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## **TLR2 activation causes tachyphylaxis to $\beta_2$ -agonists *in vitro* and *ex vivo*: modeling bacterial exacerbation**

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## Abstract

**Background:** Asthma is a widespread chronic health problem exacerbated by common viral and bacterial infections. Further research is required to understand how infection worsens asthma control in order to advance therapeutic options in the future. Recent research has revealed that  $\beta_2$ -adrenergic receptor ( $\beta_2$ -AR) agonists lose bronchodilatory efficacy because the receptor-mediated molecular pathways responsible for their beneficial actions are desensitized by infection. To date, most studies have focussed on viral infection, leaving the impact of bacterial infection on  $\beta_2$ -AR desensitization relatively under-investigated. We address this in this study. **Methods & Results:** Utilizing an *in vitro* model of bacterial exacerbation in airway smooth muscle (ASM) cells we show that activation of toll-like receptor 2 (TLR2)(mimicking bacterial infection) in the presence of an inflammatory stimulus leads to  $\beta_2$ -AR desensitization. This occurs via TLR2-dependent upregulation of COX-2 mRNA expression and increased secretion of PGE<sub>2</sub>. Importantly, PGE<sub>2</sub> causes heterologous  $\beta_2$ -AR desensitization and reduces cAMP production in response to short-acting (salbutamol) and long-acting (formoterol)  $\beta_2$ -agonists. Thus, bacterial infectious stimuli act in a PGE<sub>2</sub>-dependent manner to severely curtail the beneficial actions of  $\beta_2$ -agonists. The impact of  $\beta_2$ -AR desensitization is demonstrated by reduced gene expression of the critical anti-inflammatory molecule MKP-1 in response to  $\beta_2$ -agonists, as well as impaired bronchodilation in a mouse lung slices. **Conclusions:** Taken together our results show that, like viruses, bacteria induce prostanoid-dependent  $\beta_2$ -AR desensitization on ASM cells.

Notably, COX-2 inhibition with the specific inhibitor celecoxib represses PGE<sub>2</sub> secretion presenting a feasible pharmacological option for treatment of infectious exacerbation in asthma in the future.

## Introduction

When a person with asthma experiences a respiratory infection their lung function worsens and they require greater amounts of their asthma medicines, including  $\beta_2$ -agonists, to achieve asthma control. Exacerbations can range from mild to life-threatening, and they contribute substantially to urgent health care utilisation. Although the frequency of exacerbations can be reduced by good medical management and asthma action plans, infectious exacerbations cannot be completely eliminated. Treatment options are currently limited and thus further research into the molecular basis of infectious exacerbation is urgently required.

Both viruses and bacteria are associated with asthma exacerbation. Viruses are identified in 80% of asthma exacerbations in children and 45-80% in adults (1). Rhinovirus, which causes the majority of common colds, is responsible for at least half of all asthma exacerbations (2). Bacterial infections are increasingly recognised as playing an important role in asthma exacerbations (reviewed in (3)), although they have been less well investigated to date.

Why are asthma medicines such as  $\beta_2$ -agonists less effective in infectious exacerbations? Put simply, when a person with asthma suffers from a viral or bacterial infection the molecular pathways by which asthma medicines act are changed making them less sensitive. In well-controlled asthma,  $\beta_2$ -agonists relax the airways by interacting with  $\beta_2$ -adrenergic receptors ( $\beta_2$ -AR) rapidly increasing adenosine monophosphate (cAMP) levels in airway smooth muscle (ASM); however these molecular pathways are changed by infection. Our recent studies have revealed that viral infection desensitizes the  $\beta_2$ -AR and reduces cAMP generation in response to  $\beta_2$ -agonists (4). These changes are due to interaction of viral

products with specific toll-like receptor (TLRs), resulting in cyclooxygenase 2 (COX-2)-mediated prostaglandin production (including PGE<sub>2</sub>) and repression of  $\beta_2$ -AR activity (5). To date however, whether bacterial infection directly affects  $\beta_2$ -AR function in a similar, TLR/COX-2-dependent manner has not been investigated. This is the aim of the current study.

## **Materials and Methods**

### **Human ASM cell culture**

Human bronchi were obtained from patients undergoing surgical resection for carcinoma or from 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.

### **Chemicals**

Pam3CSK4 was purchased from InvivoGen, tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) from R&D Systems and celecoxib and PGE<sub>2</sub> from Cayman Chemical Company. All other chemicals were purchased from Sigma-Aldrich.

### **Real-time RT-PCR**

See on-line supplement.

### **PGE<sub>2</sub> and cAMP assay**

Measured by enzyme immunoassay (Prostaglandin E<sub>2</sub> EIA 514010 and cAMP EIA 58100: Cayman).

## **Preparation of mouse lung slices**

Lung slices were prepared as previously described (7-9), with all experimental procedures approved by the Animal Ethics Committees of the University of Melbourne. See on-line supplement for experimental design.

## **Results**

### **TLR2 ligand engagement upregulates TNF $\alpha$ -induced COX-2 mRNA expression and increases PGE<sub>2</sub> secretion**

Our previous studies have modelled rhinovirus-induced asthma exacerbation *in vitro* in ASM cells to reveal the molecular events responsible for  $\beta_2$ -AR desensitization (4, 5, 10). To date however, whether bacterial infection directly affects  $\beta_2$ -AR function had not been explored. We addressed this by modelling bacterial exacerbation *in vitro*. ASM cells were treated with the synthetic bacterial lipoprotein Pam3CSK4 to mimic bacterial infection via TLR2 activation and with TNF $\alpha$  to simulate inflammation. We then measured the temporal kinetics of COX-2 mRNA expression and PGE<sub>2</sub> secretion. Upregulation of COX-2 mRNA expression by TNF $\alpha$  was sustained in the presence of Pam3CSK4, with TLR2 activation significantly increasing TNF $\alpha$ -induced COX-2 expression at 24 h (Figure 2A:  $P<0.05$ ). Secretion of the COX-2 product PGE<sub>2</sub> increased in a similar manner (Figure 1B); TLR2 ligand engagement by Pam3CSK4 significantly upregulated TNF $\alpha$ -induced PGE<sub>2</sub> secretion at 24 h ( $P<0.05$ ).

