discussion

Cav-1 KO mice exhibit lung remodelling and vascular defects, suggesting a widespread effect on the physiology and function of the respiratory system (Drab *et al.*, 2001; Jasmin *et al.*, 2004; Murata *et al.*, 2007). Nonetheless, before our study, the contractility of intact airways from Cav-1 KO mice had not been assessed. To our knowledge, our findings that COX-2 and 5-LO expression is increased in the lungs of Cav-1 KO mice and that in airways this increase is chiefly associated with the epithelium have not been previously reported. Our functional findings indicate that this increase in expression appears to provide a platform for modified control of airway responsiveness in situations where the COX-2 pathway of the arachidonic acid cascade is inhibited. We found that pharma-

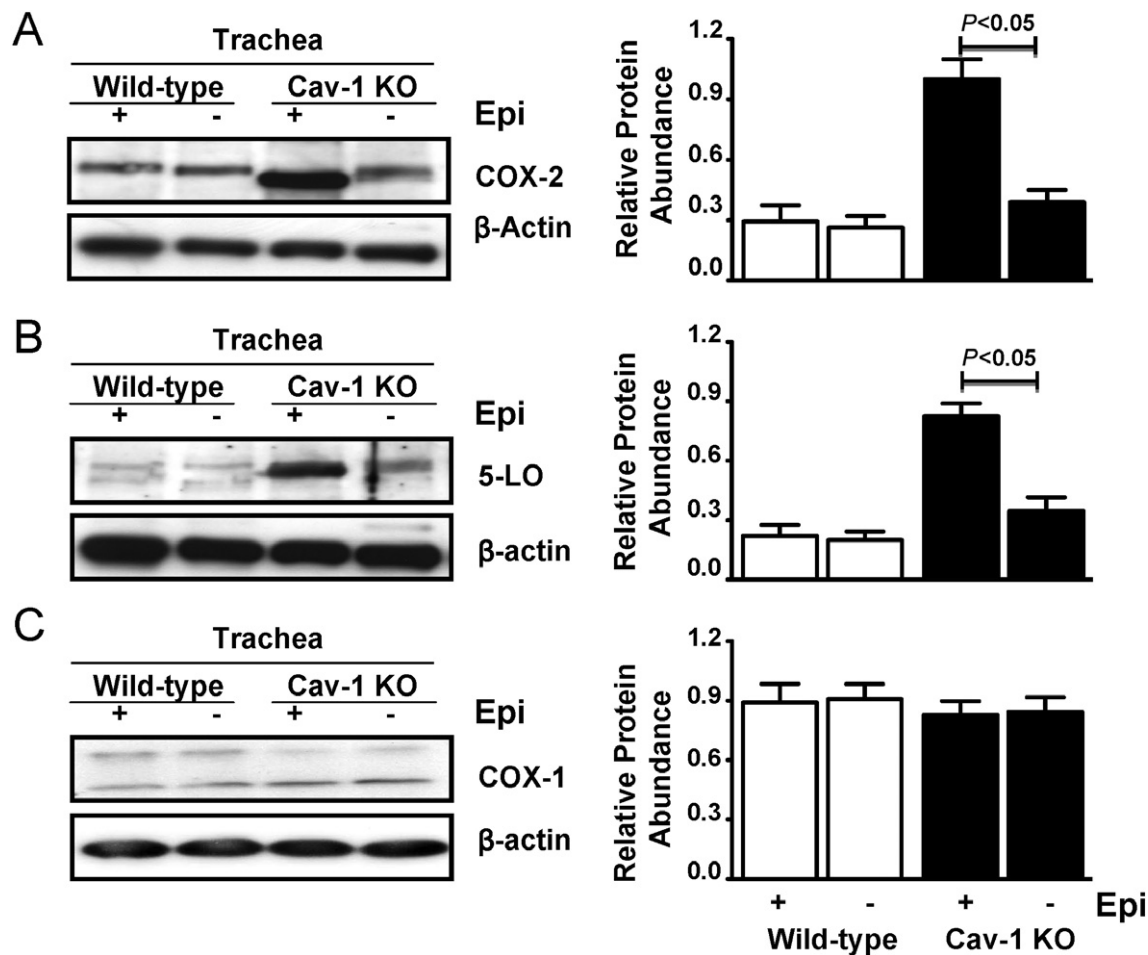


Figure 3

Increased expression of COX-2 and 5-LO in tracheas from Cav-1 KO mice. (A) Representative protein immunoblot and corresponding densitometry analysis (right column) for COX-2, (B) 5-LO and (C) COX-1 in lysates of tracheas with (+) and without (–) epithelium (epi) from Cav-1 KO and wild-type mice. Densitometry results represent replicates of scans of samples from 12–16 tracheal rings pooled from three to four mice from each strain. The *P*-values shown were obtained after one-way ANOVA with Tukey's multiple comparison test.

colological suppression of COX with indomethacin produced hyperreactivity in Cav-1 KO airways when compared with wild type airways (despite identical intrinsic contractile capacity of tracheal smooth muscle in both types). The effects of COX-2 inhibition in Cav-1-deficient airways correlated with an increased expression of both COX-2 and 5-LO almost exclusively found in the airway epithelium. Notably, the indomethacin-induced hyperreactivity of tracheal rings was sensitive to blockade of 5-LO signalling events. This functional effect seems most likely to be the result of shunting of PLA₂-derived arachidonic acid through the 5-LO pathway to produce contractile cysteinyl leukotrienes when the COX pathway was blocked (Maxis *et al.