

Theophylline Represses IL-8 Secretion from Airway Smooth Muscle Cells Independently of Phosphodiesterase Inhibition

Novel Role as a Protein Phosphatase 2A Activator

Brijeshkumar S. Patel^{1*}, Md. Mostafizur Rahman^{1*}, Nowshin N. Rumzhum¹, Brian G. Oliver^{2,3}, Nicole M. Verrills⁴, and Alaina J. Ammit¹

¹Faculty of Pharmacy, University of Sydney, New South Wales, Australia; ²Woolcock Institute of Medical Research, University of Sydney, New South Wales, Australia; ³School of Life Sciences, University of Technology, Sydney, New South Wales, Australia; and ⁴School of Biomedical Sciences and Pharmacy, Faculty of Health and Medicine, University of Newcastle, New South Wales

Abstract

Theophylline is an old drug experiencing a renaissance owing to its beneficial antiinflammatory effects in chronic respiratory diseases, such as asthma and chronic obstructive pulmonary disease. Multiple modes of antiinflammatory action have been reported, including inhibition of the enzymes that degrade cAMP–phosphodiesterase (PDE). Using primary cultures of airway smooth muscle (ASM) cells, we recently revealed that PDE4 inhibitors can potentiate the antiinflammatory action of β_2 -agonists by augmenting cAMP-dependent expression of the phosphatase that deactivates mitogen-activated protein kinase (MAPK)–MAPK phosphatase (MKP)-1. Therefore, the aim of this study was to address whether theophylline repressed cytokine production in a similar, PDE-dependent, MKP-1-mediated manner. Notably, theophylline did not potentiate cAMP release from ASM cells treated with the long-acting β_2 -agonist formoterol. Moreover, theophylline (0.1–10 μ M) did not increase formoterol-induced

MKP-1 messenger RNA expression nor protein up-regulation, consistent with the lack of cAMP generation. However, theophylline (at 10 μ M) was antiinflammatory and repressed secretion of the neutrophil chemoattractant cytokine IL-8, which is produced in response to TNF- α . Because theophylline's effects were independent of PDE4 inhibition or antiinflammatory MKP-1, we then wished to elucidate the novel mechanisms responsible. We investigated the impact of theophylline on protein phosphatase (PP) 2A, a master controller of multiple inflammatory signaling pathways, and show that theophylline increases TNF- α -induced PP2A activity in ASM cells. Confirmatory results were obtained in A549 lung epithelial cells. PP2A activators have beneficial effects in *ex vivo* and *in vivo* models of respiratory disease. Thus, our study is the first to link theophylline with PP2A activation as a novel mechanism to control respiratory inflammation.

Keywords: protein phosphatase 2A; phosphodiesterase; IL-8; cAMP; airway smooth muscle

Theophylline is an old drug, first used over 80 years ago as a bronchodilator. It has been largely superseded by β_2 -agonists, but has generated much interest recently, as it exerts positive effects in chronic respiratory

diseases, especially asthma and chronic obstructive pulmonary disease (COPD). Theophylline has been reported to have multiple modes of beneficial action (*see* review in Ref. 1), and molecular

mechanisms have begun to be revealed. First, theophylline may enhance bronchodilatory action of β_2 -agonists as it is a nonspecific (albeit relatively weak) inhibitor of the enzymes that break down

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*These authors contributed equally to this work.

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Correspondence and requests for reprints should be addressed to Alaina J. Ammit, Ph.D., Faculty of Pharmacy, University of Sydney, NSW 2006 Australia. E-mail: alaina.ammit@sydney.edu.au

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Clinical Relevance

Theophylline is an old drug experiencing a renaissance owing to its beneficial antiinflammatory effects in chronic respiratory diseases, such as asthma and chronic obstructive pulmonary disease. Multiple modes of antiinflammatory action have been reported. Our study is the first to link theophylline with protein phosphatase 2A activation as a novel mechanism to control respiratory inflammation.

cAMP (phosphodiesterases [PDEs]); second, it may exert antiinflammatory actions in its own right and, notably, can inhibit inflammation by up-regulating many of the same antiinflammatory proteins considered important for the repressive effects of corticosteroids (e.g., IL-10) (2, 3), underscoring the promise of theophylline as a nonsteroidal, antiinflammatory, add-on therapy; third, theophylline at low doses has been reported to improve corticosteroid responsiveness in a manner dependent on reinstatement of corepressors, such as histone deacetylase 2 activity (1, 4). To date, whether theophylline enhances the function of a master controller of multiple inflammatory signaling pathways, protein phosphatase (PP) 2A, was unknown.

We address this question herein using primary cultures of airway smooth (ASM) cells. We show that theophylline (at 10 μ M) is antiinflammatory and significantly represses ASM synthetic function induced by TNF- α to inhibit secretion of the neutrophil chemoattractant cytokine, IL-8. Intriguingly, the mechanism of action responsible differs from those we recently reported for PDE4 inhibitors (5). Notably, theophylline does not increase formoterol-induced cAMP, nor expression of the antiinflammatory mitogen-activated protein kinase (MAPK) deactivator, MAPK phosphatase (MKP)-1 (5). Instead, we show, for the first time, that theophylline acts to significantly increase TNF- α -induced enzymatic activity of the ubiquitous serine/threonine phosphatase, PP2A. We also performed confirmatory experiments in A549 lung epithelial cells to demonstrate that theophylline acts on A549 cells in a similar manner to ASM cells; that

is, theophylline (10 μ M) increases TNF- α -induced PP2A enzymatic activity and represses TNF- α -induced IL-8 secretion in A549 lung epithelial cells. PP2A activators have proven pharmacotherapeutic potential in respiratory disease (6, 7). Thus, our study reveals that this may be one of the ways in which theophylline may exert clinical benefits in asthma and COPD.

Material and Methods

Cell Culture

Human bronchi were obtained from patients undergoing surgical resection for carcinoma or lung transplant donors in accordance with procedures approved by the Sydney South West Area Health Service (Sydney, NSW, Australia) and the Human Research Ethics Committee of the University of Sydney (Sydney, NSW, Australia). ASM cells were dissected, purified, and cultured as previously described by Johnson and colleagues (8). A minimum of three different ASM primary cell lines were used for each experiment.

The human alveolar epithelial cell line (A549) was cultured in accordance with previously established protocols (9, 10). A minimum of three experimental replicates performed on separate days were used for each experiment.

