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INDUCTION OF THE INFLAMMATORY REGULATOR A20 BY GIBBERELIC ACID IN AIRWAY EPITHELIAL CELLS

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AUTHOR CONTRIBUTIONS

JR started the experiments, supervised the day-to-day experiments, performed statistical analyses, and provided the first manuscript draft.

BM performed PNECs experiments and some of the analyses.

AB performed p65 NF- κ B activity ELISA.

SC performed p65 Western Blot.

AC performed cell lines experiments.

RB performed cell lines experiments.

JSE was responsible for ethics, clinical governance, patient selection, clinical sampling.

HD advised on interpretation of A20 protein results.

BH supplied A20 antibody and advised on interpretation of results.

ME helped supervise the students, assisted with the data analysis and manuscript preparation.

CK identified Gibberellic acid as an A20 inducer, overlooked the experiments, analyses and manuscript preparation together with BCS.

BCS identified Gibberellic acid as an A20 inducer, supervised students and PDRA, overlooked experiments, analyses and manuscript preparation together with CK

ABBREVIATIONS

16HBE14o-	Human bronchial epithelial cells
CF	Cystic Fibrosis
Dex	Dexamethasone
ELISA	Enzyme linked immunoabsorbant assay
GA ₃	Gibberellic Acid 3
HGF	Hepatocyte growth factor
I κ B	Inhibitor of κ B
IL-8	Interleukin 8
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
NEC	Nasal epithelial cells
NEMO	NF- κ B essential modulator
NF- κ B	Nuclear Factor Kappa B
NIK	NF- κ B inducing kinase, non-canonical NF- κ B pathway
OUT	Ovarian tumour domain

OVA	Ovalbumin
PNEC	Primary nasal epithelial cells
PRR	Pattern recognition receptor
TLR4	Toll-like receptor 4
TNF α	Tumour Necrosis Factor alpha
TRAF6	TNF Receptor Associated Factor 6

ABSTRACT

Background and purpose: NF- κ B driven inflammation is negatively regulated by the zinc finger protein, A20. Gibberellic acid (GA₃) is a plant-derived diterpenoid with documented anti-inflammatory activity, which is reported to induce A20-like zinc finger proteins in plants. Here, we sought to investigate the anti-inflammatory effect of GA₃ in airway epithelial cells and determine if the anti-inflammatory action relates to A20 induction.

Experimental approach: Primary nasal epithelial cells (n=7), and a human bronchial epithelial cell line (16HBE14o-) were used. Cells were pre-incubated with GA₃ (30 μ M, 1h), stimulated with *Pseudomonas aeruginosa* LPS (10 μ g/ml), IL-6 and IL-8 release, A20, NF- κ B and I κ B α expression determined. To determine if any observed anti-inflammatory effect occurred via an A20-dependent mechanism, A20 was silenced using siRNA.

Key results: GA₃ pre-incubation significantly induced A20 mRNA (4h) and protein (24h), resulting in a significant reduction in IL-6 and IL-8 release. This effect was mediated via reduced I κ B α degradation and reduced NF- κ B (p65) expression. Furthermore, the anti-inflammatory action of GA₃ was abrogated in A20-silenced cells.

Conclusions and implications: Here we show that A20 induction by GA₃ attenuates inflammation in airway epithelial cells, at least in part through its effect on NF- κ B and I κ B α . GA₃ or gibberellin-derived derivatives could potentially be developed into anti-inflammatory drugs for the treatment of chronic inflammatory diseases associated with A20 dysfunction.

INTRODUCTION

The airway mucosa is under constant attack from invading pathogens, which are recognised by pattern recognition receptors (PRR) on the surface of airway epithelial cells and circulating immune cells. Toll-like receptor 4 (TLR4) recognises the LPS component of Gram-negative bacterial cell walls. Activation of TLR4 triggers an innate immune response leading to an acute inflammatory response that is largely mediated by the transcription factor NF- κ B. Following receptor-ligand binding with the plasma membrane, the intracellular stages of the NF- κ B signalling cascade are tightly regulated to ensure timely termination of the inflammatory response. A20 (*TNFAIP3*, (tumour necrosis factor, alpha-induced protein 3)) is an endogenous negative regulator of NF- κ B signalling that is rapidly and transiently induced in response to bacterial and viral stimuli. A20 terminates NF- κ B driven inflammation in response to LPS by inhibiting the polyubiquitination and activation of the central adaptor protein TNF receptor-associated factor (TRAF)6 (Lin *et al.* 2008).