*, 2006; Marnett, 2009). We did not detect an augmentation of contraction in airways from wild-type mice in the presence of indomethacin, indicating that the substrate shunting that occurs in airways with normal expression of COX-2 and 5-LO in the airway epithelium was not sufficient to drive a measurable change in contraction. Thus, our study indicates that Cav-1 limits the outcome of COX inhibition on the contractile response of airways, but reduction in Cav-1 abundance (in particular in

the airway epithelium) facilitates COX-2 inhibition-mediated airway hyperresponsiveness.

We did not expect that tracheal smooth muscle from Cav-1 KO mice would exhibit normal contractile responses to methacholine (in the absence of indomethacin). Previous studies using *in vitro* and *ex vivo* models with acute molecular and pharmacological interventions have shown that Cav-1 plays an important role in supporting agonist-induced Ca²⁺ mobilization and associated contraction of isolated human ASM (Darby *et al.*, 2000; Gherghiceanu and Popescu, 2006; Gosens *et al.*, 2007b; Kamishima *et al.*, 2007; Prakash *et al.*, 2007; Sharma *et al.*, 2010). Thus, our new findings that contractile function was not compromised in Cav-1-deficient tracheal rings were at odds with evidence showing that caveolae facilitate contraction. Significantly, earlier studies have all used molecular and pharmacological interventions to induce acute, transient inhibition or silencing of Cav-1. Thus, our current findings using tissues from mice with a constitutive Cav-1 null phenotype suggest that compensatory mechanisms, for example, augmentation of Ca²⁺-independent pathways involving RhoA or PKC that have

