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Doxofylline does not increase formoterol-induced cAMP nor MKP-1 expression in ASM cells resulting in lack of anti-inflammatory effect

Brijeshkumar S. Patel [a], Michael J. Kugel [b, c], Gina Baehring [b], Alaina J. Ammit, Ph.D. [b, c, *]

[a] Faculty of Pharmacy, University of Sydney, NSW, Australia
[b] Woolcock Emphysema Centre, Woolcock Institute of Medical Research, University of Sydney, NSW, Australia
[c] School of Life Sciences, University of Technology, Sydney, NSW, Australia

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ABSTRACT

The xanthine doxofylline has been examined in clinical trials and shown to have efficacy and greater tolerability than theophylline in asthma and chronic obstructive pulmonary disease. The 'novofylline' doxofylline has demonstrated bronchodilatory and anti-inflammatory actions in *in vivo* and *ex vivo* experimental models of respiratory disease. However, there are limited studies *in vitro*. We address this herein and examine whether doxofylline has anti-inflammatory impact on primary cultures of airway smooth muscle (ASM) cells. We conduct a series of investigations comparing and contrasting doxofylline with the archetypal xanthine, theophylline, and the specific phosphodiesterase (PDE) 4 inhibitor, cilomilast. We confirm that the xanthine drugs do not have action as PDE inhibitors in ASM cells. Unlike cilomilast, doxofylline (and theophylline) do not increase cAMP production in ASM cells induced by long-acting β_2-agonist formoterol. Similar to theophylline, and consistent with the lack of cAMP potentiation, doxofylline does not augment formoterol-induced upregulation of the anti-inflammatory protein mitogen-activated protein kinase phosphatase 1 (MKP-1). However, when we examine the effect of doxofylline on secretion of the interleukin 8 from ASM cells stimulated by tumour necrosis factor (an *in vitro* surrogate measure of inflammation), there was no repression of inflammation. This is in contrast to the anti-inflammatory impact exerted by theophylline and cilomilast in confirmatory experiments. In summary, our study is the first to examine the effect of doxofylline on ASM cells *in vitro* and highlights some distinct differences between two key members of xanthine drug family, doxofylline and theophylline.

1. Introduction

Interest in xanthine drugs, such as theophylline, has resurfaced based on demonstrated clinical benefits in asthma and COPD (reviewed in Ref. [1]) and the potential of theophylline to restore corticosteroid insensitivity. First utilised in the early 1900's for its bronchodilatory efficacy, now largely superseded by β_2-agonists, theophylline is a relatively weak, but non-specific, inhibitor of the phosphodiesterase (PDE) enzyme family. Importantly, the non-specific inhibition of PDEs is considered responsible for the unwanted effects of theophylline and limits its therapeutic utility [1]. Thus, there is considerable interest in understanding and exploiting the PDE-independent effects of theophylline in respiratory disease, as well as investigating the role and function of theophylline derivatives that are devoid of PDE inhibition, such as doxofylline (reviewed in Ref. [2]).

Many of the beneficial effects of theophylline, such as reversal of corticosteroid resistance [3,4] and reduction of airway inflammation [5–7] have been shown to be independent of PDE inhibition. Furthermore, we recently demonstrated that the anti-inflammatory impact of theophylline in airway smooth muscle (ASM) cells was independent of PDE inhibition [8]; but instead due to a novel mechanism of action not previously ascribed to theophylline, that of protein phosphatase 2A (PP2A) activation. PDE4, not PDE3 [9], are the predominant PDE enzymes in ASM cells. We showed that theophylline did not inhibit PDE4, as it did not potentiate cAMP release from ASM cells treated with the long-

Abbreviations: ASM, airway smooth muscle; MAPK, mitogen-activated protein kinase; MKP-1, MAPK phosphatase 1; PDE, phosphodiesterase; PP2A, protein phosphatase 2A; TNF, tumour necrosis factor.

* Corresponding author. Woolcock Emphysema Centre, Woolcock Institute of Medical Research, University of Sydney, NSW, Australia.
E-mail address: Alaina.Ammit@uts.edu.au (A.J. Ammit).

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acting β_2-agonist formoterol, nor augment cAMP-dependent expression of anti-inflammatory phosphatase that deactivates mitogen-activated protein kinase (MAPK) phosphatase (MKP)-1. Instead, we showed for the first time that theophylline activates PP2A, a master controller of multiple inflammatory signalling pathways, and theophylline represses secretion of the neutrophil chemokine interleukin 8 (IL-8) via PP2A activation [8].

