ORIGINAL RESEARCH

Formaldehyde Induces Rho-Associated Kinase Activity to Evoke Airway Hyperresponsiveness

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Abstract

Formaldehyde, a common indoor air pollutant, exacerbates asthma and synergizes with allergen to induce airway hyperresponsiveness (AHR) in animal models. The mechanisms mediating formaldehyde-induced AHR remain poorly understood. We posit that formaldehyde modulates agonist-induced contractile response of human airway smooth muscle (HASM) cells to elicit AHR. HASM cells were exposed to formaldehyde or vehicle and agonist-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) and myosin light-chain phosphatase (MYPT1) phosphorylation were determined. Air–liquid interface–differentiated human bronchial epithelial (HBE) cells were exposed to formaldehyde or vehicle and cocultured with HASM cells. Agonist-induced $[Ca^{2+}]_i$ and MYPT1 phosphorylation were determined in the cocultured HASM cells. Precision-cut human lung slices were exposed to PBS or varying concentrations of formaldehyde, and then carbachol-induced airway narrowing was determined 24 hours after exposure. HASM cells were transfected with nontargeting or nuclear factor erythroid-derived 2, like 2 (Nrf-2)-targeting small interfering RNA and exposed to formaldehyde or vehicle, followed by determination of antioxidant response (quinone oxido-reductase 1 and thioredoxin 1) and basal and agonist-induced MYPT1 phosphorylation. Formaldehyde enhanced the basal Rho-kinase activity and MYPT1 phosphorylation with little effect on agonist-induced $[Ca^{2+}]_i$ in HASM cells. Formaldehyde induced Nrf-2–dependent antioxidant response in HASM cells, although the MYPT1 phosphorylation was independent of Nrf-2 induction. Although HBE cells exposed to formaldehyde had little effect on agonist-induced $[Ca^{2+}]_i$ or MYPT1 phosphorylation in cocultured HASM cells, formaldehyde enhanced carbachol-induced airway responsiveness in precision-cut human lung slices. In conclusion, formaldehyde induces phosphorylation of the regulatory subunit of MYPT1, independent of formaldehyde-induced Nrf-2 activation in HASM cells. The findings suggest that the Rho kinase-dependent Ca^{2+} sensitization pathway plays a role in formaldehyde-induced AHR.

Keywords: formaldehyde; airway hyperresponsiveness; asthma; airway smooth muscle; Ca^{2+} sensitization

Clinical Relevance

Formaldehyde, an indoor air pollutant, enhances asthma symptoms, although the mechanisms involved remain poorly understood. We examined whether formaldehyde modulates procontractile signaling in human airway smooth muscle (ASM) cells. We report that formaldehyde modulates a key Ca^{2+} sensitization pathway in ASM cells and enhances agonist-induced airway narrowing in precision-cut human lung slices. The study sheds light on airway structural cells as the potential mediators of formaldehyde-induced airway hyperresponsiveness.

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Asthma, a chronic airway disorder, manifests as airway inflammation, hyperresponsiveness, and remodeling. Higher incidence of airway disorders, such as asthma, in some populations is attributed to increased air pollution (1). Evidence also shows that air pollution adversely affects lung health in healthy individuals and in patients with asthma (2, 3). Although outdoor air pollutants, such as ozone, nitric oxide, and particulate matter of 2.5 μm or less, induce exacerbations of asthma and chronic obstructive pulmonary disease (4–6); formaldehyde, an indoor air pollutant, exacerbates asthma in children and adults (7–10). In animal models of asthma, formaldehyde exposure enhances the allergic airway inflammation and airway hyperresponsiveness (AHR) (11, 12). Studies in animal models suggest that airway inflammation from irritation and oxidative stress may be the mechanisms enhancing formaldehyde-induced AHR. Whether formaldehyde modulates airway structural cell functions, airway epithelium, and airway smooth muscle (ASM) remains unknown.

Shortening of ASM cells directly regulates airway resistance and hyperresponsiveness in asthma (13, 14). Agonist-induced Ca^{2+} mobilization and subsequent activation of actin–myosin cross-bridge formation are required for ASM cell shortening. In ASM cells isolated from subjects with asthma, expression and function of proteins involved in Ca^{2+} homeostasis are dysregulated (14–16). A second pathway that does not directly involve Ca^{2+} mobilization can also amplify ASM responses to contractile agonists.

Ca^{2+} sensitization pathways amplify the contractile response to agonists through signaling entities, such as RhoA/RhoA-associated kinase (ROCK) (17). ROCK signaling pathway regulates Ca^{2+} sensitization in smooth muscle cells by phosphorylating the regulatory subunit of myosin light-chain phosphatase (myosin phosphatase target [MYPT] subunit 1) (18, 19). Phosphorylation of T696 in the regulatory subunit MYPT1 inactivates the phosphatase activity of myosin light chain phosphatase (20), thus maintaining the phosphorylation of myosin light chain (MLC) and enhancing bronchomotor tone. In animal models of allergen-induced AHR, ROCK-dependent Ca^{2+} sensitization plays an important role in enhancing airway resistance (19). Fungal allergens from *Aspergillus fumigatus* (*Af*) enhance MYPT1 phosphorylation in human ASM (HASM) cells, suggesting that ROCK-dependent Ca^{2+} sensitization is an important mechanism in *A. fumigatus*–induced AHR (21). Evidence suggests that toxicants can impact signaling pathways in airway structural cells, such as airway epithelial and smooth muscle cells, to modulate lung functions. In addition to the protective role, airway epithelial cells also act as mediators of intercellular signals to modulate the other cells, such as ASM cells (22, 23). Environmental toxicants also can directly act on ASM cells, especially in pathophysiological conditions that disrupt the epithelial barrier (24). The environmental toxicants, acrolein and ozone, modulate agonist-induced Ca^{2+} homeostasis, which impacts the contractile functions of ASM, thereby altering pulmonary function (25, 26). These observations underscore the importance of airway structural cells and in mediating the adverse effects of respiratory toxicants. We hypothesized that formaldehyde modulates agonist-induced contractile response of HASM cells to elicit AHR. Our findings show that formaldehyde enhances agonist-induced airway narrowing in human lung slices without significant changes in HASM cellular Ca^{2+} response. Collectively, these findings suggest that ROCK activation mediates formaldehyde-induced AHR in human small airways.

Materials and Methods

Reagents

Ham's F-12 medium, PBS, FBS, 0.05% trypsin and EDTA, Lipofectamine RNAimax, Opti-MEM, cDNA synthesis kit, TriZol, SYBR green quantitative PCR reaction mixture, and all PAGE/immune blotting supplies were purchased from Life Technologies (Grand Island, NY). Primers for ROCK1, ROCK2, and cyclophilin were purchased from IDT (Coralville, IA). Carbachol, bradykinin, and thrombin were purchased from Sigma-Aldrich (St. Louis, MO). Nontargeting small interfering RNA (siRNA) and nuclear factor erythroid-derived 2, like 2 (Nrf-2)–targeting siRNA were purchased from ThermoFisher Scientific (Dharmacon, Lafayette, CO). Antibodies against phosphorylated MYPT1 (pMYPT1) were obtained from Millipore (Billerica, MA). pMLC and α-tubulin were from Cell Signaling Technology (Danvers, MA) and quinone oxido-reductase (NQO) 1 and thioredoxin (Trx) 1 were from Santa Cruz Biotechnology (Santa Cruz, CA;). Y27642 was purchased from Cayman Chemicals (Ann Arbor, MI). Chemiluminescent reagent was obtained from ThermoFisher Scientific (Pierce, Rockford, IL).