### **PGE<sub>2</sub> induces heterologous $\beta_2$ -AR desensitization as measured by inhibition of $\beta_2$ -agonist-induced cAMP production**

Because COX-2 products, such as PGE<sub>2</sub>, are responsible for rhinovirus-induced  $\beta_2$ -AR desensitization (5), our results suggest that bacteria and viruses may induce a common molecular mechanism to down-regulate  $\beta_2$ -AR on ASM cells in a heterologous manner. To

confirm this, we pretreated ASM cells for 24 h with a range of concentrations of PGE<sub>2</sub> (0.1-100 nM) and measured cAMP production in response to two  $\beta_2$ -agonists; short-acting  $\beta_2$ -agonist salbutamol (Figure 2A) and long-acting  $\beta_2$ -agonist formoterol (Figure 2B). These experiments were performed in parallel with control experiments to demonstrate homologous  $\beta_2$ -AR desensitization by  $\beta_2$ -agonists.

In confirmation of earlier reports (11), salbutamol induced a 5-fold increase in cAMP production that was sensitive to homologous  $\beta_2$ -AR desensitization (Figure 2A:  $P<0.05$ ). Notably, pretreatment with PGE<sub>2</sub> induced heterologous  $\beta_2$ -AR desensitization in a concentration-dependent manner ( $P<0.05$ ) (5, 12). Similarly, formoterol or PGE<sub>2</sub> also significantly repressed cAMP generation in response to formoterol (Figure 2B:  $P<0.05$ ).

### **Celecoxib inhibits PGE<sub>2</sub> secretion but not COX-2 mRNA expression**

Thus, we are first to show that TLR2 activation also leads to  $\beta_2$ -AR desensitization in ASM cells. As this occurs in a COX-2-dependent, PGE<sub>2</sub>-mediated manner we now wished to test the effectiveness of the specific COX-2 inhibitor celecoxib. Celecoxib inhibits COX-2 enzymatic activity, not expression; therefore we confirmed that celecoxib had no effect on COX-2 mRNA expression (Figure 3A). In contrast, celecoxib did have the expected inhibitory effect on PGE<sub>2</sub> secretion. This is shown in Figure 3B, where celecoxib pretreatment completely represses PGE<sub>2</sub> secretion induced by Pam3CSK4 + TNF $\alpha$  (Figure 3B:  $P<0.05$ ).

### **$\beta_2$ -agonist-induced MKP-1 gene expression is repressed by $\beta_2$ -AR desensitization**

In addition to bronchodilatory actions in the airways,  $\beta_2$ -agonists also have anti-inflammatory effects via cAMP-dependent upregulation of immunomodulatory molecules. One such molecule is the MAPK-deactivating protein MKP-1 (13, 14). MKP-1 expression is mediated

via  $\beta_2$ -AR/cAMP-dependent mechanisms (13); thus we propose that upregulation of this critical anti-inflammatory molecule by  $\beta_2$ -agonists would be severely curtailed in bacterial infection. To examine this possibility *in vitro*, we measured the impact of  $\beta_2$ -AR desensitization on  $\beta_2$ -agonist-induced MKP-1 mRNA expression. Salbutamol-induced MKP-1 mRNA expression was significantly repressed by pretreatment with  $\beta_2$ -agonists via homologous desensitization (Figure 4A). Heterologous desensitization by PGE<sub>2</sub> also inhibited the MKP-1 upregulation induced by the short-acting  $\beta_2$ -agonist at all concentrations tested ( $P<0.05$ ). We then examined the effect of  $\beta_2$ -AR desensitization on formoterol-induced MKP-1 mRNA expression. As a full agonist, formoterol appeared to be less sensitive to desensitization than salbutamol. This could be observed in Figure 4B, where PGE<sub>2</sub> only had a significant effect on formoterol-induced MKP-1 at 100 nM ( $P<0.05$ ).

#### **Pam3CSK4 + TNF $\alpha$ cause tachyphylaxis of $\beta_2$ -AR agonists mediating MKP-1 gene expression and this can be reversed by celecoxib**

We next examined the direct effects of TLR2 activation on MKP-1 responses. Notably, pretreating cells with Pam3CSK4 + TNF $\alpha$  resulted in a significant reduction in  $\beta_2$ -AR agonist-induced MKP-1 gene expression (Figure 5A:  $P<0.05$ ). Figure 5B represents control experiments confirming PGE<sub>2</sub>-mediated  $\beta_2$ -AR desensitization. These data demonstrate that Pam3CSK4 + TNF $\alpha$  causes tachyphylaxis of  $\beta_2$ -AR agonist-induced MKP-1 gene expression in a PGE<sub>2</sub>-dependent manner. This could be reversed by celecoxib to allow  $\beta_2$ -agonist-induced MKP-1 gene expression to be reinstated (Figure 5C).

#### **PGE<sub>2</sub> induces heterologous $\beta_2$ -AR desensitization as measured by inhibition of $\beta_2$ -agonist-induced airway relaxation**

To test the influence of exposure to PGE<sub>2</sub> and  $\beta_2$ -agonists on dilator responses of airway

smooth muscle, experiments were conducted using mouse lung slices. This permits assessment of the reactivity of intrapulmonary airways (150-300  $\mu$ m diameter), with contraction and relaxation visualized *in situ* using phase-contrast microscopy. Under control conditions, a submaximal concentration of methacholine (MCh) reduced airway lumen area by  $46\pm4\%$ , with both  $\beta_2$ -agonists producing partial relaxation (% relaxation: salbutamol  $41\pm4\%$ ; formoterol  $33\pm8\%$ , Figure 6A & 6C). Overnight treatment with either  $\beta_2$ -agonist did not affect the level of MCh-induced precontraction, but completely abolished the subsequent relaxation to the same agonist. Airway relaxation to salbutamol, but not formoterol, was also significantly reduced by overnight incubation with PGE<sub>2</sub> (Figure 6C:  $P<0.05$ ). Overnight incubation of lung slices with Pam3CSK4 + TNF $\alpha$ , but not either agent alone, induced a greater than 10-fold increase in levels of PGE<sub>2</sub> in conditioned media (Figure 6D). However, the gradual overnight accumulation of these levels of PGE<sub>2</sub> were not sufficient to induce impairment of the relaxation response to salbutamol (Figure 6E), which was only reduced by overnight incubation with exogenous PGE<sub>2</sub> or salbutamol itself (Figure 6C).