Chemicals

TNF- α was purchased from R&D Systems (Minneapolis, MN). Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

cAMP Assay

cAMP was measured by enzyme immunoassay (cAMP EIA 581001; Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's instructions.

Real-Time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Doncaster, VIC, Australia) and reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Hanover, MD). Real-time RT-PCR was performed on an ABI Prism 7,500 with MKP-1 (DUSP1: Hs00610256_g1), TaqMan Gene Expression Assays, and the eukaryotic 18S

rRNA endogenous control probe (Applied Biosystems, Foster City, CA) subjected to the following cycle parameters: 50°C for 2 minutes, 1 cycle; 95°C for 10 minutes, 1 cycle; 95°C for 15 seconds, 60°C for 1 minutes, 40 cycles, and mRNA expression quantified by $\Delta\Delta$ threshold cycle calculations.

Western Blotting

MKP-1 was quantified by Western blotting using a rabbit polyclonal antibody against MKP-1 (C-19; Santa Cruz Biotechnology, Santa Cruz, CA). The catalytic subunit of PP2A (PP2A-C) was detected with a mouse monoclonal antibody (IgG2bk, clone 1D6; Merck Millipore, Darmstadt, Germany). α -Tubulin was used as the loading control (mouse monoclonal IgG₁, DM1A; Santa Cruz Biotechnology). Primary antibodies were detected with goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

PP2A Activity Assay

PP2A activity was determined using the PP2A immunoprecipitation phosphatase assay kit (Merck Millipore) according to the manufacturer's instructions.

ELISAs

Cell supernatants were collected and stored at -20°C for later analysis by ELISA. IL-8 ELISAs were performed according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA).

PP2A-C Small Interfering RNA Transfection

A549 cells were left untransfected, or transfected with scrambled control (ON-Target plus Control Non-targeting small interfering RNA [siRNA]) or siRNA against PP2A-C (ON-Target plus SMART pool Human PPP2CA siRNA [aka PP2A-C]; both from Dharmacon, Thermo-Fisher Scientific, Waltham, MA) by reverse transfection with RNAiMAX, according to the manufacturer's protocols (Invitrogen, Carlsbad, CA) and as published previously (10).

Statistical Analysis

Statistical analysis was performed using one-way ANOVA then Fisher's *post hoc* multiple comparison test. *P* values less than 0.05 were sufficient to reject the null hypothesis for all analyses.

Results

Theophylline Does Not Increase Formoterol-Induced cAMP Production in ASM Cells

One of the ways in which theophylline has been considered to exert beneficial effects is via its action as a nonspecific inhibitor of PDEs, the enzyme family responsible for the degradation of cAMP released in response to β_2 -agonists. However, theophylline has been reported to be a relatively weak PDE inhibitor (11) and, *in vivo*, the positive effects observed clinically with theophylline occur at doses that are lower than those necessary for PDE inhibition (reviewed in Ref. 1). We recently reported (5) that PDE4 inhibitors enhance β_2 -adrenoceptor-mediated responses in ASM cells, and that, by increasing cAMP, PDE4 inhibitors augment antiinflammatory effects of β_2 -agonists via increased expression of the antiinflammatory protein, MKP-1. Whether theophylline acts in a similar manner to exert cAMP-dependent antiinflammatory effects in ASM was unknown.

We addressed this herein, and in Figure 1 we examined the impact of pretreating ASM cells with a range of concentrations of theophylline (0.1–100 μ M) before stimulation with the long-acting β_2 -agonist, formoterol. As shown in Figure 1, formoterol significantly increased cAMP production in ASM cells, in confirmation of our early study (5). When ASM cells were treated for 15 minutes with formoterol, significant cAMP production resulted (vehicle, 11.8 ± 4.9 pmol/ml cAMP versus formoterol, 65.7 pmol/ml cAMP [$P < 0.05$]; results are expressed in Figure 1 as percentage of formoterol-induced cAMP [designated as 100%]). However, unlike other PDE inhibitors (5), theophylline does not increase formoterol-induced cAMP (Figure 1). Notably, this lack of effect exists even when the concentration of theophylline tested was increased

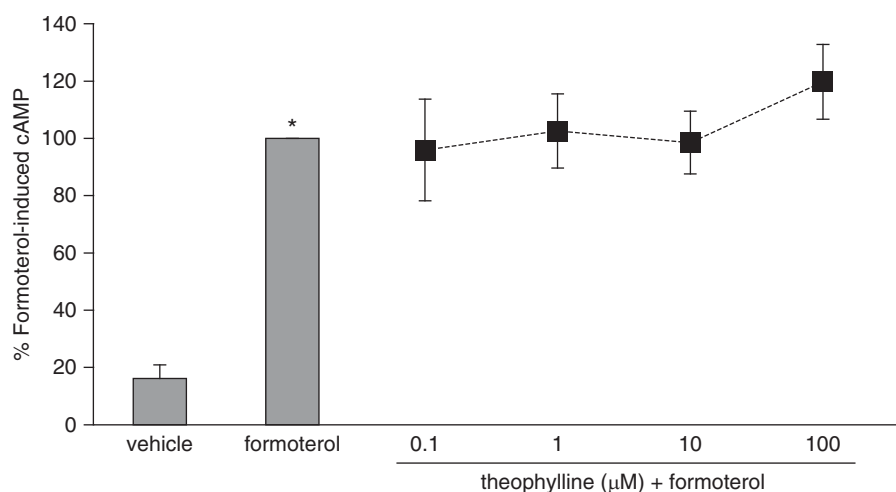


Figure 1. Theophylline does not increase formoterol-induced cAMP production in airway smooth muscle (ASM) cells. Growth-arrested ASM cells were pretreated for 30 minutes with theophylline (0.1–100 μ M), and cAMP production measured in response to stimulation with 10 nM formoterol for 15 minutes, in comparison to vehicle (results expressed as a percentage of formoterol-induced cAMP). Statistical analysis was performed using one-way ANOVA and then Fisher's *post hoc* multiple comparison test. *Significant increase in cAMP production by formoterol ($P < 0.05$). Data are mean $\pm$ SEM values from $n = 5$ primary ASM cell cultures.

to 100 μ M, 10 times higher than previously tested for specific PDE inhibitors (5). Thus, theophylline does not increase β_2 -agonist-induced cAMP.