In the current study, we extend our investigations and examine the anti-inflammatory impact of doxofylline in ASM cells. Doxofylline is a xanthine derivative of theophylline with anti-inflammatory and bronchodilatory effects that are considered independent of PDE inhibition [2,10–12]. Herein, we address whether doxofylline increases formoterol-induced production of cAMP and expression of the anti-inflammatory phosphatase MKP-1. Experiments are performed in comparison with the xanthine congener, theophylline [8], and the PDE4 inhibitor cilomilast [9]. Although doxofylline does not increase formoterol-induced cAMP, consistent with its lack of effect on PDE enzymes, our studies reveal that unlike theophylline, doxofylline does not repress production of IL-8 in ASM cells.

2. Material and methods

2.1. ASM cell culture

Human bronchi were obtained from macroscopically normal lung tissue from patients undergoing surgical resection for carcinoma or lung transplant donors in accordance with procedures approved by the Sydney South West Area Health Service and the Human Research Ethics Committee of the University of Sydney. ASM cells were dissected, purified and cultured as previously described by Johnson et al. [13]. In brief, cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 5 mM L-glutamine, 20 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B. For experiments, cells were grown to confluence, then growth-arrested for 48 h in DMEM containing 0.1% (v/v) bovine serum albumin. A minimum of three different ASM primary cell lines (passage 3–8) were used for each experiment.

2.2. Chemicals

Doxofylline was from Santa Cruz Biotechnology (Santa Cruz, CA). Tumour necrosis factor (TNF) was purchased from R&D Systems. Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

2.3. cAMP assay

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

2.4. Western blotting

MKP-1 was quantified by Western blotting using a rabbit polyclonal antibody against MKP-1 (C-19: Santa Cruz Biotechnology) with α-tubulin used as the loading control (mouse monoclonal IgG_1, DM1A: Santa Cruz Biotechnology). Primary antibodies were detected with goat anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

2.5. Real-time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen Australia, 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 7500 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 min, 1 cycle; 95 °C for 10 min, 1 cycle; 95 °C for 15 s, 60 °C for 1 min, 40 cycles and mRNA expression (fold increase) quantified by delta delta Ct calculations.

2.6. ELISA

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).

2.7. Statistical analysis

Statistical analysis was performed using Student's unpaired *t*-test or one-way ANOVA then Fisher's post-hoc multiple comparison test (StatView Software from SAS). P values < 0.05 were sufficient to reject the null hypothesis for all analyses.

3. Results

3.1. Doxofylline does not increase formoterol-induced cAMP production in ASM cells

Doxofylline effects have been demonstrated to be independent of PDE inhibition in cell types apart from ASM cells [2,11]. In Fig. 1, we investigate the impact of a range of concentrations of doxofylline (0.1–100 μM) on cAMP released from ASM cells after 15 min incubation with the long-acting β_2-agonist, formoterol. In accordance with our previous publications, formoterol induces cAMP [14] and while this can be increased with the PDE4 inhibitor, cilomilast [9], theophylline does not augment formoterol-induced cAMP [8]. Like theophylline, doxofylline also does not increase β_2-agonist-induced cAMP; these results suggest that doxofylline has no effect on PDE4 in ASM cells.

3.2. Effect of doxofylline on formoterol-induced MKP-1 protein upregulation in ASM cells

We have previously shown that a panel of PDE4 inhibitors, including cilomilast [9], increased formoterol-induced upregulation of the anti-inflammatory protein, MKP-1. This occurred in a cAMP-dependent manner. Given that doxofylline did not augment cAMP production in response to the long-acting β_2-agonist, it is unlikely that doxofylline will have an impact on MKP-1 protein upregulation. To confirm this assertion, ASM cells were pretreated with a range of doxofylline concentrations (0.1–100 μM), and compared to theophylline (10 μM) or cilomilast (10 μM). Cells were then stimulated with 10 nM formoterol (or vehicle) for 1 h. MKP-1 protein was quantified by Western blotting (with α-tubulin as a loading control). As shown in Fig. 2A, and in concordance with our prior publication [9], formoterol increased MKP-1 protein and this was enhanced by cilomilast. In contrast, the xanthine drugs - theophylline and doxofylline - did not potentiate formoterol-induced MKP-1 upregulation; this is consistent with their lack of effect on cAMP induced by the β_2-agonist in ASM cells. This was confirmed by densitometric analysis (Fig. 2B), where formoterol-