## Discussion

Utilizing an *in vitro* model of bacterial exacerbation we show that TLR2 activation in the presence of an inflammatory stimulus leads to  $\beta_2$ -AR desensitization in ASM cells. TLR2 activation induced a significant time-dependent increase in expression of COX-2 mRNA and secretion of PGE<sub>2</sub>. PGE<sub>2</sub> induced heterologous desensitization of the  $\beta_2$ -AR on ASM cells, reducing the amount of cAMP produced in response to salbutamol and formoterol, and curtailing the anti-inflammatory and bronchodilatory actions of  $\beta_2$ -agonists. Consistent with our previous findings that COX-2 products, such as PGE<sub>2</sub>, are responsible for rhinovirus-induced  $\beta_2$ -AR desensitization (4, 5), these results show that bacteria also induce a prostanoid-dependent molecular mechanism to down-regulate the  $\beta_2$ -AR on ASM cells.

In well-controlled asthma,  $\beta_2$ -agonists relax the airways by rapidly increasing cAMP levels in ASM. Unfortunately, the response to  $\beta_2$ -agonists is much less effective in virus-induced asthma exacerbations (15-17) and can be compromised by bacterial infection (3). To date, the underlying mechanisms are not completely understood and in order to ultimately improve asthma therapy we require a greater understanding of the molecular events that occur at the cellular level that manifest clinically with reduced  $\beta_2$ -agonist response during infection.

Utilising a model of rhinovirus-induced asthma exacerbation *in vitro* in ASM cells (4, 5, 10) we previously learned how key molecular steps responsible for the action of  $\beta_2$ -agonists are severely curtailed by viral infectious stimuli. Whether bacterial infection directly affects  $\beta_2$ -AR function had not been explored. To address this, we modelled bacterial exacerbation *in vitro* with the synthetic bacterial lipoprotein Pam3CSK4 (a TLR2 agonist) to mimic bacterial infection and TNF $\alpha$  to simulate inflammation. TLR2 is a pattern recognition receptor important for mediating the response to bacterial products and Pam3CSK4 has been widely used to mimic bacterial infections (gram + bacteria) (18, 19). TLR2 ligation robustly increased secretion of cytokines implicated as playing a role in asthmatic exacerbation (19). Interleukin 8, in particular, was increased by ~ 3-fold and IL-8-mediated neutrophil chemotaxis was greatly enhanced in the presence of the bacterial lipoprotein (19).

Using this model we now show that that TLR2 activation results in a time-dependent increase in TNF $\alpha$ -induced COX-2 mRNA expression. COX-2 converts arachidonic acid to prostanoids (including prostaglandins), and in confirmation of earlier reports (12), COX-2 upregulation in ASM cells consequently results in secretion of PGE<sub>2</sub>. Secreted PGE<sub>2</sub> then acts back on the ASM in an autocrine manner to cause heterologous  $\beta_2$ -AR desensitization (5, 11, 12). Homologous desensitization was confirmed in parallel experiments (20); ASM cells were pretreated with  $\beta_2$ -agonists and the resultant cAMP formed in response to salbutamol and formoterol was significantly attenuated. Moreover, it was interesting to note that the

sensitivity to heterologous  $\beta_2$ -AR desensitization induced by PGE<sub>2</sub> differed between the two  $\beta_2$ -agonists tested. Inhibition of salbutamol-induced cAMP generation was evident after pretreatment with a 10-fold lower concentration of PGE<sub>2</sub> than was required to inhibit the formoterol response, perhaps reflecting the lack of receptor reserve for salbutamol, which is a partial agonist at  $\beta_2$ -ARs.

In order to demonstrate the impact of  $\beta_2$ -AR desensitization we measured the effect on two important ways in which  $\beta_2$ -agonists induce their beneficial effects in asthma; 1) anti-inflammatory actions; 2) bronchorelaxation.

Firstly, our recent studies have demonstrated that one of the key ways in which  $\beta_2$ -agonists have an anti-inflammatory effect in airways is via upregulation of MKP-1 (13, 14). As this upregulation is mediated via  $\beta_2$ -AR/cAMP-dependent mechanisms (13), we now show that  $\beta_2$ -agonist-induced MKP-1 mRNA expression is significantly repressed when the  $\beta_2$ -AR is heterologously desensitized by PGE<sub>2</sub>. The extent of repression of the response to salbutamol and formoterol by PGE<sub>2</sub> was also consistent with their relative capacity to induce cAMP-dependent MKP-1 expression. Being a full agonist, formoterol appeared to be relatively less sensitive to  $\beta_2$ -AR desensitization than the partial agonist salbutamol. Since salbutamol lacks receptor reserve, PGE<sub>2</sub>-mediated desensitization had a greater impact on its capacity to increase expression of MKP-1, and potentially to limit airway inflammation. Importantly, we also demonstrated that TLR2 activation has a direct effect on  $\beta_2$ -agonist-induced MKP-1 response. Pam3CSK4 + TNF $\alpha$  caused tachyphylaxis of  $\beta_2$ -AR agonist-induced MKP-1 gene expression and this could be reversed by celecoxib. These results suggest that celecoxib could be therapeutic in infectious exacerbation as treatment reverses the tachyphylaxis of salbutamol and formoterol on MKP-1 gene expression.

Secondly, in order to demonstrate the impact of  $\beta_2$ -AR desensitization on reduced  $\beta_2$ -agonist bronchodilatory efficacy we have used an *ex vivo* technique that closely mimics the *in vivo*

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context. Precision-cut lung slices examines the reactivity of the clinically relevant small airways and enables investigation into bronchoconstriction in an *ex vivo* setting where cell-cell and cell-matrix relationships are maintained. Lung slices have proved to be an effective tool for assessing alterations in small airway reactivity in response to a range of stimuli, including exposure to inflammatory cytokines *in vitro* and after chronic allergen challenge *in vivo* (8, 21, 22). We have previously characterised dilator responses in mouse airways in lung slices, showing that PGE<sub>2</sub> elicits maximal relaxation with greater potency and efficacy than salbutamol in mouse airways, eliciting complete relaxation that is resistant to desensitization by salbutamol and by itself (9). In this study we were able to confirm loss of  $\beta_2$ -agonist mediated relaxation in small airways through homologous desensitization (22). The finding that PGE<sub>2</sub> was also able to impair dilator responses to the partial agonist salbutamol, but not to the full agonist formoterol, was consistent with its greater inhibition of salbutamol-mediated cAMP production in ASM also reported here. The relatively higher potency and larger receptor reserve of formoterol provide an explanation for the maintained  $\beta_2$ -AR-mediated relaxation to this agonist in the same setting. We were not able to establish that overnight incubation of lung slices with Pam3CSK4 + TNF $\alpha$  impaired subsequent dilator responses to  $\beta_2$ -AR agonists. Although Pam3CSK4 + TNF $\alpha$  induced the expected increased secretion of PGE<sub>2</sub> in this setting, the measured levels released from a very small amount of lung tissue and diluted within the surrounding media were markedly lower than the concentration of exogenous PGE<sub>2</sub> shown to induce desensitization of salbutamol-mediated relaxation. Nevertheless, we propose that the local concentrations of PGE<sub>2</sub> generated within the whole lung *in vivo* following infection would be sufficient to contribute to the loss of dilator responsiveness. This is supported by a previous *in vivo* study in an infection model where influenza A infection induced sustained increases in bronchoalveolar lavage (BAL)