Effect of Theophylline on MKP-1 mRNA Expression and Protein Up-Regulation in ASM Cells

MKP-1 is an antiinflammatory protein that we have shown can be up-regulated in a cAMP-dependent manner in ASM cells (5, 12, 13). Because theophylline did not increase cAMP production in response to formoterol, this suggests that theophylline will not augment MKP-1 mRNA expression and protein up-regulation in response to the long-acting β_2 -agonist. To test this, we initially examined the effect of theophylline on β_2 -agonist-induced MKP-1 mRNA expression. As shown in Figure 2A, formoterol significantly increases MKP-1 mRNA expression ($P < 0.05$), and these data are in accord with our previous studies (5, 12, 13). We then examined the impact of increasing concentrations of theophylline (0.1–100 μ M) on formoterol-induced MKP-1 mRNA expression (Figure 2A). Notably, potentiation of formoterol-induced MKP-1 mRNA expression and protein up-regulation was not achieved with 0.1–10 μ M theophylline; theophylline concentrations as high as 100 μ M were required to achieve a significant increase ($P < 0.05$). We then performed Western

blotting and, as shown in Figures 2B and 2C, the pattern of MKP-1 protein up-regulation is consistent with the mRNA data (Figure 2A). That is, formoterol increases MKP-1 protein up-regulation, and this is unaffected by pretreatment with theophylline (0.1–10 μ M), but high concentrations of theophylline (100 μ M) resulted in formoterol-induced MKP-1 protein up-regulation (Figure 2C; $P < 0.05$). As this concentration (100 μ M) is 100–1000 times higher than those necessary for MKP-1 potentiation by PDE4 inhibitors (cilomilast, piclamilast, rolipram) reported in our previous publication (5), these data are consistent with the relatively weak PDE inhibition achieved with theophylline.

Theophylline (10 μ M) Represses TNF- α -Induced IL-8 Secretion in a Manner that Is Independent of Antiinflammatory MKP-1 Up-Regulation in ASM Cells

Thus far, we have shown that, unlike other PDE inhibitors, theophylline doesn't increase formoterol-induced cAMP, nor does it increase formoterol-induced MKP-1 mRNA expression and protein up-regulation within a similar concentration range (0.1–10 μ M). However, when theophylline is used clinically, it has recognized antiinflammatory actions. Thus,

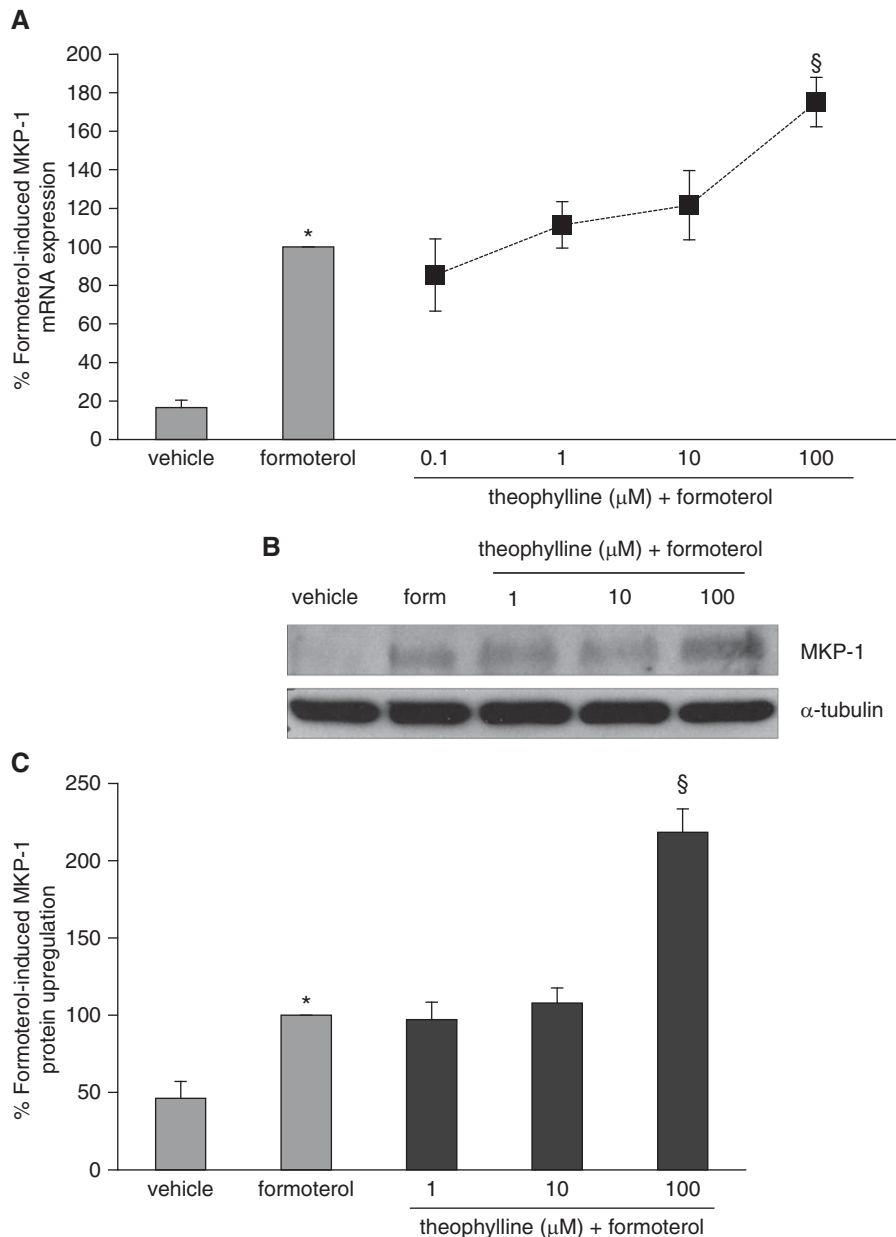


Figure 2. Effect of theophylline on formoterol-induced MAPK phosphatase (MKP)-1 mRNA expression and protein up-regulation in ASM cells. Growth-arrested ASM cells were pretreated for 30 minutes with the indicated theophylline concentrations and then stimulated with 10 nM formoterol for 1 hour, in comparison to vehicle. (A) MKP-1 mRNA expression was measured by RT-PCR (results expressed as a percentage of formoterol-induced MKP-1 mRNA expression). (B and C) MKP-1 protein was quantified by Western blotting, using α -tubulin as the loading control. Results are shown as (B) representative Western blots and (C) densitometric analysis (results expressed as a percentage of formoterol-induced MKP-1 protein up-regulation). Statistical analysis was performed using one-way ANOVA and then Fisher's *post hoc* multiple comparison test. *Significant induction of MKP-1 by formoterol; §Significant potentiation of formoterol-induced MKP-1 by theophylline ($P < 0.05$). Data are mean $\pm$ SEM values from (A) $n = 5$ and (B and C) $n = 3$ primary ASM cell cultures.