In the lung, Gram-negative *Pseudomonas aeruginosa* (*P. aeruginosa*) challenge rapidly induces A20 in mice (Tiesset *et al.* 2009), while A20 is essential for termination of TLR2/4 mediated IL-8 release from primary airway epithelial cells (Gon *et al.* 2004). Furthermore, A20 protects against ovalbumin (OVA)-induced ‘asthma’ in mice (Kang *et al.* 2009). Li *et al.* (2013) showed that A20 is involved in antigen degradation by facilitating the fusion between the endosome and lysosome, resulting in reduced antigenicity upon absorption of the antigen. We recently demonstrated that A20 is significantly reduced in the chronically inflamed Cystic Fibrosis (CF) airway epithelium and that basal mRNA expression correlates strongly with lung function (FEV₁ % predicted) in people with CF (Kelly *et al.* 2013a, 2013b). In addition to observations in the airways, A20 is known to be important in the pathogenesis of various inflammatory diseases (rheumatoid arthritis, systemic lupus erythematosus, Type 1 Diabetes, multiple sclerosis, psoriasis, colitis, chronic inflammatory bowel disease and Crohn’s disease), where it may also serve as a susceptibility gene and biomarker of disease development (Martin and Dixit 2011, Barmada *et al.* 2004, Wellcome Trust Case Consortium 2007). A20 knockout in epithelial cells promotes severe mucosal inflammation (Vereecke *et al.* 2011). The therapeutic potential of targeting the A20 gene and resulting protein has been widely recognized but a therapeutic agent is not currently available.

Although a critical regulator of mammalian immune responses, A20-like zinc finger proteins are also important in controlling plant stress responses. Liu *et al.* reported that gibberellic acid (GA₃), a tetracyclic diterpene, induces A20-like zinc finger proteins in rice crops (Liu *et al.* 2011). Interestingly, GA₃ has previously been used in the treatment of human lung cancer and in a rodent model of diabetes, and was shown to exert anti-inflammatory and analgesic effects (Miklussak *et al.* 1980, Muthuraman and Srikumar 2009). Furthermore, a recently developed gibberellin derivative possesses potent anti-tumor and anti-angiogenic activity in various tumour derived cell lines (Zhang *et al.* 2012). Gibberellins comprise a large family of tetracyclic diterpenoid plant hormones biosynthesized via *ent*-kaurene intermediates, which have diverse biological roles in plant growth and development. In plants, gibberellins play signalling roles in responses to environmental changes (temperature, stress and light) and are involved in the control of stem and root elongation, leaf expansion, seed germination and flowering (Yamaguchi 2008). Many terpenes, including diterpenes, triterpenes, and sesquiterpenes, possess anti-inflammatory activity both *in vivo* and *in vitro*. Furthermore, most terpenes inhibit NF-κB activity, although the precise mechanisms of action have not been fully characterized. In particular, forskolin has been used successfully in the treatment of asthma to reduce inflammation and histamine release through activation of cAMP-dependent mechanisms (Huerta *et al.* 2010). In the case of kaurenes, Castrillo *et al.* (2001) have identified relevant targets for this inhibition, showing that the inhibition of NIK (NF-κB inducing kinase, non-canonical NF-κB pathway) and p38 and/or ERK1 and ERK2 activation abrogates the inflammatory response, of genes dependent on NF-κB activation in particular. The fact that kaurenes are intermediates in the biosynthesis of plant hormones, such as gibberellins, offers the possibility of envisaging further analysis of the interaction of these molecules in several aspects of mammalian cell biology (Castrillo *et al.* 2001).

Several gibberellin-based preparations are under patent for the treatment of diabetes (Patent reference US 20050215496 A1), psoriasis and prostatitis (Patent reference WO 1991008751 A1), highlighting the increasing interest in developing gibberellins for pre-clinical and clinical trials. However, gibberellins have not yet been used clinically as anti-inflammatory drugs and the mechanism of action has not been reported. Here, we seek to investigate if GA₃ can induce A20 and thereby reduce the innate inflammatory response to bacterial LPS in airway epithelial cells (16HBE14o- and primary nasal epithelial cells).

EXPERIMENTAL PROCEDURES

Cell Culture

The immortalised bronchial epithelial cell line 16HBE14o- (obtained from D. Gruenert UCSF, USA) was grown as described (Kelly *et al.* 2013c). Primary nasal epithelial cells (NEC) were obtained from healthy volunteers (n=7) and grown as previously described (de Courcey *et al.* 2012). The participants did not have any acute airways disease at the time of sampling, or a history of any chronic airways inflammation. The study was approved by the Research Ethics Committee of Northern Ireland (07/NIR02/23) and all participants provided informed consent (de Courcey *et al.* 2012).