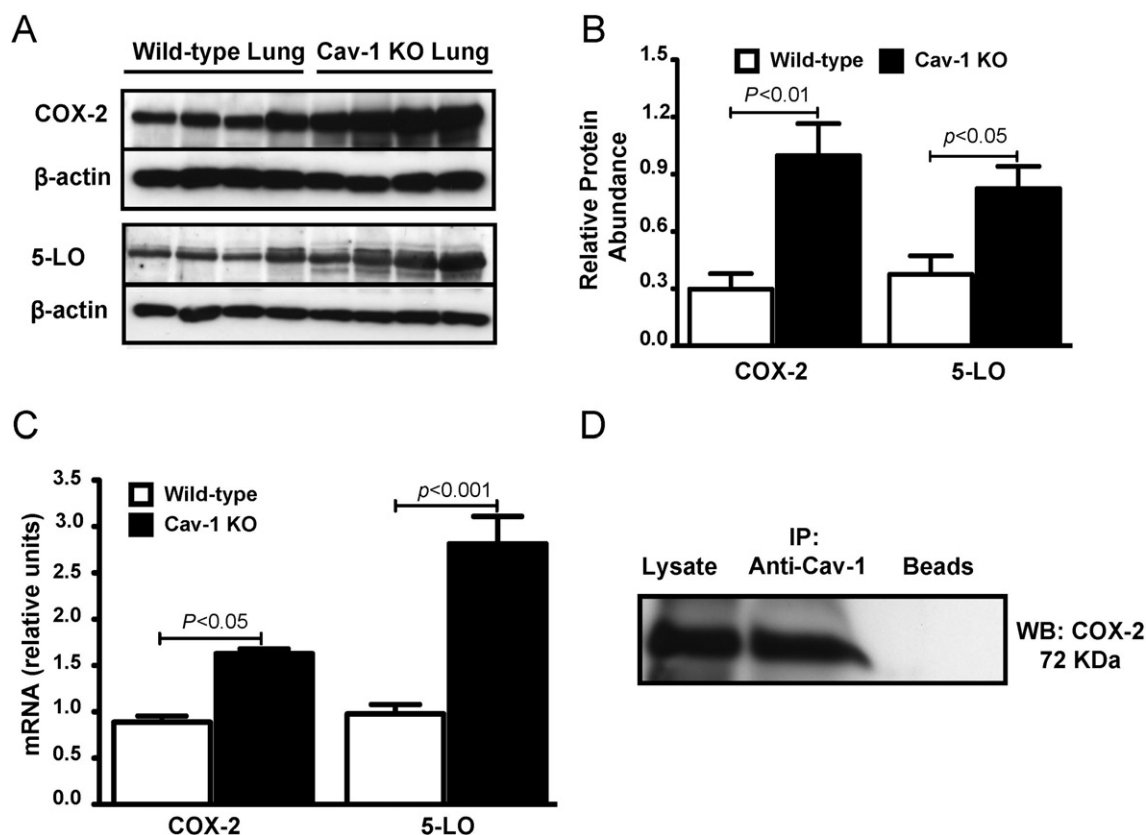


Figure 4

Increased expression of COX-2 and 5-LO in lungs from Cav-1 KO mice. (A) Representative Western blots and (B) corresponding densitometric analyses showing COX-2 and 5-LO abundance in whole lung lysates from Cav-1 KO and wild-type mice ($n = 4-5$ for each group). P -values shown were obtained after one-way ANOVA with Tukey's multiple comparison test. (C) Histogram showing results of quantitative RT-PCR for COX-2 and 5-LO mRNA in lysates from lungs of Cav-1 KO and wild-type mice. 18S RNA was used as an internal control ($n = 4-5$ for each group). P -values shown were obtained after one-way ANOVA with Tukey's multiple comparison test. (D) Western blot (WB) showing COX-2 abundance after immunoprecipitation (IP) with Cav-1 antibody and protein-G-conjugated sepharose beads from lung homogenates of wild-type mice. The lane labelled 'Beads' included sample but no antibody.

been linked to caveolae (Shakirova *et al.*, 2006; Somara *et al.*, 2007), may be altered in ASM from Cav-1 KO mice. The data in this current study do not provide direct insight into such mechanisms but do suggest that further work is needed to dissect the interplay between Cav-1 regulated Ca^{2+} -dependent and Ca^{2+} -independent pathways in ASM cells.

Cyclooxygenase is the key enzyme in prostanoid biosynthesis. One isozyme, COX-1, is expressed constitutively, whereas COX-2 is induced in inflammatory conditions including lung cancer and chronic airway diseases, such as asthma and COPD (Wasserman, 1988; Walls *et al.*, 1991; Wenzel, 1997; Gavett *et al.*, 1999; Taha *et al.*, 2000; Peebles *et al.*, 2002; Khanapure *et al.*, 2007; Torres *et al.*, 2008; Sala *et al.*, 2010). COX-2 is expressed in abundance by airway epithelial cells, thus allowing the synthesis of a wide profile of prostanoids (Eling *et al.*, 1986; Churchill *et al.*, 1989; Duniec *et al.*, 1989; Holtzman, 1992). Prostaglandins such as PGE_2 and PGI_2 that are synthesized abundantly by the airway epithelium typically suppress ASM contraction or promote relaxation, though this is species dependent and related to the expression profile of prostaglandin EP receptor subtypes (Backlund *et al.*, 2005; Ruan *et al.*, 2008; Clarke