we were intrigued to examine the effect of increasing theophylline concentrations (0.1–100 μ M) on the secretion of IL-8 from ASM stimulated with TNF- α . As shown in

Figure 3A, there was little evidence of a concentration-dependent effect of theophylline on TNF- α -induced IL-8 secretion. That is, although 0.1, 1.0, or 100

μ M theophylline were without effect on ASM cytokine production, TNF- α -induced IL-8 secretion was significantly inhibited by 10 μ M theophylline ($P < 0.05$). This repression was independent of antiinflammatory MKP-1 up-regulation. This is shown in Figure 3B, where the representative Western blot confirms that TNF- α increases MKP-1 protein up-regulation by 60 minutes, and that this level of MKP-1 protein induced by TNF- α was unaffected by pretreatment with 10 μ M theophylline. This was supported by densitometric analysis, seen in Figure 3C, where there was no significant difference between TNF- α (18.9 ± 3.2 -fold) versus theophylline (10 μ M) plus TNF- α (22.2 ± 4.4 -fold) at 60 minutes.

Theophylline (10 μ M) Increases TNF- α -Induced PP2A Enzymatic Activity in ASM cells, but Not PP2A-C Protein Levels

Because we have shown that theophylline's antiinflammatory effects are independent of PDE inhibition or up-regulation of antiinflammatory MKP-1, how is it mediating its repressive effects? A number of molecular explanations may exist, but whether theophylline modulates PP2A activity had not yet been explored. PP2A is a serine/threonine phosphatase that dephosphorylates a number of kinases that drive inflammatory cell signaling (14). Importantly, its activation has been shown to be beneficial in *ex vivo* and *in vivo* models of asthmatic inflammation (6, 7, 15, 16). PP2A is ubiquitously expressed, and, in Figure 4A, we show that the basal PP2A enzymatic activity in ASM cells is $616.2 (\pm 53.7)$ pmol free phosphate, and this can be significantly increased (to $1,034.2 \pm 54.5$ pmol free phosphate) by treatment for 60 minutes with TNF- α ($P < 0.05$). When cells are pretreated with 10 μ M theophylline, there is no increase in the level of basal PP2A activity (627.5 ± 82.1 pmol free phosphate), but, when added together with TNF- α , theophylline significantly augments TNF- α -induced PP2A activity to $1,319.5 (\pm 57.6)$ pmol free phosphate (Figure 4A; $P < 0.05$). Intriguingly, these significant changes to PP2A enzyme activity occur in the absence of any changes in the protein levels of the catalytic subunit for PP2A (PP2A-C), as TNF- α or theophylline plus TNF- α do not affect PP2A-C protein up-regulation (up to 60 min; Figure 4B).