levels of PGE<sub>2</sub>, and that COX2<sup>-/-</sup> mice had reduced BAL inflammatory cells and cytokines compared to wild type mice (23).

Thus, we have begun to achieve a better understanding of the molecular mechanisms of infection-induced asthma exacerbations that will ultimately allow the development of new therapeutics to specifically treat infection-induced asthma exacerbations. Because COX-2 products, including PGE<sub>2</sub>, are also responsible for rhinovirus-induced  $\beta_2$ -AR desensitization (5) our results suggest that bacteria and virus induce a common molecular mechanism responsible for  $\beta_2$ -agonist tachyphylaxis. Importantly, our data reveals that COX-2 inhibition with celecoxib completely abrogates PGE<sub>2</sub> secretion. Thus, celecoxib may represent a feasible pharmacological option for treatment of infection-induced asthma exacerbations in the future.

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## Contributorship Statement

HA: Performed MKP-1 mRNA experiments and co-ordinated experimental plan

NNR: Performed MKP-1 mRNA and cAMP experiments

MMR: Performed COX-2 and MKP-1 mRNA experiments and PGE<sub>2</sub> secretion

MF: Performed mouse lung experiments

MdP: Performed cAMP experiments

BGG: Advised on experimental design for cAMP and PGE<sub>2</sub> assays and helped to conceive the project

JEB: Conceived experimental design for mouse lung experiments and performed statistical analysis

AJA: Conceived the project, designed experiments, analysed data and wrote the paper and is responsible for the overall content

## Figure Legends

**Figure 1. TLR2 ligand engagement upregulates TNF $\alpha$ -induced COX-2 mRNA expression and increases PGE<sub>2</sub> secretion.** Growth-arrested ASM cells were pretreated with vehicle or Pam3CSK4 (1  $\mu$ g/ml) for 1 h, followed by treatment with vehicle or TNF $\alpha$  (10 ng/ml) for 0, 1, 2, 4, 8, and 24 h. (A) COX-2 mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase compared to vehicle-treated cells at 0 h) and (B) PGE<sub>2</sub> secretion was measured by EIA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where \* denotes a significant effect of Pam3CSK4 on TNF $\alpha$ -induced COX-2 mRNA expression and PGE<sub>2</sub> secretion ( $P<0.05$ )). Data are mean+SEM values from (A) n=7 and (B) n=4 primary ASM cell cultures.

**Figure 2. PGE<sub>2</sub> induces heterologous  $\beta_2$ -AR desensitization as measured by inhibition of  $\beta_2$ -agonist-induced cAMP production.** Growth-arrested ASM cells were pretreated for 24 h

with vehicle, salbutamol (10  $\mu$ M), formoterol (0.01  $\mu$ M), or a range of PGE<sub>2</sub> concentrations (0.1-100 nM). Desensitization of the  $\beta_2$ -AR was assessed by measuring production of cAMP in response to stimulation with (A) 10  $\mu$ M salbutamol or (B) 0.01  $\mu$ M formoterol for 15 min, compared to vehicle, in the presence of the pan-phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX: 30 min pretreatment at 10  $\mu$ M). Results are expressed as a percentage of  $\beta_2$ -agonist-induced cAMP. Statistical analysis was performed using one-way ANOVA then Bonferroni's post-test (where § or \* denotes a significant effect of  $\beta_2$ -agonists or PGE<sub>2</sub> on cAMP production, respectively ( $P<0.05$ )). Data are mean+SEM values from n=5 primary ASM cell cultures.

**Figure 3. Celecoxib inhibits PGE<sub>2</sub> secretion but not COX-2 mRNA expression.** Growth-arrested ASM cells were pretreated with vehicle or 10  $\mu$ M celecoxib for 1 h. Cells were then treated with vehicle or Pam3CSK4 (1  $\mu$ g/ml) for 1 h, followed by vehicle or TNF $\alpha$  (10 ng/ml) for 24 h. (A) COX-2 mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase compared to vehicle-treated cells) and (B) PGE<sub>2</sub> secretion was measured by EIA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where \* denotes a significant effect of celecoxib on PGE<sub>2</sub> secretion ( $P<0.05$ )). Data are mean+SEM values from n=4 primary ASM cell cultures.

**Figure 4. Salbutamol- and formoterol-induced MKP-1 gene expression is repressed by  $\beta_2$ -AR desensitization.** Growth-arrested ASM cells were pretreated for 24 h with vehicle, salbutamol (10  $\mu$ M), formoterol (0.01  $\mu$ M), or a range of PGE<sub>2</sub> concentrations (1-100 nM). The impact of  $\beta_2$ -AR desensitization on anti-inflammatory actions of  $\beta_2$ -agonists was assessed by measuring MKP-1 mRNA expression by RT-PCR in response to stimulation with (A) 10  $\mu$ M salbutamol or (B) formoterol (0.01  $\mu$ M) for 1 h, in comparison to vehicle (results

expressed as percentage of  $\beta_2$ -agonist-induced MKP-1). Statistical analysis was performed using one-way ANOVA then Bonferroni's post-test (where § or \* denotes a significant effect of  $\beta_2$ -agonists or PGE<sub>2</sub> on MKP-1 mRNA expression, respectively ( $P<0.05$ )). Data are mean+SEM values from n=5 primary ASM cell cultures.