Cell culture stimulations

All cells were treated with LPS (*P. aeruginosa*, Sigma-Aldrich, 10 µg/ml) for up to 24 h. Cells were exposed to a range of GA₃ concentrations (Sigma- Aldrich, G7645, lot BCBB9751, 3–300 µM) or dexamethasone (Dex, Sigma-Aldrich, D4902, lot BCBH2988V, 1 µM) for 1 h prior to LPS stimulation.

Determination of LPS induced cytokine release (IL-6 and IL-8)

The concentrations of IL-6 and IL-8 in cell-free culture supernatants were measured by a commercially available ELISA (PeproTech EC Ltd., UK) according to the manufacturer's instructions.

LDH Cytotoxicity assay

The effect of GA₃ on membrane integrity was determined by quantification of lactate dehydrogenase (LDH) in cell culture supernatants (LDH-Cytotoxicity Assay Kit, BioVision Ltd. USA) according to the manufacturer's instructions.

Proliferation Assay

Cell proliferation was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, UK) following 72h exposure to GA₃ and according to the manufacturer's recommendations. The assay determines the activity of mitochondrial NAD(P)H-dependent oxidoreductases, which reduces the tetrazolium dye MTS (3-(4,5-di-

methylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) to insoluble purple formazan, which is measured at $\lambda=490$ nm.

Transfection studies

Transfections were performed at 70–80% confluency. A20 was silenced in 16HBE14o- cells using a commercially available human A20 siRNA (GenomeWide siRNA, Qiagen) and RNAiFect™ Transfection Reagent (Qiagen). Experiments included mock (transfection reagent only) and scrambled (Allstars Neg siRNA, Qiagen) controls. A20 knockdown was confirmed by qPCR (transfection efficiency over 70%) and Western Blot.

RNA extraction and real time qPCR

Total RNA was extracted using an RNeasy kit (Qiagen) and quantified on a Nanodrop (Thermo Scientific). Equal amounts of RNA were reverse transcribed into cDNA (Sensiscript Reverse Transcription Kit, Qiagen). Primers were designed using gene accession numbers and Primer3 open-source PCR primer design software and obtained from Invitrogen Ltd. (Paisley, UK). Quantitative PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics Ltd, UK). Multiple house-keeping genes were tested and β -actin chosen for consistency within cycles and between different samples.

Expression of A20, p65 and β -actin (housekeeping gene) were assessed by qPCR. All primer sequences are given in Table S1. Relative expression to β -actin was calculated using the $\Delta\Delta C_t$ method. To overcome inter-patient variability in basal gene expression levels in NECs, mRNA expression after LPS stimulation (24h) was compared with mRNA expression at 0h (standardized to 1) for each individual sample. cDNA obtained from Jurkat cells acted as an internal calibrator for all experiments and was used to determine differences in basal gene expression.

Western Blotting

Protein expression was determined by Western Blotting after extraction of total proteins in RIPA buffer. Cell lysates were diluted in nuclease free water and Laemmli loading buffer, loaded onto Tris-HCl polyacrylamide gels (Thermo Scientific), separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were incubated with 1 μ g/ml primary antibodies (Ber-A20 (Hirsch *et al.* 2012), A20 C-terminal (IMGENEX, IMG-161A), p65

(Santa Cruz Biotechnology, C-20), I κ B α (Santa Cruz Biotechnology, C-21) washed, incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Thermo Scientific, UK) and visualized on a BioRadChemi Doc XRS system.

P65 activation ELISA

Nuclear protein was extracted from 16HBE14o- cells using the Ne-Per™ assay (Thermo Scientific, cat no.78833). Total protein was determined in the extracts using the BCA protein assay (Pierce, cat no. 23225) and equal amounts nuclear protein (10 μ g) were used in the p65 activation ELISA (Activ Motif, TransAM®, NF- κ B Family Transcription factor Assay kit, Cat no. 43296). Briefly, each ELISA well contains the immobilized NF- κ B consensus site (5'-GGGACTTTCC-3') to which active NF- κ B binds. The primary antibody (here anti-p65) can only bind to the epitope on the active p65 that is bound to its target DNA. Then an HRP-conjugated secondary antibody is used to provide a sensitive colorimetric readout that is spectrophotometrically quantified. All assays were performed according to the manufacturer's instructions.