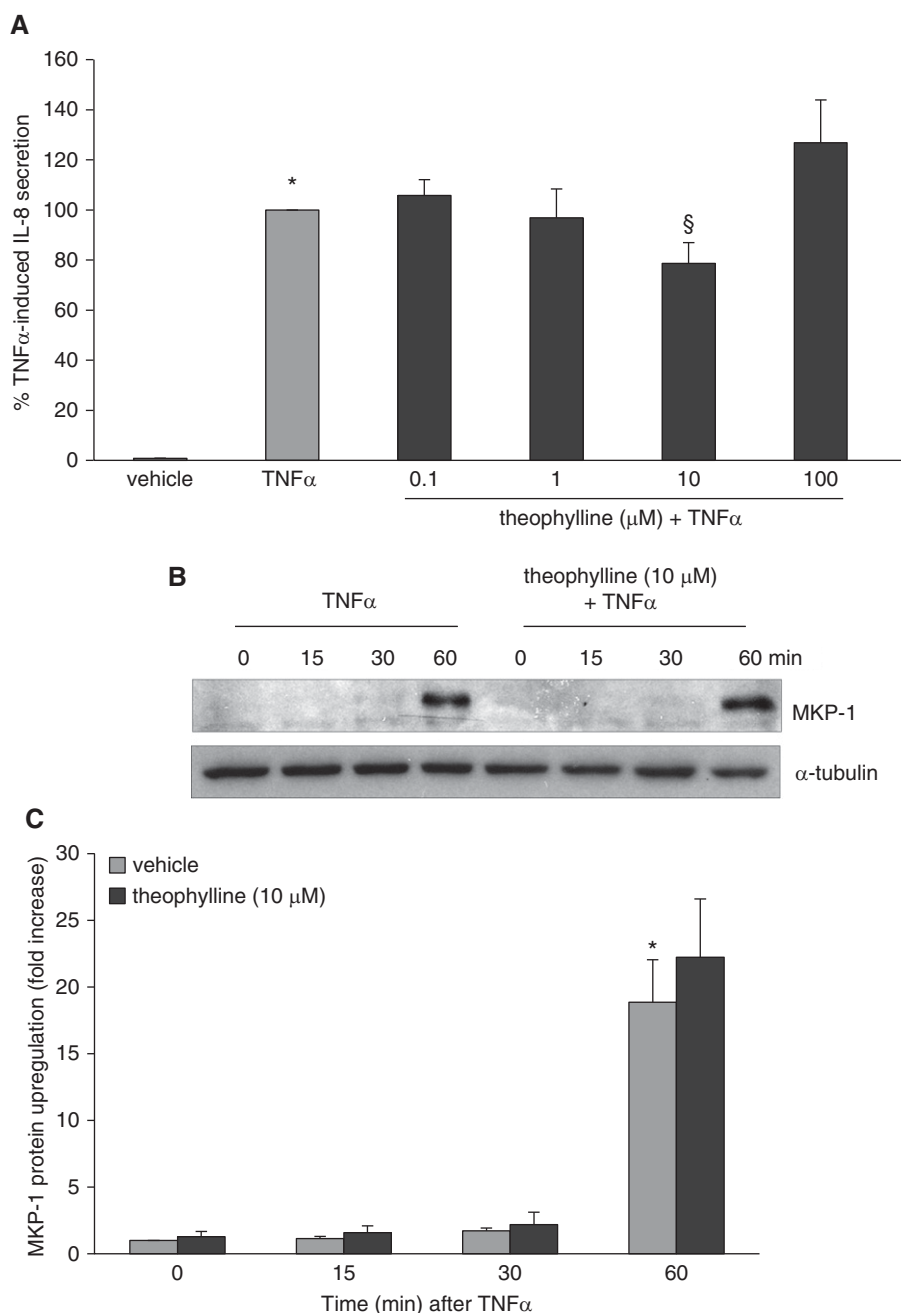


Figure 3. Theophylline (10 μ M) represses TNF- α -induced IL-8 secretion in a manner that is independent of antiinflammatory MKP-1 up-regulation in ASM cells. (A) Growth-arrested ASM cells were pretreated for 30 minutes with theophylline (0.1–100 μ M) or vehicle. Cells were stimulated for 24 hours with TNF- α (10 ng/ml), and IL-8 secretion was measured by ELISA (results expressed as a percentage of TNF- α -induced IL-8 secretion). Statistical analysis was performed using one-way ANOVA and then Fisher's *post hoc* multiple comparison test. *Significant up-regulation of IL-8 by TNF- α ; $\S$ significant repression of TNF- α -induced IL-8 by theophylline ($P < 0.05$). (B and C) Growth-arrested ASM cells were pretreated for 30 minutes with vehicle or theophylline (10 μ M) and then stimulated for 0, 15, 30, and 60 minutes with TNF- α (10 ng/ml). MKP-1 protein was quantified by Western blotting, using α -tubulin as the loading control. Results are shown as (B) representative Western blots and (C) densitometric analysis (results expressed as fold increase in MKP-1 protein up-regulation compared with vehicle). Statistical analysis was performed using one-way ANOVA then Fisher's *post hoc* multiple comparison test. *Significant induction of MKP-1 by TNF- α ($P < 0.05$). Data are mean $\pm$ SEM values from (A) $n = 10$ and (B and C) $n = 3$ primary ASM cell cultures.

Theophylline (10 μ M) Increases TNF- α -Induced PP2A Enzymatic Activity and Represses TNF- α -Induced IL-8 Secretion in A549 Cells

To examine the impact of theophylline in airway structural cells apart from ASM, we performed additional experimentation in a lung epithelial cell line (A549) widely used in *in vitro* studies of airway inflammation (9, 10, 17, 18). We first examined the impact of theophylline, alone or in combination with TNF- α , on PP2A enzymatic activity (Figure 5A). In confirmation of our recent study (10), and in accordance with the ubiquitous nature of PP2A phosphatase activity, A549 cells demonstrate a basal level of enzymatic activity (671.9 ± 14.6 pmol free phosphate), which can be significantly increased by TNF- α to $1,249.6 (\pm 45.7)$ pmol free phosphate (Figure 5A; $P < 0.05$). Notably, in alignment with data obtained in ASM cells (Figure 4A), pretreatment with theophylline (10 μ M) significantly increased TNF- α -induced PP2A enzymatic activity in A549 cells (Figure 5A; $P < 0.05$). We then investigated whether this increased PP2A activity exerted by theophylline resulted in a greater degree of repression of IL-8 secretion in response to TNF- α . As shown in Figure 5B, TNF- α -induced IL-8 secretion was significantly repressed by theophylline in A549 cells ($P < 0.05$).

Specific Knockdown of PP2A-C with siRNA Reverses Theophylline-Mediated Repression of IL-8 Protein Secretion from A549 Cells

We then employed A549 cells to demonstrate a link between the antiinflammatory impact of theophylline and PP2A activity. A549 cells are amenable to transfection, and we used siRNA against the catalytic subunit of PP2A (PP2A-C) to confirm that the anti-IL-8 effects of theophylline are mediated via PP2A. A549 cells were transfected with siRNA against PP2A-C, and examined in comparison with cells transfected with scrambled siRNA or untransfected controls. Cells were then pretreated for 30 minutes with vehicle or theophylline (10 μ M), and the impact of specific knockdown of PP2A-C on TNF- α -induced IL-8 secretion examined. We first confirmed