**Figure 5. Pam3CSK4 + TNF $\alpha$  cause tachyphylaxis of  $\beta_2$ -AR agonists mediating MKP-1 gene expression and this can be reversed by celecoxib.** Growth-arrested ASM cells were pretreated for 24 h with vehicle, Pam3CSK4 (1  $\mu$ g/ml) + TNF $\alpha$  (10 ng/ml), or PGE<sub>2</sub> (100 nM). (A) The impact of direct TLR2-mediated  $\beta_2$ -AR desensitization on anti-inflammatory actions of  $\beta_2$ -agonists was assessed by measuring MKP-1 mRNA expression by RT-PCR in response to stimulation with 10  $\mu$ M salbutamol or formoterol (0.01  $\mu$ M) for 1 h, in comparison to vehicle (results expressed as fold increase compared to vehicle- or Pam3CSK4 + TNF $\alpha$ -treated cells). (B) Control experiments confirm PGE<sub>2</sub>-mediated  $\beta_2$ -AR desensitization results in reduced  $\beta_2$ -agonist-mediated MKP-1 expression, in comparison to vehicle-treated cells. (C) In parallel experiments, cells were pretreated with vehicle or 10  $\mu$ M celecoxib for 1 h, and treated under identical conditions as above (A) to demonstrate that tachyphylaxis of  $\beta_2$ -AR agonists was reversed by celecoxib allowing  $\beta_2$ -agonist-induced MKP-1 gene expression to be reinstated. Statistical analysis was performed using one-way ANOVA then Bonferroni's post-test (where \* denotes a significant repression of  $\beta_2$ -agonist-induced MKP-1 mRNA expression and § denotes a significant effect of celecoxib ( $P<0.05$ )). Data are mean+SEM values from n=3 primary ASM cell cultures.

**Figure 6. PGE<sub>2</sub> and  $\beta_2$ -AR desensitization inhibit  $\beta_2$ -agonist-mediated relaxation of mouse intrapulmonary airways.** (A) Lung slices were incubated overnight in the absence (upper panels) or presence of  $\beta_2$ -agonists (middle panels), or PGE<sub>2</sub> (lower panels). Airways

were pre-contracted with methacholine (MCh: 100 nM) before assessment of the dilator actions of maximally effective concentrations of salbutamol (SAL (10  $\mu$ M): left panels) or formoterol (FORM (0.01  $\mu$ M): right panels). (A) Representative traces show data collected at 2 sec intervals normalized to the initial airway lumen area. (B) Representative images show impaired dilator response to SAL after overnight treatment with PGE<sub>2</sub>. (C) Grouped dilator responses averaged over the final minute of 5 min perfusion under each condition are expressed as % relaxation of the relevant precontraction. Lung slices were also incubated overnight in the absence (vehicle) or presence of Pam3CSK4 (1  $\mu$ g/ml) and TNF $\alpha$  (10 ng/ml) alone or in combination. Grouped responses show (D) levels of PGE<sub>2</sub> in conditioned media and (E) relaxation to SAL (10  $\mu$ M). Results are mean $\pm$ SEM values from n=4-6 slices from separate mice. Statistical analysis was performed using one-way ANOVA then Bonferroni's post-test (where §§§ or \*\*\* denotes a significant effect of overnight treatment with  $\beta_2$ -agonists or PGE<sub>2</sub> on relaxation, respectively ( $P<0.01$ )). PGE<sub>2</sub> values were transformed to log values prior to similar analysis (where \* denotes significant effect of treatment with Pam3CSK4 + TNF $\alpha$  ( $P<0.05$ )).