et al., 2009; Park *et al.*, 2010). Several studies have shown that COX-2 can form a complex with Cav-1 with subsequent localisation to caveolae (Liou *et al.*, 2001; Perrone *et al.*, 2007). Interestingly, association of COX-2 with Cav-1 facilitates its degradation (Chen *et al.*, 2010a), Cav-1 can down-regulate β -catenin-Tcf/Lef-dependent transcription of COX-2 (Rodriguez *et al.*, 2009), and it modulates post-transcriptional mechanisms such as augmented proteosomal degradation (Folley-Bosco *et al.*, 2000; 2002) that regulate protein stability. Cav-1 can also regulate COX-2 activity, suppress PGE_2 synthesis and modulate transport of PGE_2 -containing vesicles to the plasma membrane for release (Kojima *et al.*, 2004; Rodriguez *et al.*, 2009). These observations are significant because we observed that COX-2 and 5-LO abundance was markedly increased in Cav-1 KO lung and trachea, and that COX-2 was associated with Cav-1 in wild-type lungs. Thus, COX-2 expression and protein half-life are likely to be increased in Cav-1 KO mice due to the absence of Cav-1-supported degradation. This concept correlates with our data showing that indomethacin-mediated suppression of COX activity promoted hyperreactivity selectively in Cav-1 KO tracheas.