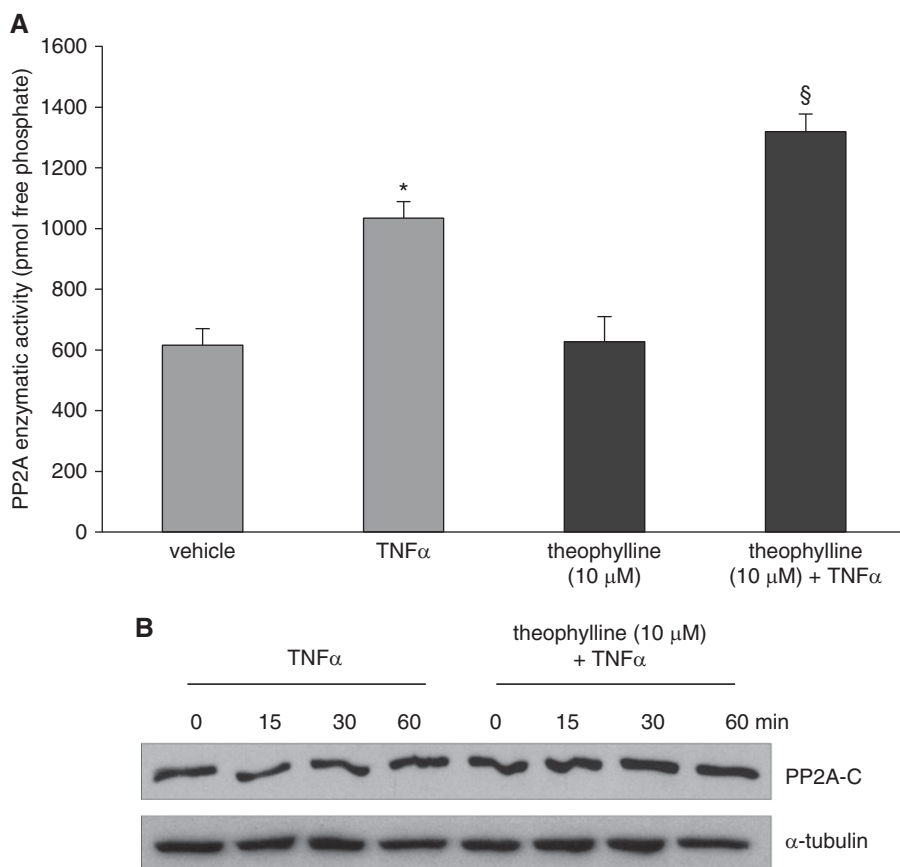


Figure 4. Theophylline (10 μ M) increases TNF- α -induced protein phosphatase 2A (PP2A) enzymatic activity but not PP2A-C protein levels in ASM cells. (A) Growth-arrested ASM cells were pretreated for 30 minutes with vehicle or theophylline (10 μ M) and then stimulated for 60 minutes with TNF- α (10 ng/ml) compared with vehicle. PP2A enzymatic activity was measured as picomoles of free phosphate. Statistical analysis was performed using one-way ANOVA and then Fisher's *post hoc* multiple comparisons test. *Significant increase in PP2A enzymatic activity by TNF- α ; $\S$ significant potentiation of TNF- α -induced PP2A enzymatic activity by theophylline ($P < 0.05$). Data are mean $\pm$ SEM values from $n = 5$ primary ASM cell cultures. (B) Growth-arrested ASM cells were pretreated for 30 minutes with vehicle or theophylline (10 μ M) and then stimulated for 0, 15, 30, and 60 minutes with TNF- α (10 ng/ml). PP2A-C protein was quantified by Western blotting, using α -tubulin as the loading control (results are representative Western blots of $n = 3$ primary ASM cell cultures).

knockdown of PP2A-C, and, as shown in Figure 6A, siRNA successfully reduced PP2A-C protein levels as demonstrated by Western blotting. We then measured PP2A enzymatic activity under identical conditions and confirmed that theophylline significantly increases TNF- α -induced PP2A enzyme activity (Figure 6B; $P < 0.05$). As expected, knocking down PP2A-C with siRNA reduced PP2A enzymatic activity in both the absence and presence of theophylline (Figure 6B; $P < 0.05$). Finally, we measured IL-8 secretion, as shown in Figure 6C. When PP2A-C is knocked down, there is a small, but significant, increase in TNF- α -induced

IL-8 secretion (in confirmation of our previous publication and consistent the antiinflammatory role of PP2A [10]). Importantly, theophylline-mediated repression of TNF- α -induced IL-8 secretion in A549 cells is lost when PP2A-C is specifically knocked down with siRNA (Figure 6C; $P < 0.05$). Taken together, these data link theophylline with PP2A activation as a novel mechanism to control respiratory inflammation modeled *in vitro*. That is, specific knockdown of PP2A-C with siRNA reverses theophylline-mediated repression of IL-8 protein secretion from A549 cells.

Discussion

In this study, we demonstrate that theophylline represses the secretion of IL-8 from ASM cells stimulated with TNF- α . We then explored the molecular mechanisms responsible and consistent with the notion that theophylline is a relatively weak PDE inhibitor; theophylline does not increase formoterol-induced cAMP production in ASM cells. Because of this lack of cAMP potentiation, the mechanism of antiinflammatory action is not MKP-1 dependent. Instead, we reveal a novel function for theophylline by showing that it can significantly enhance enzymatic activity of the phosphatase PP2A stimulated by TNF- α . Confirmatory experiments were performed in A549 lung epithelial cells and, importantly, specific knockdown of PP2A-C with siRNA reversed theophylline-mediated repression of IL-8 protein secretion from A549 cells, confirming that the anti-IL-8 effects of theophylline are mediated via PP2A. Thus, these data suggest that theophylline may serve as a PP2A activator in respiratory inflammation.

Theophylline is an old drug that was first used over 80 years ago. It has recognized beneficial effects in asthma and COPD, restoring corticosteroid responsiveness in particular (reviewed in Ref. 1). In this study, we were intrigued to extend our understanding of the molecular mechanism responsible for its antiinflammatory action. We used our well established method of stimulating primary cultures of ASM cells with a cytokine found elevated in asthma—TNF- α (19)—and measuring ASM synthetic function. We focused on IL-8 secretion from ASM cells, as antiinflammatory strategies targeting repression of this neutrophil-chemoattractant cytokine may have relevance in both asthma and COPD—chronic respiratory diseases where neutrophils can be key contributors. When we explored the antiinflammatory impact of theophylline *in vitro*, we observed that only 10 μ M theophylline significantly repressed TNF- α -induced IL-8 secretion. Notably, repression of TNF- α -induced IL-8 secretion was independent of PDE inhibition and cAMP-dependent MKP-1 up-regulation; molecular mechanisms recently reported for PDE4 inhibitors in ASM cells (5).