Culture of HASM and Human Bronchial Epithelial Cells

Primary HASM cells were isolated from donor lung trachea as described previously (27, 28). HASM cells (passage 2–4) were grown in Ham's F-12 medium containing 10% FBS to confluence. Before experiments, the cells were serum starved for 48 hours. Human bronchial epithelial (HBE) cells were cultured and differentiated in air–liquid interface, as previously described (29). Differentiated cells were exposed to vehicle or formaldehyde for 1 hour and placed on serum-starved HASM cells for 24 hours. pMLC/pMYPT1 or agonist-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) were determined in cocultured HASM cells.

Formaldehyde Exposure

Neutral buffered formalin solution (10%; VWR International, Radnor, PA) in PBS was placed in a glass impinger and atmospheric air was drawn by vacuum force (5 L/min) to generate formaldehyde vapor. Formaldehyde vapor was delivered into a chamber containing precision-cut human lung slice (PCLSs) or cells. Various concentrations of formalin solutions were used in preliminary trials to obtain vapor concentrations of 0.2, 0.8, or 2 ppm formaldehyde within the exposure chamber. Chamber formaldehyde levels were measured by passive formaldehyde monitors (product no. 3721; 3M, St. Paul, MN) placed in the chamber and submitted for analysis (Bureau Veritas, Novi, MI). PCLSs or cells were exposed to PBS or formaldehyde vapor (0.2, 0.8, or 2 ppm) for 1 hour and readouts were determined 24 hours after exposure.

Determination of $[Ca^{2+}]_i$ in HASM Cells

Agonist-induced $[Ca^{2+}]_i$ in HASM cells was determined as previously described (21) with some modifications. Bradykinin (1 nM) or histamine (1 μM) was used as agonist.

Mediator Release from PCLSs and HASM Cells

IL-6 and IL-8 levels were determined in culture supernatants of lung slices or HASM cells following the manufacturer's instructions (R&D Systems, Minneapolis, MN). Cytokine levels were normalized to the total protein of the supernatants.

ROCK Assay

Lysates were collected from HASM cells in Tris-sucrose lysis buffer with protease and phosphatase inhibitors. Protein (10 μg) was used in duplicate to determine ROCK activity following the manufacturer's instructions (catalog no. CSA001; Millipore).

Immunoblotting

Lysates were collected from HASM cells in Tris-sucrose buffer with protease and phosphatase inhibitors. Protein (10 μg) was run in SDS-PAGE for immunoblotting. The membrane was blocked with 3% BSA solution and probed for nicotinamide adenine dinucleotide phosphate reduced–NQO1, Trx, and α-tubulin. To detect phosphoproteins, lysates were collected in 0.6 N $HClO_4$.

siRNA Transfection

HASM cells were transfected with nontargeting or Nrf-2 siRNA using Lipofectamine RNAiMAX as previously described (30). Nrf-2 silencing was confirmed by NQO1 and Trx1 expression. HASM cells were exposed to formaldehyde at 72 hours after transfection and cell lysates were collected 24 hours after formaldehyde exposure.

Human PCLS and Carbachol Dose–Response

Human lungs were obtained through NDRI (Philadelphia, PA) or IIAM (Edison, NJ). Samples were deidentified and therefore exempted by the University of Pennsylvania (Philadelphia, PA) Institutional Review Board. PCLSs were prepared and carbachol (10^{-8}–10^{-4} M) dose–response parameters (half-maximal effective concentration [log EC_{50}], area under the curve, and maximal effect) were determined as previously described (31).

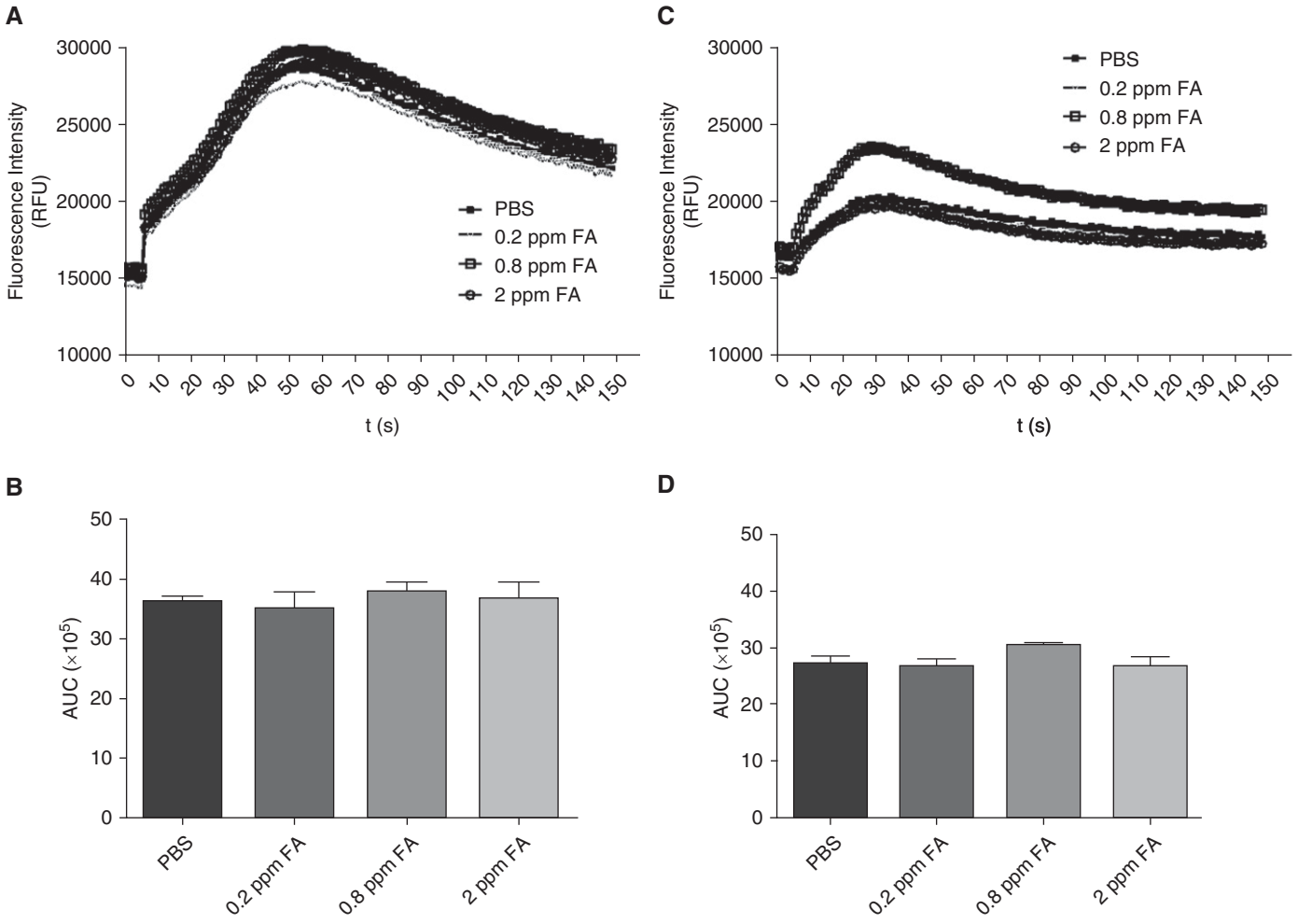

Figure 1. Formaldehyde has little effect on agonist-induced intracellular Ca^{2+} $[Ca^{2+}]_i$ in human airway smooth muscle (HASM) cells. HASM cells were loaded with the Ca^{2+}-binding dye fluo-8 and stimulated with agonists to determine the global changes in $[Ca^{2+}]_i$. There was no significant effect on the (*A*) bradykinin-induced (1 nM) or (*C*) histamine-induced (1 μM) $[Ca^{2+}]_i$ in cells exposed to formaldehyde. Area under the curves (AUCs) of $[Ca^{2+}]_i$ transients induced by (*B*) bradykinin or (*D*) histamine were comparable between PBS-treated and formaldehyde-treated HASM cells ($n = 3$ donors with 3 technical replicates per condition); *A* and *C*, mean RFU; *B* and *D*, mean ± SEM. FA, formaldehyde; ppm, parts per million; RFU, relative fluorescence unit.