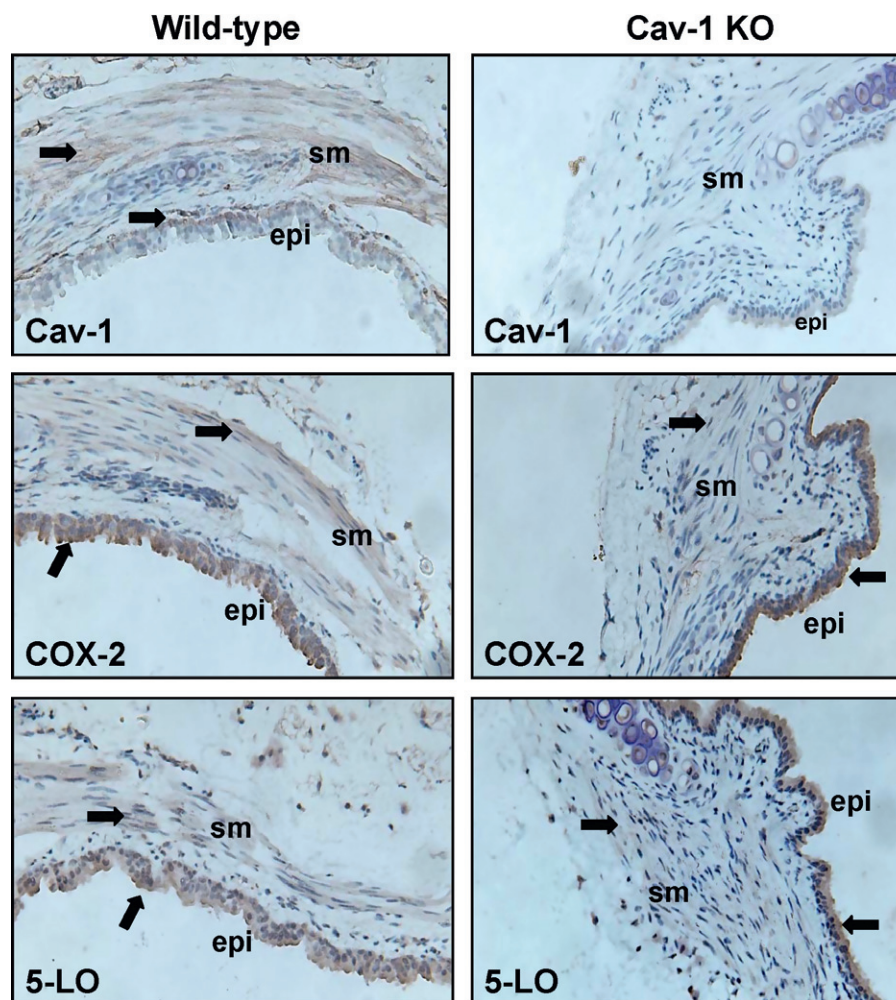


Figure 5

Cellular expression profile of Cav-1, COX-2 and 5-LO in airways from wild-type and Cav-1 KO mice. Representative immunohistological sections showing staining (brown colour highlighted with arrows) for Cav-1, COX-2 and 5-LO are shown. sm, airway smooth muscle layer; epi, epithelium.

As confirmed by our immunohistochemical evidence, multiple cell types, including epithelia and smooth muscle, express COX-2 and 5-LO. However, our current study suggests that an increased expression of these proteins in the airway epithelium is the primary mechanism for the altered contractility of Cav-1 deficient airways in the presence of indomethacin. Firstly, physical removal of the airway epithelium eliminates differences in COX-2 and 5-LO abundance (as measured by immunoblotting) between Cav-1 KO and wild-type airways. This observation implies that if the proteins are increased in ASM cells, immunoblotting is not sensitive enough to detect this trend. Secondly, we show that in the absence of epithelium there is no functional consequence of indomethacin treatment on the contractility of airways from Cav-1-deficient mice. On this basis, we conclude that although 5-LO and COX-2 are expressed by ASM, any functional effect of COX inhibition of muscle-expressed protein on contraction is too small to be detected by the bioassays we employed. Thirdly, there were no differences in the depolarization- or methacholine-induced contraction of wild-type and Cav-1-deficient airways with intact epithelium.

This observation suggests there was no intrinsic change in control of ASM responses. Thus, the measurable changes in airway contractility in response to COX inhibition appear to be linked to the epithelial dysfunction in Cav-1 KO tissues.

One of the novel findings of our study is that 5-LO levels were elevated, chiefly in the airway epithelium, in mice lacking Cav-1. However, to our knowledge there are no prior reports that indicate Cav-1 can interact with, or is linked to, pathways that regulate the expression of 5-LO, in contrast to the interactions between Cav-1 and COX-2 described above. Thus, the mechanism for increased 5-LO in Cav-1 KO lungs is not clear and further investigation is warranted, primarily because the LTs (i.e. LTB_4 , LTC_4 , LTD_4 and LTE_4) are potent bronchoconstrictors, chiefly mediating contraction of airway smooth muscle via the G-protein coupled receptor, $CysLT_1$ (Lynch *et al.*, 1999). We have shown that inhibition of 5-LO or blockade of $CysLT_1$ receptors was sufficient to reverse the indomethacin-induced hyperreactivity in tracheal rings with intact epithelium from Cav-1 KO mice. However, treatment of tracheal rings with montelukast or zileuton alone had no effect on the contractility of tissues from Cav-1 KO or wild-type