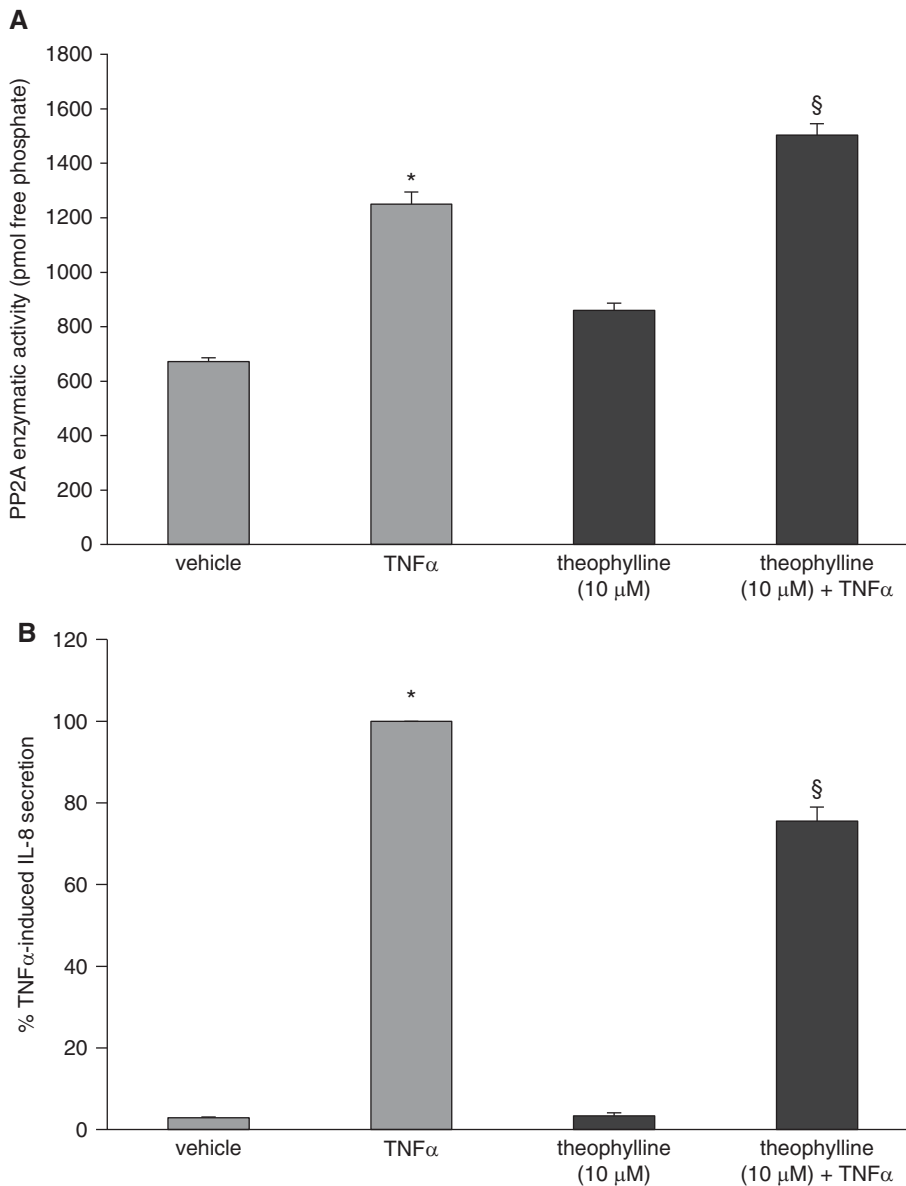


Figure 5. Theophylline (10 μ M) increases TNF- α -induced PP2A enzymatic activity and represses TNF- α -induced IL-8 secretion in A549 cells. A549 cells were pretreated for 30 minutes with vehicle or theophylline (10 μ M) and then (A) stimulated for 60 minutes with TNF- α (4 ng/ml) compared with vehicle. PP2A enzymatic activity was measured as picomoles of free phosphate and (B) stimulated for 24 hours with TNF- α (4 ng/ml), and IL-8 secretion was measured by ELISA (results expressed as a percentage of TNF- α -induced IL-8 secretion). Statistical analysis was performed using one-way ANOVA and then Fisher's *post hoc* multiple comparisons test. *Significant effect of TNF- α ; §significant impact of theophylline on TNF- α -induced responses ($P < 0.05$). Data are mean $\pm$ SEM values from $n = 3$ independent experiments.

Instead, we show that theophylline significantly increases TNF- α -induced PP2A activity and reduces IL-8 secretion in ASM cells. These data were confirmed in A549 cells, a lung epithelial cell line widely used in *in vitro* studies of airway inflammation (9, 10, 17, 18) and used to perform siRNA experiments (as they are

amenable to transient transfection) to demonstrate a direct cause-effect relationship and show that the anti-IL-8 effects of theophylline are mediated via PP2A. These results are notable for two main reasons: (1) demonstrating that theophylline has antiinflammatory capacity independent of PDE inhibitory

activity is important, as a number of the unwanted effects of theophylline are considered related to PDE inhibition (20); and (2) supporting recent preclinical studies that have underscored the key role played by PP2A in the control of inflammation in respiratory disease. Thus, our study is the first to show that PP2A activity may be one of the molecular mechanisms by which theophylline may achieve its beneficial effect.

PP2A is a ubiquitously expressed serine/threonine phosphatase that has generated much excitement recently as a target for anticancer therapy (21). It is a tumor suppressor known to regulate a range of cellular signaling pathways involved in proliferation, survival, and migration in cancer. Despite these developments in the cancer field, investigations into the clinical utility of PP2A in respiratory inflammation had lagged behind somewhat until recent *ex vivo* and *in vivo* investigations. PP2A levels (and therefore activity) were reduced in mouse models of allergen- and rhinovirus-induced asthma (6) and, significantly, PP2A activity could be reinstated pharmacologically to repress hallmark features of allergic and rhinovirus-induced airway disease (6, 7). Notably, defects in PP2A cause corticosteroid insensitivity in severe asthma (15). Reduced PP2A activity induces corticosteroid resistance, and this can be reversed by the long-acting β_2 -agonist, formoterol (16). Thus, PP2A has been shown to play a key role in the control of respiratory inflammation, infectious exacerbation, and corticosteroid responsiveness, conditions that have been reported to be improved by theophylline (1). The role that PP2A plays in mediating theophylline's effects warrants further investigation.

PP2A is a trimolecular holoenzyme consisting of a catalytic subunit (C), a structural subunit (A), and a variable regulatory subunit (B), of which there are at least three different families (B55, B56, B''), each with several isoforms (22). PP2A is ubiquitously expressed and, with this study, we are the first to measure the basal activity of PP2A in primary ASM cells. We show that this activity can be significantly increased by TNF- α , but not by theophylline on its own, and that theophylline increases TNF- α -induced PP2A enzymatic activity. This increase in enzymatic activity is independent of increases in the protein level for the