Statistical Analysis

HASM cells from at least three donors or PCLSs from at least five donors were used in the studies. In PCLS studies, from each donor, a minimum of three slices per experimental group were used. Data are expressed as mean or mean (±SEM). GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA) was used for statistical analysis (one way ANOVA or Student's *t* test) and means were considered significantly different when P was less than or equal to 0.05.

Results

Formaldehyde Has Little Effect on Agonist-Induced $[Ca^{2+}]_i$, but Enhances ROCK Activity in HASM Cells

Mobilization of $[Ca^{2+}]_i$ in ASM cells is a pivotal signaling event in regulating agonist-induced ASM shortening. To determine whether formaldehyde exposure alters agonist-induced $[Ca^{2+}]_i$ in HASM cells, bradykinin or histamine-induced $[Ca^{2+}]_i$ was determined in HASM cells 24 hours after exposure to formaldehyde. The magnitude of bradykinin-induced $[Ca^{2+}]_i$ (area under the curve of the Ca^{2+} transients) was comparable in PBS and formaldehyde-treated HASM cells (Figures 1A and 1B). Similarly, histamine (1 μM) evoked comparable magnitudes of $[Ca^{2+}]_i$

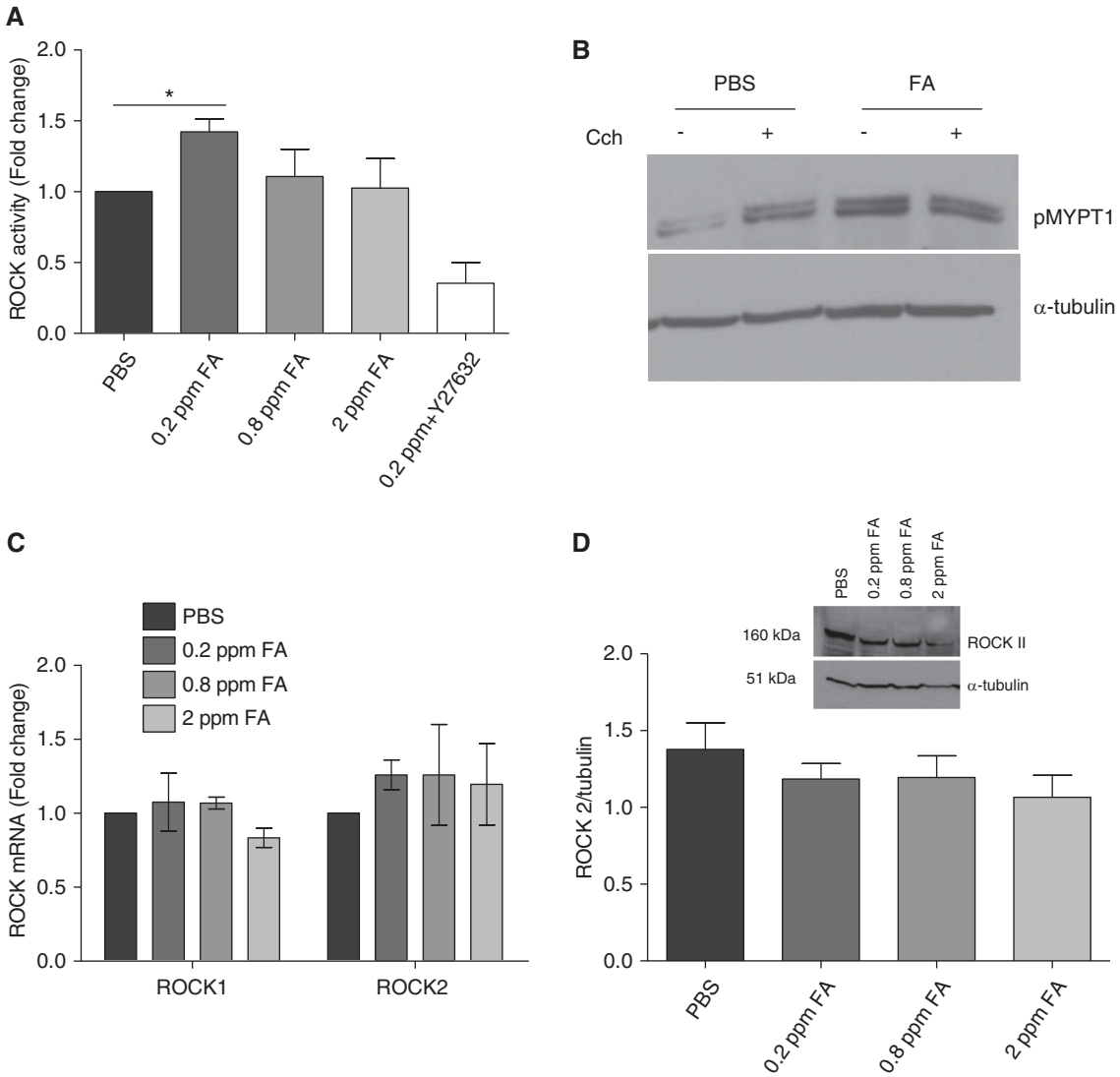

Figure 2. Formaldehyde enhances myosin light-chain phosphatase (MYPT1) phosphorylation in HASM cells. Rho-associated kinase (ROCK) activity was determined in HASM cell lysates by assessing phosphorylated (p) MYPT1 (pMYPT1) levels, either by ELISA or immune blotting. (*A*) Formaldehyde (0.2 ppm) increased the basal ROCK activity in HASM cells compared with PBS-treated control (mean ± SEM; $n = 3$ donors; $^*P < 0.05$). (*B*) Phosphorylation of MYPT1, the downstream target of ROCK, was increased in HASM cells exposed to 0.2 ppm formaldehyde. Stimulation with the contractile agonist carbachol (10 μM for 10 min) increased the pMYPT1 in PBS-treated HASM cells but not in formaldehyde-treated cells (blot representative of five experiments). Formaldehyde exposure has little effect on the expression of (*C*) ROCK1/2 mRNA (mean ± SEM, $n = 3$) or (*D*) ROCK2 protein in HASM cells (representative blot for $n = 3$; mean ± SEM). CCh, carbachol concentration.