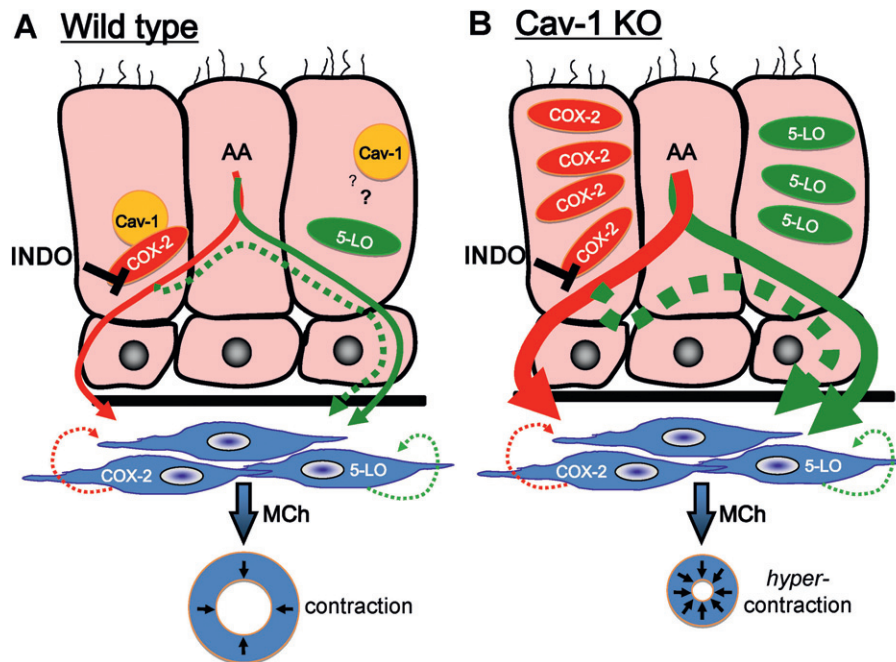


Figure 6

Schematic representation of the proposed effect of COX-2 inhibition on the production of arachidonic acid (AA) metabolites in epithelial cells and its association with disparate effects of airway contractility in wild-type and Cav-1 KO airways. (A) In wild-type airways, relatively low levels of COX-2 and 5-LO are expressed in the epithelium (pink cells) due to Cav-1-associated protein turnover. This is likely because of direct interaction of COX-2 with Cav-1; however, whether 5-LO interaction with Cav-1 is necessary is unknown (question mark). In wild-type tissues (in the absence of indomethacin; INDO), relatively modest basal levels of COX-2 and 5-LO metabolites are released by the epithelium (thin red and green arrows respectively), and act equally on underlying airway smooth muscle. Airway smooth muscle is also likely to produce small amounts of prostanooids (green hatched line) and leukotrienes (red hatched line) that have autocrine effects. Inhibition of the COX-2 pathway with indomethacin suppresses the generation of prostanooids, thereby promoting shunting of AA metabolites through the 5-LO arm of the cascade (hatched green arrow); however, this imbalance is not sufficient to affect methacholine (MCh)-induced contraction of airway smooth muscle (blue cells, blue ring below represents an intact airway). (B) For Cav-1 KO airways, the lack of caveolin-1 leads to selective accumulation (and increased activity) of both COX-2 and 5-LO in airway epithelial cells, and increases basal capacity to generate prostanooids and leukotrienes (thick red and green arrows respectively); however, in the absence of indomethacin, these metabolites act equally on underlying airway smooth muscle, thus no difference in airway response from wild-type tissues is evident. As in wild-type tissue, airway smooth muscle also produces small amounts of prostanooids (green hatched line) and leukotrienes (red hatched line), but unlike Cav-1 KO epithelium COX-2 and 5-LO in smooth muscle cells is not significantly elevated. In contrast to basal conditions, inhibition of the COX-2 pathway with indomethacin leads to significant unbalancing of AA metabolite production through shunting via the 5-LO branch of the cascade (thick, hatched green arrow). Due to the elevated levels of COX-2 and 5-LO, with indomethacin treatment sufficient levels of leukotrienes are synthesized and released to promote hypercontraction of the underlying airway smooth muscle (blue), an effect that is evident by excessive contraction in response to methacholine (depicted with blue ring at the bottom of the panel).

mice. These findings suggest that 5-LO signalling only contributed to enhanced contractility of Cav-1 KO tissues when COX was inhibited. Although inhibition of 5-LO by zileuton might have caused the shunting of arachidonic acid through the COX-2 pathway, we observed no measurable effect on airway contractile responses. This observation suggests that there was insufficient shunting to produce function-altering prostanooid concentrations, or that the prostanooids generated did not augment methacholine-induced contraction, as has been reported for some exogenously added prostanooids such as $\text{PGF}_{2\alpha}$ (Schaafsma *et al.*, 2005).