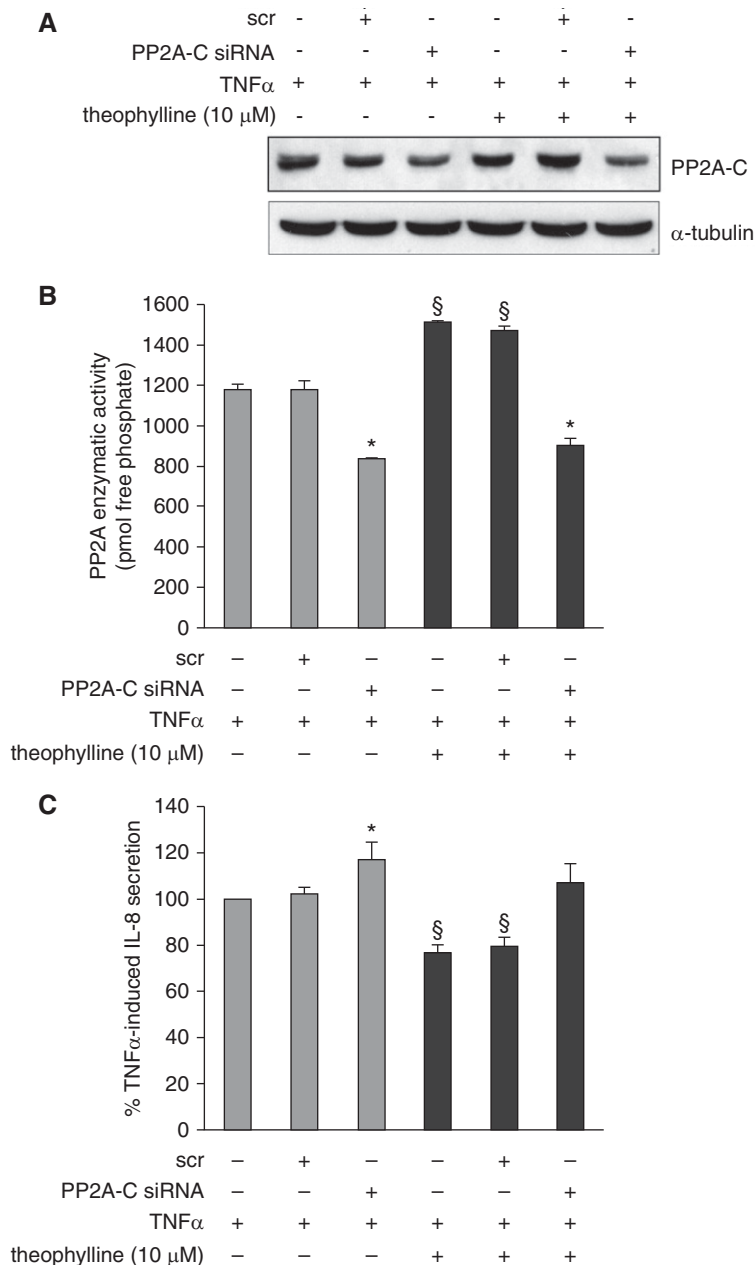


Figure 6. Specific knockdown of PP2A-C with small interfering (siRNA) reverses theophylline-mediated repression of IL-8 protein secretion from A549 cells. A549 cells were either left untransfected or transfected with scrambled control (scr) or siRNA against PP2A-C, then pretreated for 30 minutes with vehicle or theophylline (10 μ M) and stimulated with TNF- α (4 ng/ml) before: (A) knockdown of PP2A-C protein expression confirmed at 1 hour by Western blotting (compared with α -tubulin as loading control; representative Western blot shown); (B) effect on PP2A activity detected at 1 hour (PP2A enzymatic activity was measured as pmol free phosphate); (C) IL-8 protein secretion (results expressed as a percentage of TNF- α -induced IL-8 secretion in untransfected controls pretreated with vehicle). Statistical analysis was performed using one-way ANOVA then Fisher's *post hoc* multiple comparison test. *The impact of knocking down PP2A-C with siRNA; §significant effect of theophylline ($P < 0.05$) compared with untransfected controls pretreated with vehicle. Data are mean $\pm$ SEM values from (A and B) $n = 3$ and (C) $n = 6$ independent experiments.

PP2A catalytic subunit (PP2A-C). PP2A is highly regulated at multiple levels (23), including transcriptional and post-transcriptional regulation, control by

post-translational modifications, regulation by endogenous inhibitory proteins, and by the nature and identity of the PP2A subunits. How theophylline (and TNF- α)

regulates PP2A is currently unknown. Interestingly, we show that TNF- α itself increases PP2A enzyme activity. This is consistent with the notion that PP2A is part of a feedback loop that TNF- α uses to terminate its signal. To the best of our knowledge, this appears to be the first demonstration of PP2A forming part of the negative-feedback mechanisms in ASM cells. PP5 has been shown to be a driver of cytokine-induced glucocorticoid insensitivity in ASM cells (24), but the role of PP2A as a negative-feedback effector in ASM cells has not been examined. We have previously published that the MAPK deactivator, MKP-1, is an important negative-feedback mechanism regulating TNF- α signaling in ASM cells (25, 26). That is, TNF- α activates p38 MAPK to increase expression of MKP-1 mRNA and rapid protein expression that then feeds back to dephosphorylate p38 MAPK and terminate the inflammatory signal. Thus, in this way, TNF- α controls both the “on” switch (kinase; specifically, p38 MAPK) and “off” switch (phosphatase; specifically, MKP-1) to regulate cytokine production in ASM cells. Herein, we extend our understanding of negative regulators of cytokine production in ASM cells, and show that TNF- α also increases the activity of another important phosphatase—PP2A. Both phosphatases dephosphorylate different phosphoprotein targets: MKP-1 has protein tyrosine/threonine phosphatase activity and PP2A has serine/threonine phosphatase activity. Thus, by stimulating phosphatase expression/activity, TNF- α exerts control over the intensity and duration of a broad range of cellular signaling pathways in ASM cells.

The widespread clinical use of theophylline is hampered by its modest clinical benefit when given orally, and severe unwanted effects, including nausea and vomiting, and adverse cardiac events, such as tachycardia and cardiac arrhythmias (27). It has a narrow therapeutic range and, thus, theophylline toxicity can be observed with even therapeutic doses, as observed in two recent systematic reviews (28, 29). The mechanisms responsible for the unwanted effects of theophylline warrant further investigation. Theophylline dose is critical, and it is considered that PDE inhibition is

responsible for the nausea, vomiting, and headaches, whereas, at higher doses, antagonism of the adenosine A₁ receptor occurs, and is responsible for severe cardiac unwanted effects and seizures (see review in Ref. 1). Thus, clinical use of theophylline may require therapeutic drug monitoring for safe and effective treatment. Inhaled formulations of theophylline offer the potential for greater safety (30). Other xanthines (such as doxofylline) have also been reported as having an improved therapeutic window over theophylline (31), in addition to antiinflammatory efficacy

in vivo (32). Interestingly, the mechanism of action for doxofylline has been reported to be unrelated to PDE inhibition or antagonism at the adenosine receptors at therapeutic doses (11).

Collectively, our study extends our understanding of the molecular mechanisms responsible for the antiinflammatory actions of theophylline, and shows that theophylline represses airway cell synthetic function in a manner independent of PDE inhibitory activity, but linked to capacity as an activator of PP2A. ■

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