responses in HASM cells exposed to PBS and formaldehyde (Figures 1C and 1D). Furthermore, formaldehyde had little effect on basal and thrombin-induced $[Ca^{2+}]_i$ in HASM cells (Figure E1). Ca^{2+} sensitization is a complementary mechanism that sensitizes the contractile apparatus to an existing $[Ca^{2+}]_i$ level so that the contractile response is enhanced without an increase in agonist-induced $[Ca^{2+}]_i$. ROCK pathway is a key modulator of Ca^{2+} sensitization in smooth muscle cells (19). We next determined ROCK activity in HASM cell lysates by assessing the pMYPT1 level, either by ELISA or by immunoblotting. In ELISA assays, ROCK activity was significantly increased in HASM cells exposed to 0.2 ppm formaldehyde compared with PBS-treated cells (Figure 2A). We confirmed increased MYPT1 phosphorylation by immunoblotting lysates from HASM cells exposed to formaldehyde. The basal MYPT1 phosphorylation was increased by formaldehyde exposure, even in the absence of a contractile agonist (Figure 2B). To determine whether formaldehyde alters ROCK gene expression, we examined ROCK I and 2 mRNA expression levels in HASM cells 24 hours after exposure to PBS or formaldehyde. Formaldehyde has little effect on ROCK1 or ROCK2 mRNA levels in HASM cells (Figure 2C). Similar to ROCK mRNA levels, formaldehyde had little effect on ROCK2 protein expression in HASM cells (2 D). In ASM cells, ROCK activation is regulated by the upstream small molecular weight G protein RhoA. To determine whether formaldehyde induces MYPT1 phosphorylation via Rho activation, HASM cells were exposed to formaldehyde in the presence of Rho Inhibitor I (CT04 or exoenzyme C3 transferase of *C.botulinum*). Rho Inhibitor has little effect on

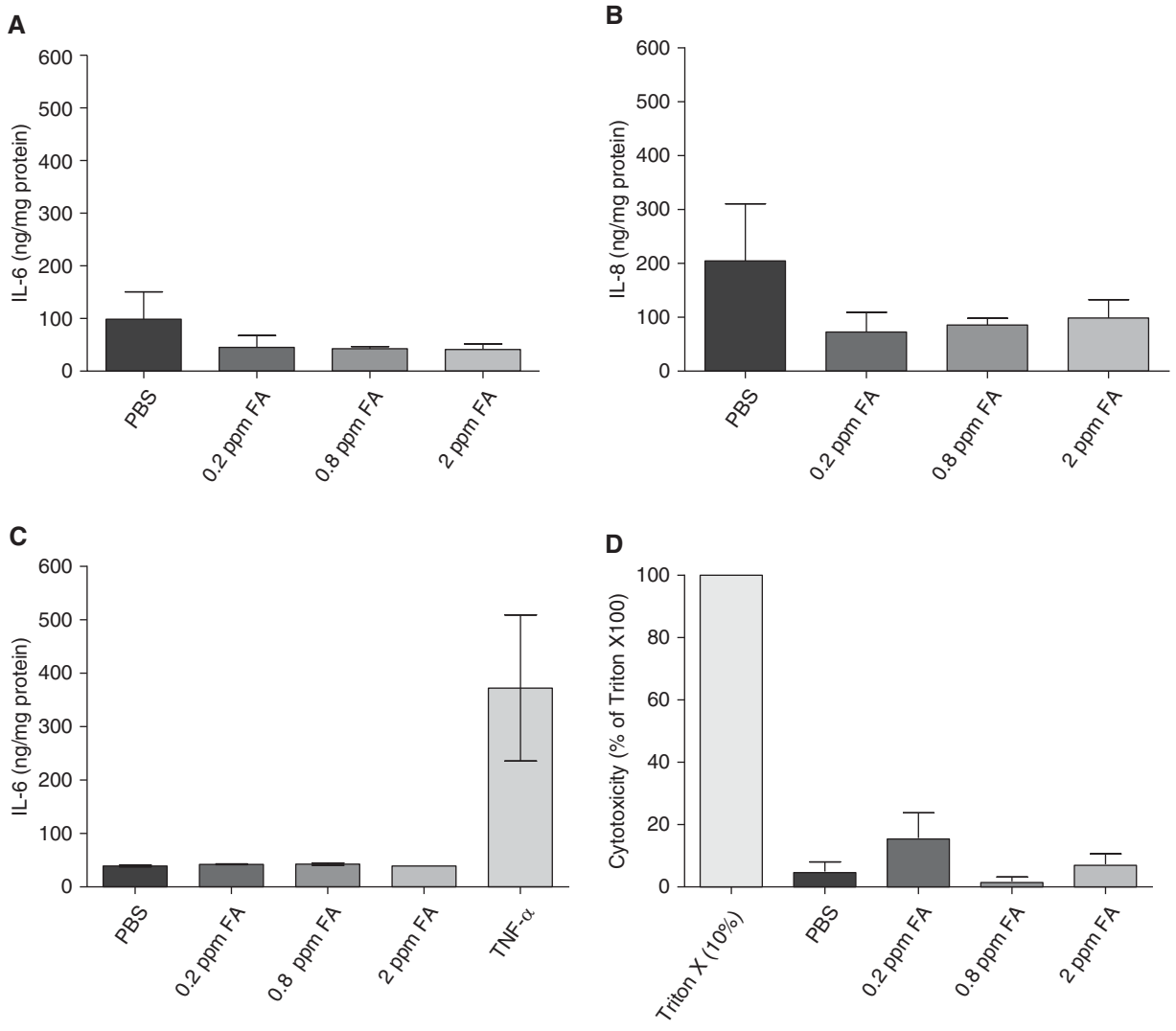

Figure 3. Formaldehyde has little effect on inflammatory mediator release from precision-cut human lung slices (PCLSs) or HASM cells. In the supernatants of lung slices, formaldehyde reduced the (*A*) IL-6 and (*B*) IL-8 levels, although the reduction was not statistically significant (mean ± SEM; $n = 3$ donors). (*C*) IL-6 levels in the culture supernatants of HASM cells were not altered by formaldehyde exposure. TNF-α (10 ng/ml, 24 h) induced a robust IL-6 level in the HASM cells (mean ± SEM; $n = 4$ donors). To confirm whether formaldehyde adversely affects the viability of slices, lactate dehydrogenase (LDH) activity was determined in supernatants. (*D*) Formaldehyde caused marginal LDH release compared with 10% Triton X 100 (six slices from two independent donors).