As depicted schematically in Figure 6, we suggest that increased epithelial expression of both COX-2 and 5-LO in Cav-1 KO tissues was a key predisposing factor for the selective indomethacin-induced hyperreactivity. Moreover, it seems likely that the augmented generation of cysteinyl LTs (and their subsequent binding to CysLT_1 receptors on ASM) in

Cav-1 KO airways was associated with a shunting of PLA_2 -derived arachidonic acid through the 5-LO pathway when COX-2 was inhibited (Maxis *et al.*, 2006; Chen *et al.*, 2010b). This effect may also occur in wild-type airways, but our functional evidence indicates that it is not sufficient to alter airway contractility, probably because of the much lower levels of COX-2 and 5-LO in the wild-type airway epithelium. These data suggest that Cav-1 acts as a determinant for the expression of 5-LO and COX-2, which in turn mediates the increased potential for epithelial control of airway contraction if COX-2 activity is modulated. However, in the absence of COX-2 modulation, airway contractility was unchanged in Cav-1 KO mice, this may have been because, although both COX-2 and 5-LO protein levels and activity were increased, the normal balance (ratio) of 5-LO and COX metabolites generated was maintained. We conclude that increased expression of COX-2 and 5-LO in Cav-1 KO airways predis-

posed these tissues to exaggerated shunting of arachidonic acid via 5-LO in the presence of indomethacin, yielding a functional enhancement of contraction. Consistent with this conclusion are our observations that epithelial denudation by itself was insufficient to produce tracheal smooth muscle hyperreactivity in Cav-1 KO preparations, thereby demonstrating that elevated epithelial COX-2 activity alone could not account for the indomethacin-induced hyperreactivity in Cav-1 deficient airways. One caveat to our interpretation is that our studies do not preclude the possibility of direct negative regulation of LT-induced contraction by PGs, a mechanism that will require further experimentation.

Our data also showed that wild-type murine airways were refractory to the effects of indomethacin, indicating that Cav-1 is likely to contribute to the inhibition of pathways that can impart control of airway responsiveness to the epithelium. Our experiments do not allow precise identification of these pathways, but revealing that Cav-1 is a regulator of these pathways is of relevance. For instance, smooth muscle contractility in chronic airway diseases is regulated by the epithelium, a rich source of lipid mediators and other types of contracting and relaxing factors (Barnes *et al.*, 1988; Fernandes *et al.*, 1990; Holtzman, 1992; Raeburn and Webber, 1994; Chung and Barnes, 1999; Fortner *et al.*, 2001). In asthma, aberrant biosynthesis of lipid mediators by COX-2 or 5-LO has been linked with changes in airway reactivity and bronchial inflammation (Wenzel, 1997; Wasserman, 1988; Gavett *et al.*, 1999; Peebles *et al.*, 2002; Peters-Golden and Henderson, 2007; Torres *et al.*, 2008). In bovine tracheal preparations, contraction is refractory to epithelial denudation (Schaafsma *et al.*, 2005), whereas prostanoids are released in abundance in guinea pig airways (Walls *et al.*, 1991). Thus, our finding that Cav-1 modulates expression and activity of COX-2 and 5-LO in the airway epithelium indicates that Cav-1 should be considered as a potential regulator of mechanisms that lead to changes in bronchial constriction in pathophysiological conditions.

In summary, our findings suggest that differences in Cav-1 expression can regulate the degree to which the airway epithelium is able to control bronchoconstriction (e.g. when COX is inhibited). Cav-1 contributes in this capacity through effects on expression and activity of both epithelial COX-2, which drives the synthesis of prostaglandins such as PGE₂, and 5-LO, which mediates synthesis of cysteinyl LTs. Moreover, our findings suggest that the ability of ASM to contract in response to other GPCR ligands (i.e. methacholine) is unchanged in Cav-1 KO mice, a result that differs from data showing acute transient silencing of Cav-1 suppresses Ca²⁺ mobilization and contraction. Our new findings suggest that other mechanisms that maintain contractility may be enhanced with long-term Cav-1 depletion. This study also reveals that Cav-1 and caveolae offer a complex control of airway responses via effects in different cell types that lead to alterations in both intrinsic cellular responses and intercellular communication.

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Conflicts of interest

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