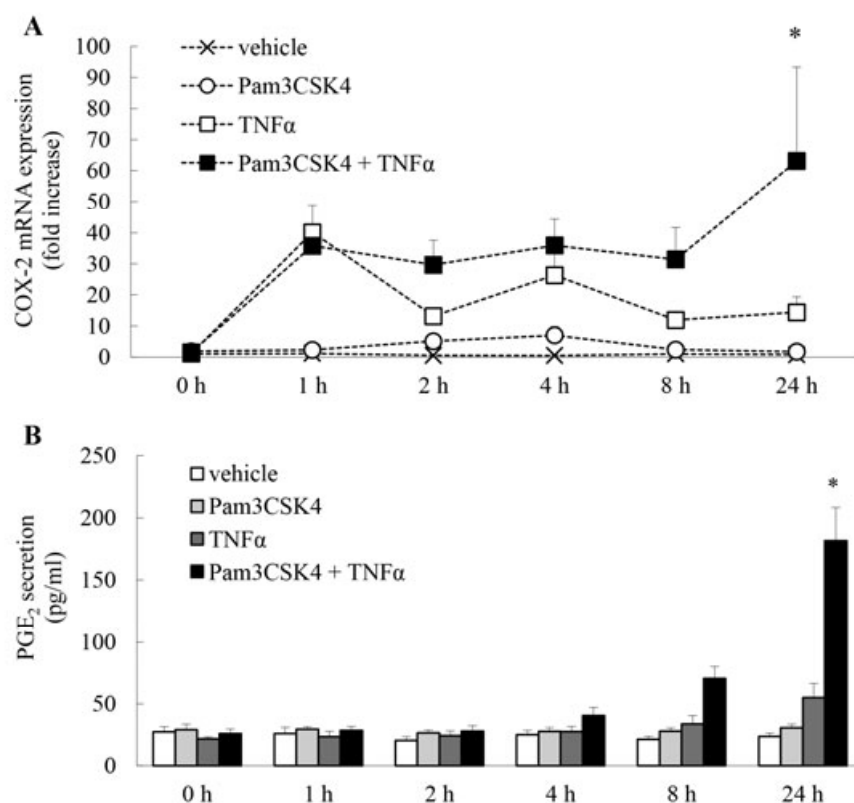
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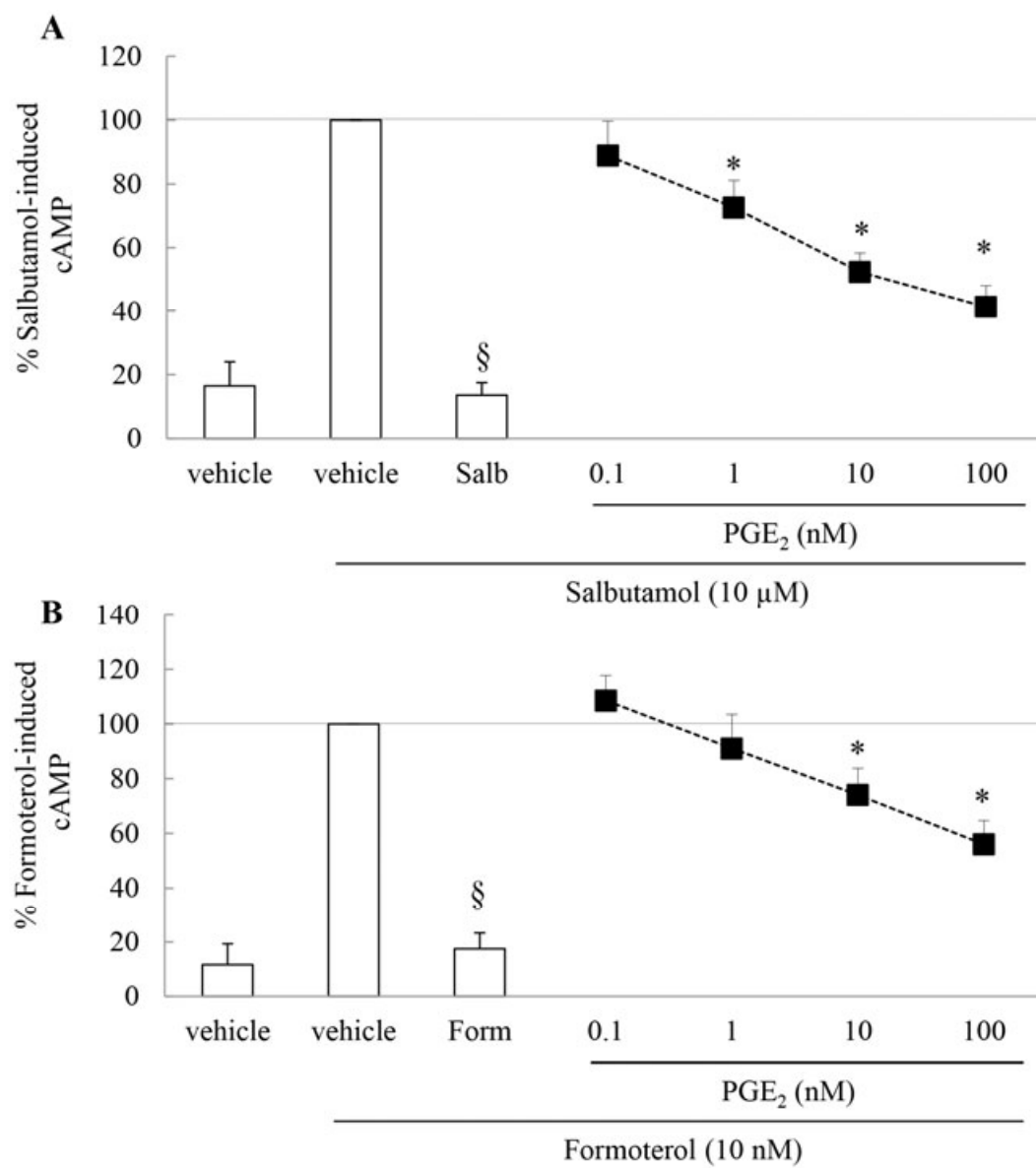
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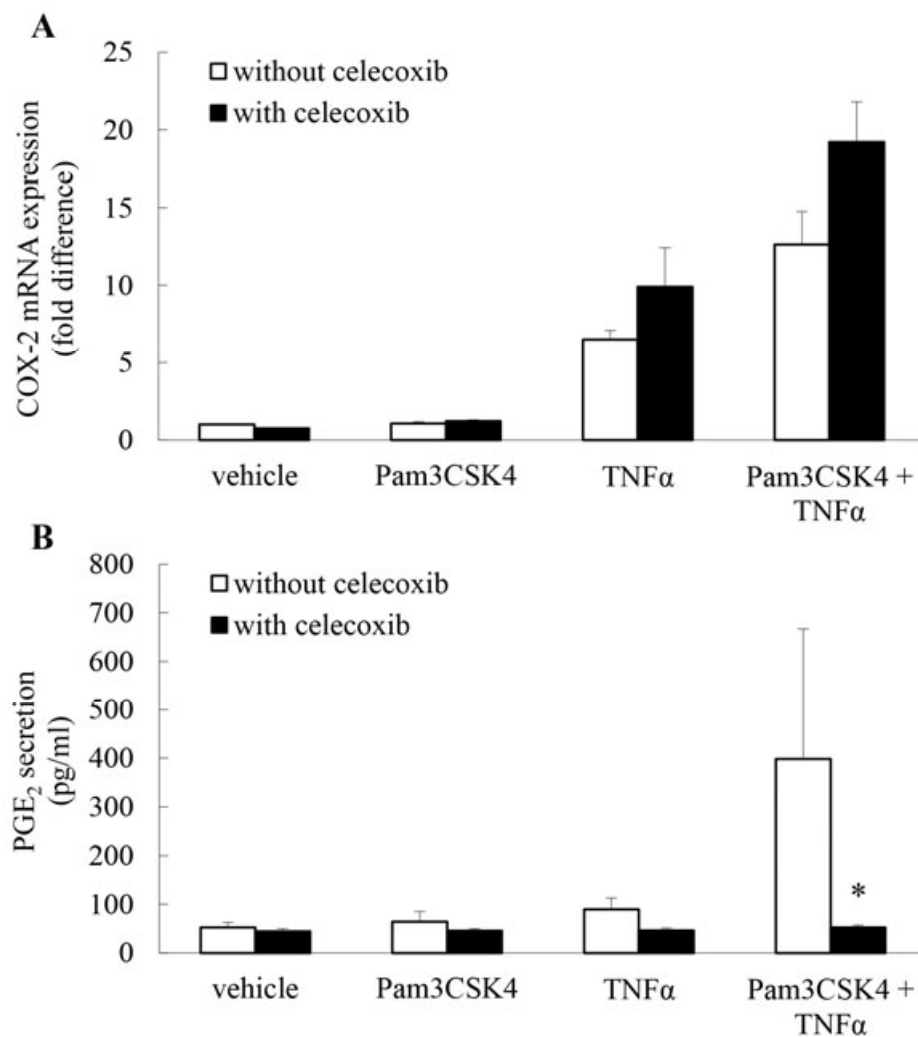
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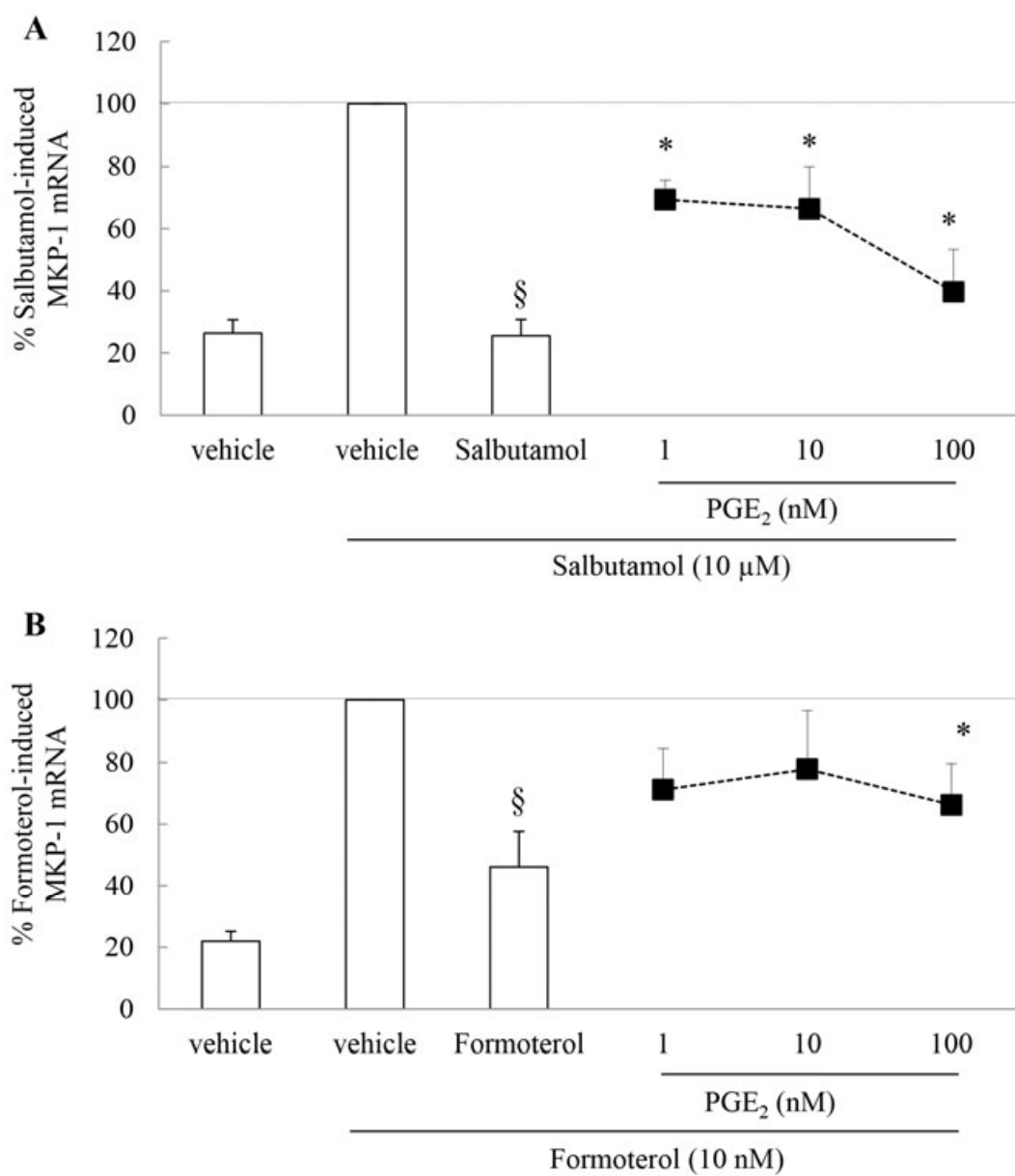
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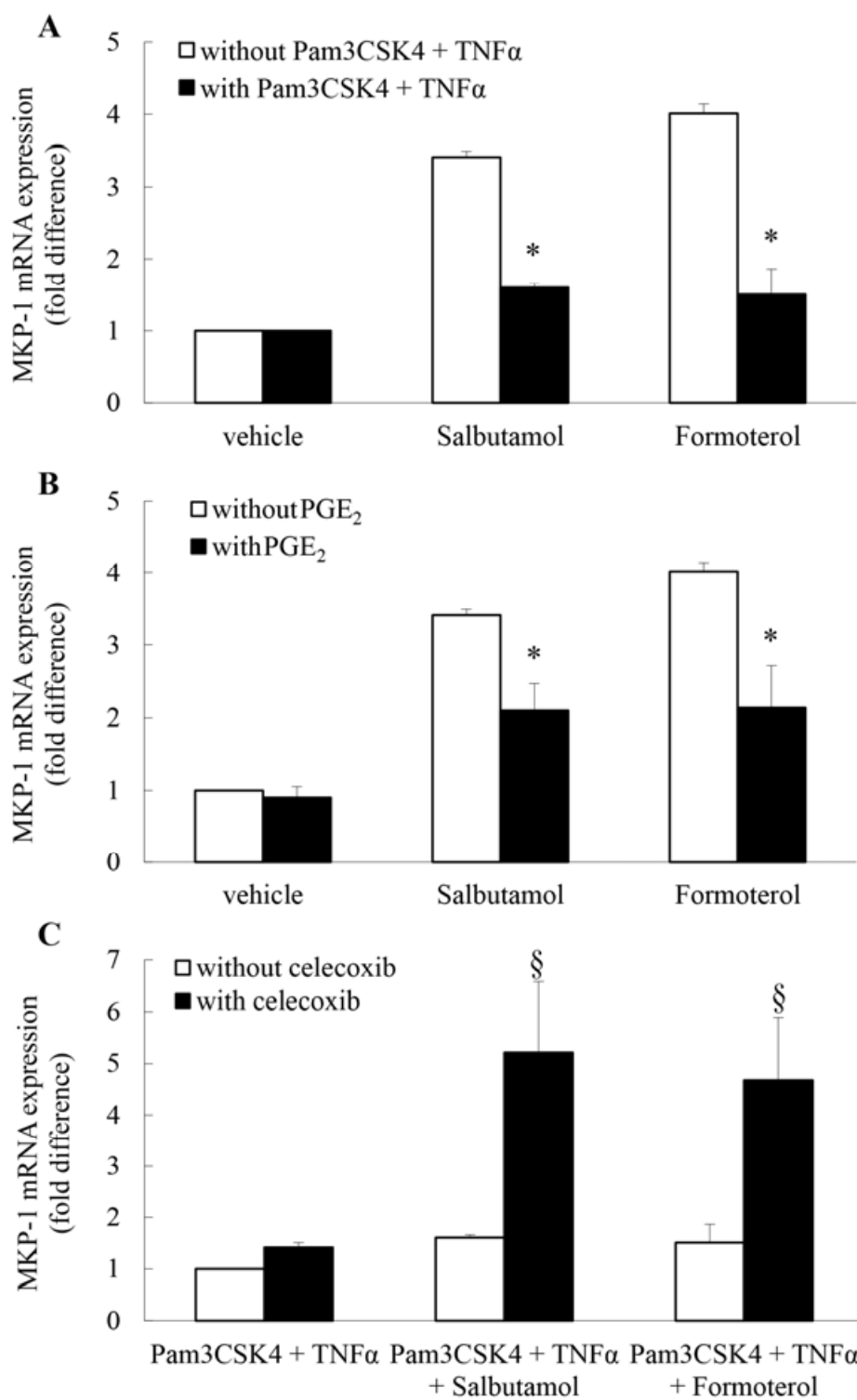
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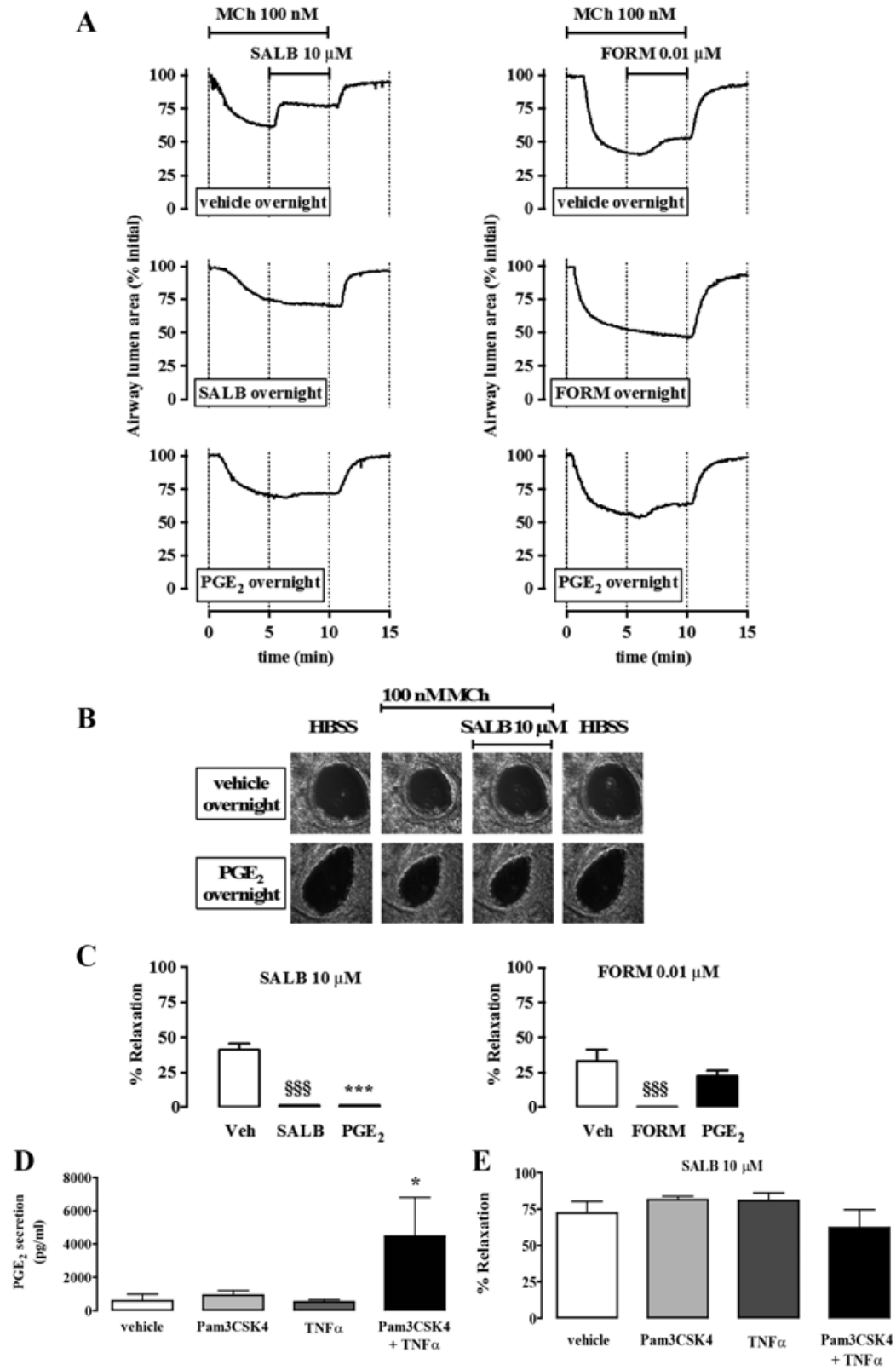












## **Online Supplement**

**TLR2 activation causes tachyphylaxis to  $\beta_2$ -agonists *in vitro* and *ex vivo*:**

### **modeling bacterial exacerbation**

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## **Materials and Methods**

### **Real-time RT-PCR**

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the RevertAid First strand cDNA Synthesis Kit (Fermentas Life Sciences). Real-time RT-PCR was performed on an ABI Prism 7500 with COX-2 (Hs0015133\_m1) and MKP-1 (DUSP1: Hs00610256\_g1) TaqMan® Gene Expression Assays and the eukaryotic 18S rRNA endogenous control probe (Applied Biosystems) 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.

### **Design of experiments utilizing mouse lung slices**

All experiments were conducted in slices perfused under gravity through 8 separate channels by a valve system under manual control (Warner Instruments Inc., LFS Lee Company). Following overnight incubation in the absence or presence of PGE<sub>2</sub> (100 nM), salbutamol (10 µM) or formoterol (0.01 µM), slices were washed with 1X HBSS/HEPES for > 5 min prior to commencement of experiments at room temperature. Dilator responses to salbutamol (10 µM) or formoterol (0.01 µM) were measured in airways after submaximal precontraction with methacholine (100 nM).