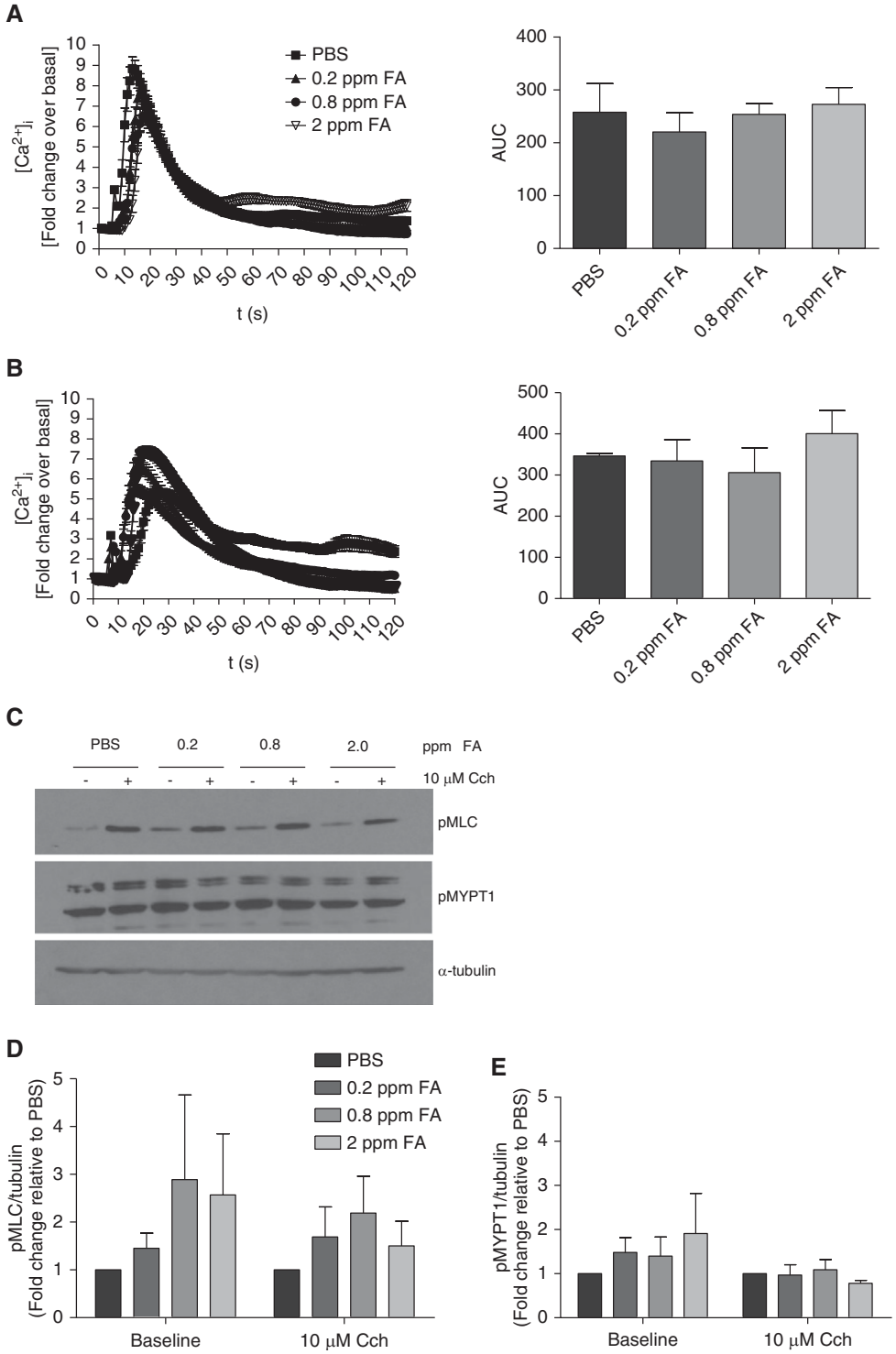

Figure 4. Formaldehyde-treated human bronchial epithelial (HBE) cells have little effect on agonist-induced $[Ca^{2+}]_i$ or MYPT1 phosphorylation in HASM cells. Air–liquid interface–differentiated HBE cells were exposed to formaldehyde and co-incubated with HASM cells for 24 hours. Agonist-induced $[Ca^{2+}]_i$ and phosphorylation status of myosin light chain (MLC) and MYPT1 were determined in the HASM cells. Co-culture of HASM cells with formaldehyde-treated HBE cells had little effect on (*A*) histamine- or (*B*) thrombin-induced $[Ca^{2+}]_i$ in the HASM cells (*left panels*, baseline-corrected fluorescence intensity; *right panels*, AUCs of the traces; mean ± SEM, $n = 3$ donors, 60 cells/experimental condition). (*C*) There were marginal elevations in the baseline phosphorylation of MLC and MYPT1 in the HASM cells cocultured with formaldehyde-treated HBE cells (immune blot representative of five experiments). (*D*) At the baseline (without carbachol), phosphorylated MLC levels trended toward an increase in HASM cells cocultured with formaldehyde-treated HBE cells, although the increase was not statistically significant (mean ± SEM; $n = 5$ donors). (*E*) At baseline, phosphorylated MYPT1 levels were marginally elevated in HASM cells upon coculture with formaldehyde-treated HBE cells, without statistical significance (mean ± SEM; $n = 5$ donors).

formaldehyde-induced MYPT1 phosphorylation (Figure E2).

Formaldehyde Has Little Effect on Inflammatory Mediator Release from PCLSs or HASM Cells

ASM cells contribute to asthma pathogenesis, not only by modulating bronchomotor tone, but also by releasing inflammatory mediators. IL-8 and IL-6 are two major inflammatory mediators released by ASM cells. Ozone, an important environmental pollutant, induces IL-6 release from HASM and airway epithelial cells (29). To determine whether formaldehyde induces cytokine or chemokine secretion, PCLSs or HASM cells were exposed to PBS or formaldehyde and the culture supernatants were analyzed for IL-6 and IL-8. In PCLSs, supernatant IL-6 or IL-8 levels were unaffected in response to formaldehyde exposure (Figures 3A and 3B). Furthermore, formaldehyde has little effect on IL-6 release from HASM cells, while TNF-α (10 ng/ml) increased IL-6 levels (Figure 3C). IL-8 levels followed a similar pattern in HASM cell culture supernatant after formaldehyde exposure (data not shown). To address whether formaldehyde induces cytotoxicity in lung slices, lactate dehydrogenase (LDH) activity was determined in the slice supernatants. Formaldehyde-induced LDH release was minimal in slices when compared with the Triton X-100, which was the positive inducer of toxicity (Figure 3D).

Formaldehyde-Treated HBE Cells Have Little Effect on Agonist-Induced $[Ca^{2+}]_i$ or MYPT1 Phosphorylation in HASM Cells

Epithelial cells that line the airway are likely some of the first cells exposed to inhaled formaldehyde. *In vitro*, airway epithelial cells exposed to formaldehyde exhibit altered cell structure and functions (32). To determine whether epithelial cells mediate formaldehyde effects on the HASM cells, air–liquid interface–differentiated HBE cells were exposed to formaldehyde and then co-cultured with HASM cells for 24 hours. Coculture of formaldehyde-treated HBE cells with HASM cells has little effect on agonist-induced $[Ca^{2+}]_i$ (Figures 4A and 4B). There was a trend toward enhancement of basal MLC phosphorylation in HASM cells co-cultured with formaldehyde-treated HBE cells (Figures 4C and 4D). Formaldehyde-treated HBE cells had little effect on the phosphorylation of MYPT1 (Figures 4C and 4E).

Formaldehyde Elicits Nrf-2–Dependent Antioxidant Response in HASM Cells

In other cells types, formaldehyde effects are partly attributed to oxidative injury (33). Typical cellular antioxidant response is mediated by stabilization and activation of nuclear factor erythroid-derived 2, like 2 (Nrf-2) and subsequent up-regulation of antioxidant and cytoprotective genes (34). We sought to determine whether formaldehyde induces Nrf-2 activation in HASM cells. The cytoprotective enzyme, nicotinamide adenine dinucleotide phosphate reduced–NQO1, and the antioxidant protein, Trx1, served as the surrogates of Nrf-2 activation in these experiments. NQO1 and Trx1 expression levels increased as early as 2 hours after formaldehyde exposure and remained elevated 18 hours after exposure (Figure 5A). In subsequent experiments, NQO1 and Trx1 expression levels were determined after 18 hours exposure to PBS or formaldehyde. The lowest concentration of formaldehyde (0.2 ppm) increased NQO1 and Trx1 levels in HASM cells (Figures 5C and 5D) while exposure to higher concentration (2 ppm) also increased, albeit to a lesser level (Figure 5D).

Formaldehyde-Induced MYPT1 Phosphorylation Is Independent of Nrf-2 Induction in HASM Cells

Cellular antioxidant response elicited by Nrf-2 plays a protective role against oxidants and environmental toxicants (35). To determine whether Nrf-2 has a role in formaldehyde-induced MYPT1 phosphorylation, HASM cells were exposed to PBS or formaldehyde after transiently transfecting them with Nrf-2–targeted siRNA. After transfection of Nrf-2–targeting siRNA, both basal and formaldehyde-induced NQO1 or Trx1 expressions were completely abolished (Figure 6A). Although there was a variable response to Nrf-2 silencing among the 5 donors, on average, silencing of Nrf-2 has little effect on formaldehyde-induced MYPT1 phosphorylation in HASM cells (Figure 6B).

Formaldehyde Enhances Carbachol-Induced Airway Narrowing in PCLSs

Our findings show that formaldehyde enhances a key Ca^{2+} sensitization pathway in HASM cells, potentially enhancing agonist-induced excitation-contraction coupling in HASM cells. To determine whether formaldehyde enhanced airway responsiveness, human PCLSs were exposed to PBS or formaldehyde and carbachol-induced contractile response was determined 24 hours after exposure. Exposure to 2 ppm formaldehyde enhanced carbachol-induced contractile response in PCLSs (Figure 7A). The maximal contractile response was comparable in PBS- and formaldehyde-treated slices (Figure 7B). Sensitivity to carbachol, as measured by log EC_{50}, was significantly increased in 2 ppm formaldehyde-treated slices compared with the PBS-treated slices (Figure 7C). Area under the curve of the carbachol dose–response curve was significantly increased in slices exposed to 2 ppm formaldehyde compared with that of PBS-treated slices (Figure 7D).

Discussion

Formaldehyde induces asthma exacerbations in humans and AHR in animal models of asthma (7, 9, 11). The underlying mechanisms, however, remain poorly understood. We hypothesized that formaldehyde induces AHR and asthma exacerbation through modulating the contractile response of ASM cells to agonists. Our findings show that formaldehyde enhances contractile response of human lung slices to agonist. Furthermore, formaldehyde exposure enhanced MYPT1 phosphorylation in HASM cells, suggesting Ca^{2+} sensitization as a possible mechanism causing AHR. To the best of our knowledge, this is the first report on the effect of formaldehyde on a signaling pathway that leads to enhanced contractile response in ASM cells.

Ca^{2+} mobilization and elevated $[Ca^{2+}]_i$ regulate excitation–contraction coupling in various types of smooth muscle cells (reviewed in Ref. 36). Altered

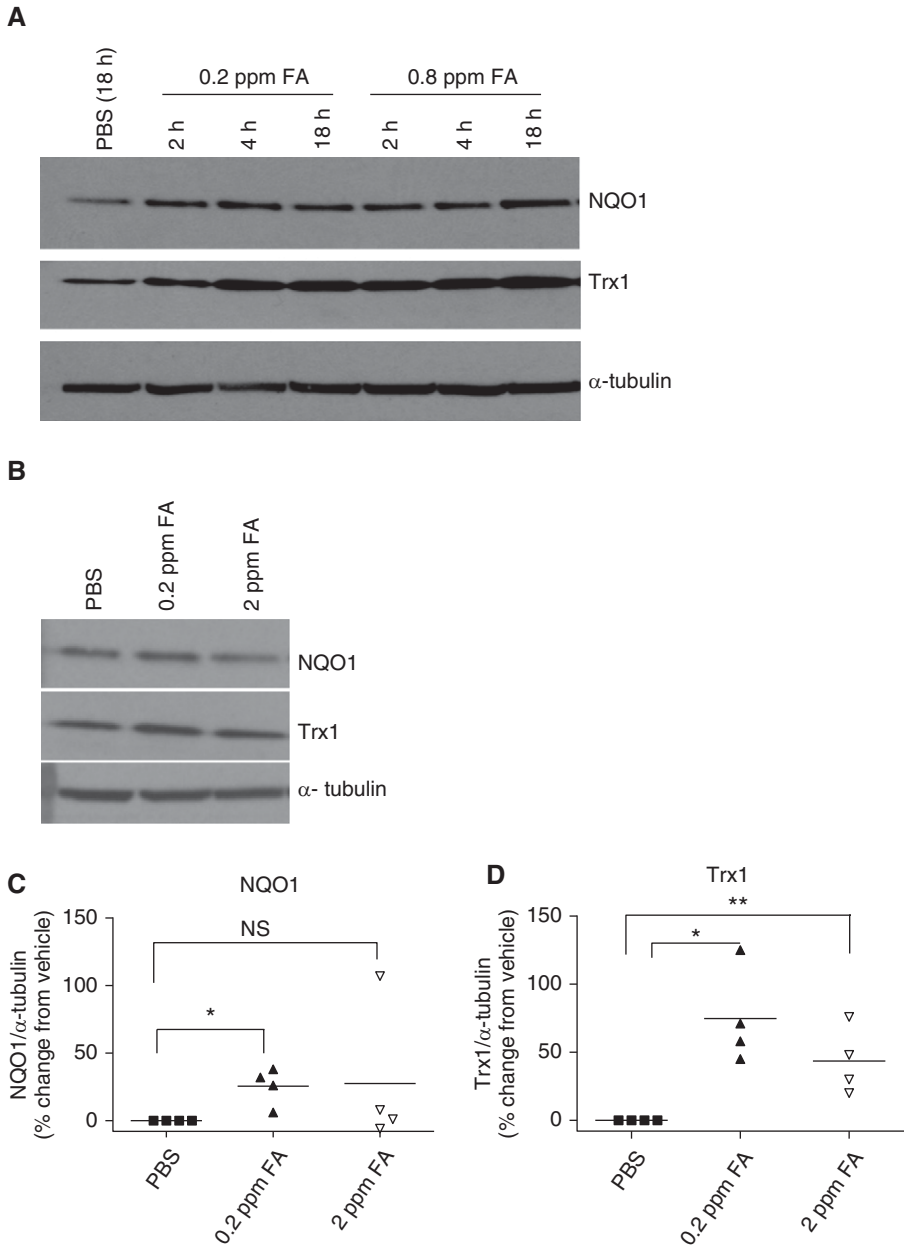

Figure 5. Formaldehyde elicits nuclear factor erythroid-derived 2, like 2 (Nrf-2)–dependent antioxidant response in HASM cells. Whole-cell lysates from HASM cells exposed to PBS or formaldehyde were immune blotted for the markers of antioxidant (thioredoxin [Trx] 1) and cytoprotective (quinone oxido-reductase [NQO] 1) responses, as surrogate measures of Nrf-2 activation. (*A*) In response to formaldehyde exposure (0.2 and 0.8 ppm), NQO1 and Trx1 were elevated compared with the PBS-treated cells. NQO1 and Trx1 increases were noticed as early as 2 hours after exposure and remained elevated 18 hours after exposure (representative immune blot, $n = 2$). (*B–D*) At 18 hours after exposure, the lowest concentration of formaldehyde (0.2 ppm) caused a significant increase in NQO1 (*$P = 0.017$, $n = 4$) and Trx1 (*$P = 0.0054$, $n = 4$ donors), whereas a higher level of formaldehyde (2 ppm) caused relatively subdued induction of NQO1 and Trx1 (**$P = 0.012$, $n = 4$ donors). (*B*) Representative immune blot for $n = 4$ donors; (*C* and *D*) densitometry ratio; *black lines* show the mean for $n = 4$ donors. NS, not significant.

Ca^{2+} mobilization mechanisms are reported in ASM cells isolated from donors with asthma, potentially accounting for enhanced agonist-induced ASM cell shortening in asthma (15, 16). Toxicants, such as acrolein and ozone, enhance agonist-induced airway narrowing by altering the Ca^{2+} mobilization in ASM cells (25, 37). Lack of significant effects by formaldehyde on ASM cell Ca^{2+} dynamics prompted us to focus on an alternate mechanism of hypercontractility in ASM cells.

Ca^{2+} sensitization, a mechanism in which the contractile apparatus of smooth muscle cells is rendered more sensitive to $[Ca^{2+}]_i$ levels, amplifies even smaller increments in $[Ca^{2+}]_i$ to cause a larger magnitude of ASM cell shortening (reviewed in Ref. 38). In smooth muscle cells, RhoA/RhoA-associated kinase (RhoA/ROCK) signaling is a key regulator of Ca^{2+} sensitization (39). Our results show that ROCK activity is increased 24 hours after formaldehyde exposure. This observation, taken together with unaltered ROCKI/II levels, implied either: (*1*) an enhancing effect on ROCK enzyme activity by formaldehyde; or (*2*) increased RhoA activity upstream of ROCK. Our studies, using a cell-permeant Rho inhibitor, suggested that formaldehyde-induced MYPT1 phosphorylation is Rho-independent. Studies in vascular smooth muscle, however, show that oxidative stress and reactive oxygen species modulate ROCK-dependent Ca^{2+} sensitization (40). Furthermore, in rodent models of AHR, formaldehyde exposure increased oxidative stress and attenuated antioxidant responses (33). These observations prompted us to determine whether similar mechanisms are functional in HASM cells exposed to formaldehyde.

Formaldehyde induced a typical Nrf-2–dependent antioxidant response in HASM cells. However, the effect of Nrf-2 silencing on formaldehyde-induced MYPT1 phosphorylation was heterogeneous among the five donors used in the experiments. The variability among donors may be due to differential basal redox capacity. It is difficult to speculate on specific signaling molecules involved in this redox status heterogeneity, although various redox-related polymorphisms have been reported in humans. For instance, polymorphisms in γ glutamyl cysteine synthetase, the key enzyme of glutathione synthesis, are known to be associated with cystic fibrosis and certain lung cancers (41, 42). Alternatively, formaldehyde-induced MYPT1 phosphorylation and oxidative injury may be independent events in HASM cells. Because formaldehyde is an electrophile, the enhancing effects on ROCK activity may be

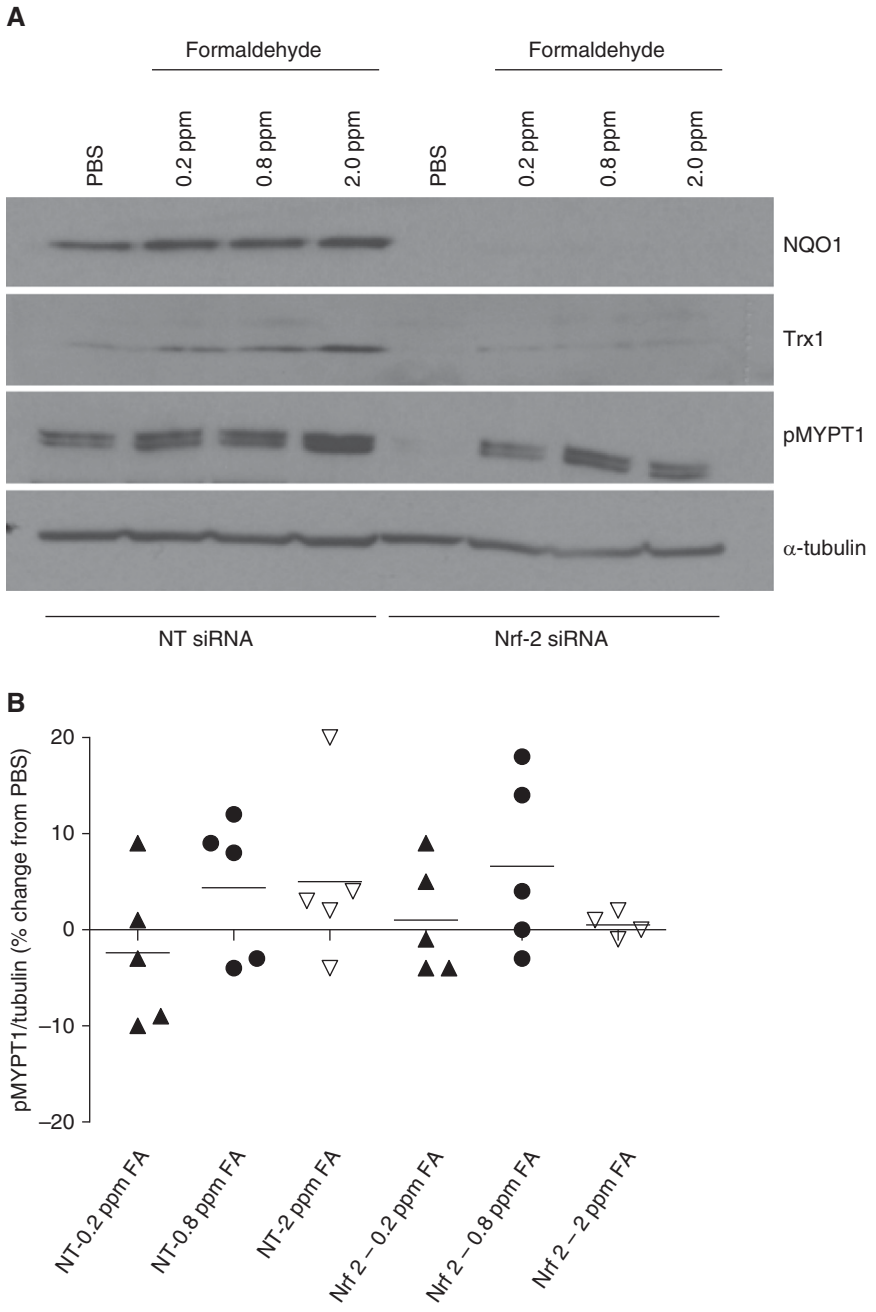

Figure 6. Formaldehyde-induced MYPT1 phosphorylation is independent of Nrf-2 induction in HASM cells. HASM cells were transiently transfected with nontargeting (NT) or Nrf-2–trageting (Nrf-2) small interfering RNA (siRNA) (40 nM) and exposed to PBS or formaldehyde 72 hours after transfection. Phosphorylated MYPT1 levels were determined at baseline 24 hours after formaldehyde exposure. (*A*) NQO1 and Trx1 expression levels were significantly down-regulated 72 hours after transfection with Nrf-2 siRNA (representative immune blot; $n = 5$ donors). (*B*) Formaldehyde-induced MYPT1 phosphorylation was comparable in HASM cells transfected with NT or Nrf-2 siRNA (each *point* denotes an independent donor; *horizontal black lines* mark means; $n = 5$ donors).

mediated directly by electrophile attack and be independent of reactive oxygen species generation. Our findings, that formaldehyde-induced Rho kinase activity is independent of Rho activation, lend further support to the aforementioned hypothesis. Although it remains to be determined whether this is the mechanism, the physicochemical properties of formaldehyde, such as higher solubility and smaller molecular weight, support this hypothesis.

Airway epithelial cell–ASM interaction is another plausible mechanism for formaldehyde-induced AHR that we observed in lung slices. HBE cell–HASM cell coculture studies were designed to test this possibility. We showed that HBE cells exposed to formaldehyde have little effect on agonist-induced Ca^{2+} mobilization in HASM cells. However, formaldehyde-treated HBE cells trended toward enhancing pMLC levels in the HASM cells, suggesting that the epithelial cells may be an integral component in the formaldehyde-enhanced airway responsiveness seen in PCLSs.

Formaldehyde is primarily an indoor air pollutant, although it is an important component in outdoor emissions from automobiles and industries (43). In the developed countries, indoor formaldehyde levels in households range between 0.005 and 0.136 ppm, depending on the method of measurement (7, 10, 44–46). In the United States, the National Institute for Occupational Safety and Health recommended exposure limit over an 8-hour period is 0.016 ppm. A recent study by Dannemiller and colleagues (7) found that a significant proportion of households in their study population had formaldehyde levels well above the National Institute for Occupational Safety and Health standard. We chose 0.2 ppm as our minimum formaldehyde level to account for the shorter exposure time (1 h) in the cells and PCLSs. The lowest level of formaldehyde (0.2 ppm) induced antioxidant response and ROCK activation in HASM cells, whereas only the highest dose (2 ppm) elicited enhanced airway narrowing in lung slices. It is likely that, compared with a monolayer of cells, the lung slices (~350 μm in thickness) had additional barriers to the distribution of formaldehyde, requiring 10-fold higher formaldehyde concentration to elicit a response.

Most of the studies examining the effects of formaldehyde have focused on *in vitro* cultures of airway epithelial cells. These studies showed that formaldehyde exposure alters gene expression profile of airway epithelial cells and enhances inflammatory mediator release (47–50). Evidence on the direct effects of

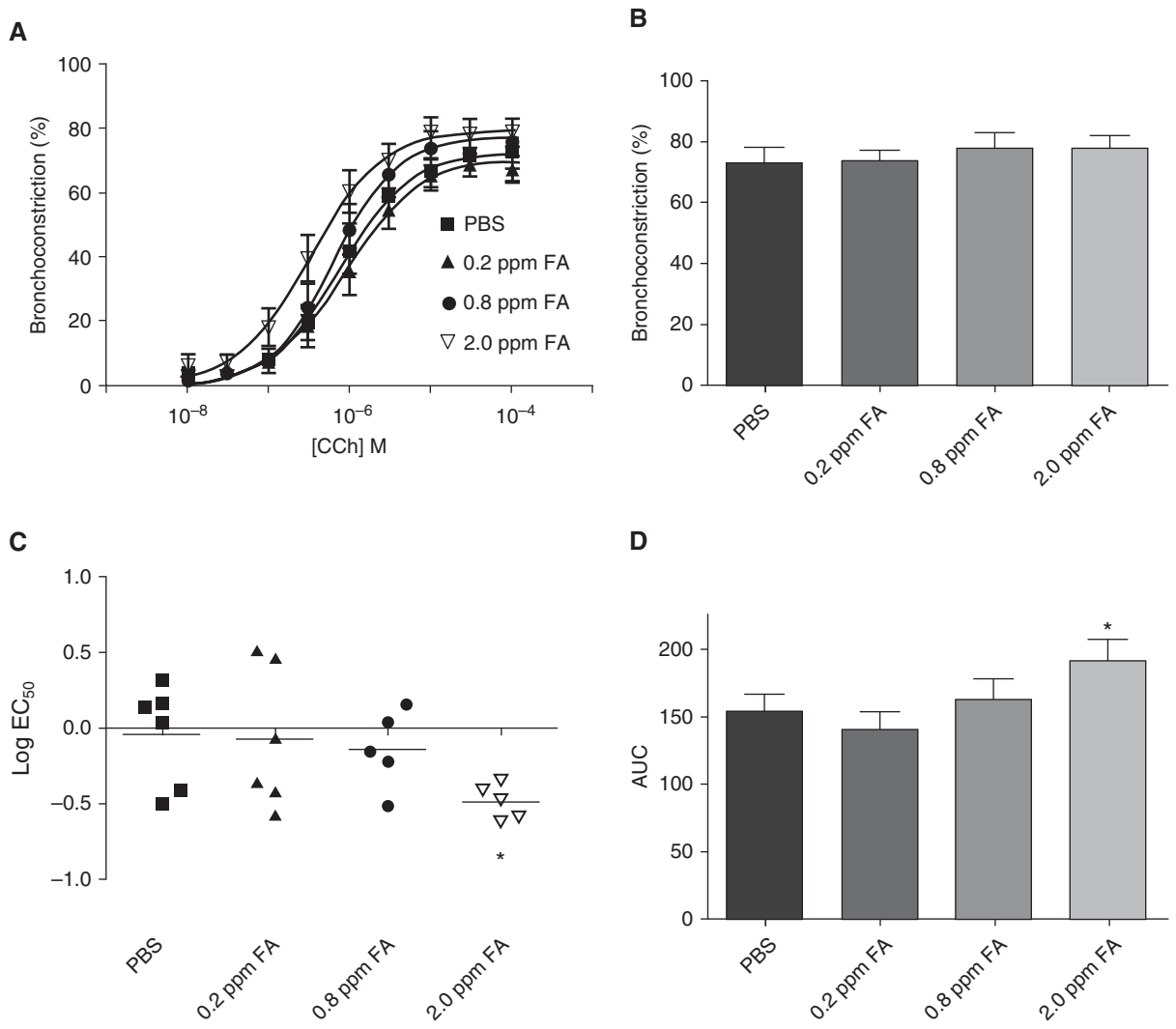

Figure 7. Formaldehyde enhances carbachol-induced airway narrowing in PCLSs. PCLSs were exposed to PBS or formaldehyde (0.2, 0.8, or 2 ppm) for 1 hour and then incubated for 24 hours before determining the carbachol-induced contractile response. (*A*) Slices exposed to 2 ppm carbachol (*inverted open triangles*) showed a left shift in the carbachol dose–response curve, indicating enhanced sensitivity (mean ± SEM; $n = 5$–6 donors). (*B*) There was little effect on the maximal response to carbachol after formaldehyde exposure (mean ± SEM; $n = 5$–6 donors). (*C*) The half-maximal effective concentration (log EC_{50}) of the carbachol dose–response curve was significantly decreased in slices exposed to 2 ppm formaldehyde compared with that of PBS-treated slices (mean denoted by *black horizontal lines*; $n = 5$–6 donors; $^*P = 0.019$). (*D*) The AUC of carbachol dose–response curve was significantly elevated in slices exposed to 2 ppm formaldehyde compared with that of slices exposed to PBS (mean ± SEM; $n = 5$–6 donors; $^*P = 0.049$).

formaldehyde on ASM cells are few (51). Studies show that inhaled toxicants, including formaldehyde, however, impair the structural integrity of the airway epithelial cells, thus allowing diffusion of toxicants to submucosal sites (32, 52, 53). These observations provide a rationale for directly exposing HASM cells to formaldehyde vapor to examine the outcomes. To account for the role of airway epithelial cells in mediating formaldehyde-induced AHR, we also used HBE–HASM cell coculture system in our studies. Furthermore, human PCLSs are an integrative platform to study the effects of inhaled toxicants, and our findings provide physiological significance to the effects of formaldehyde on AHR in humans.

In summary, the current study identifies MYPT1 phosphorylation and ROCK-dependent Ca^{2+} sensitization in HASM cells as plausible mechanisms for formaldehyde-induced AHR. Although not related to MYPT1 phosphorylation in our studies, an Nrf-2–dependent antioxidant response is elicited by formaldehyde in HASM cells. In human PCLSs, formaldehyde exposure enhanced airway narrowing, indicating that the augmented MYPT1 phosphorylation manifests into AHR. The findings suggest that formaldehyde-induced AHR, and potentially asthma exacerbations that result from formaldehyde exposure, may be therapeutically targeted at the level of ROCK-mediated Ca^{2+} sensitization. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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