Statistical analysis

All data are presented as the means \pm SEM. Differences between groups were analysed using the Kruskal-Wallis non-parametric ANOVA, which was considered to be significant if $p < 0.05$. Dunn's post-test was performed if the Kruskal Wallis test was significant. To assess the effect of GA₃ pre-treatment on LPS stimulated PNECs, Willcoxon paired T-test was applied. Statistical significance levels are indicated as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. GraphPad Prism was used to plot graphs and to analyse the data.

RESULTS

Changes in gene or protein expression are shown relative to the corresponding untreated control. Given the limited amount of material available from primary cultures, all primary NEC investigations were performed at the mRNA level initially and confirmed at the protein level where possible.

Effect of GA₃ pre-treatment on cellular viability and LPS-stimulated cytokine release

In the first instance, we sought to determine an optimal non-cytotoxic GA₃ dose that would also induce significant anti-inflammatory effects. Therefore, the effect of various GA₃ concentrations (0.03–300 μM) on cellular viability and LPS-stimulated IL-6 and IL-8 release was investigated in the bronchial epithelial cell line 16HBE14o-.

LDH release into cell culture supernatants was analysed 24 h after GA₃ treatment and used as a measure of cytotoxicity. GA₃ concentrations of 0.03–30 μM did not increase LDH release above the vehicle control, while 300 μM GA₃ caused a significant increase in LDH release ($p < 0.001$, $n = 6$; *Fig 1A*). IL-8 release was significantly increased compared with untreated cells following 24 h LPS stimulation (*Fig 1B*). In 16HBE14o- cells, GA₃ treatment alone did not have any effect on basal IL-8 release, but GA₃ pre-treatment significantly reduced the LPS-induced IL-8 release in a dose-dependent manner with a significant reduction at 30 μM ($p < 0.01$, $n = 5-9$) (*Fig 1B*). The maximal effective concentration to reduce IL-8 was 28.19 μM, while a reduction of 40% was achieved at 2.82 μM (*Fig 1C*). Similarly, GA₃ treatment alone did not have any effect on basal IL-6 release, but GA₃ pre-treatment significantly reduced the LPS induced IL-6 release with a significant reduction at 30 μM ($p < 0.05$, $n = 5-8$) (*Fig 1D*).

Based on these results and previous publications, a concentration of 30 μM GA₃ was employed in all further experiments. For comparative purposes, the anti-inflammatory effect of dexamethasone (Dex, 1 μM), was also investigated (*Fig 1B, D*). IL-8 and IL-6 release from 16HBE14o- cells pre-treated with Dex prior to LPS stimulation displayed significantly lower levels of each cytokine than cells treated with LPS alone ($p < 0.001$ for both cytokines, $n = 5$) or GA₃ and LPS ($p < 0.05$ for both cytokines, $n = 5$).

Our initial experiments in 16HBE14o- cells were confirmed in primary nasal epithelial cells (PNECs). PNECs were pre-treated with GA₃ (3-300 μM) for 1 h before LPS was added to the cultures for a further 24 h. GA₃ concentrations of 0.3–300 μM did not increase LDH release above the vehicle control, but 300 μM GA₃ caused a significant increase in LDH release compared to 0.3 μM and 3 μM ($p < 0.01$ and $p < 0.05$, respectively, $n = 6$; *Fig 2A*). In PNECs, LPS treatment significantly increased IL-8 and IL-6 release ($p < 0.05$ and $p < 0.01$ respectively, $n = 7$), (*Fig 2B-1* and *Fig 2C-1*), while GA₃ pre-treatment significantly reduced it ($p = 0.019$ and $p = 0.008$, Wilcoxon (paired t-test, $n = 7$) at 24 h (*Fig 2B-2* and *Fig 3C-2*).

GA₃ promotes an A20-driven, anti-inflammatory response in 16HBE14o- cells

LPS stimulation of 16HBE14o- cells resulted in the rapid and transient induction of A20 mRNA with peak expression observed after 1 h. Thereafter, A20 expression slowly declined and returned to basal levels 4 h after LPS stimulation (*Fig 3A*). This is consistent with the original findings of Opipari *et al.* (1990). GA₃ pre-treatment (30 μM) alone significantly induced A20 mRNA expression compared to culture medium control (1 h, $p < 0.05$). The subsequent addition of LPS further enhanced and prolonged the induction of A20 mRNA compared to culture medium control (1 h and 4 h, $p < 0.001$, 24 h, $p < 0.05$), LPS alone (4 h, $p < 0.05$) or GA₃ alone (1 h and 4 h, $p < 0.05$) and A20 expression remained above basal levels in GA₃ pre-treated cells even 24 h after LPS treatment (*Fig 3A*, $n = 5-9$). A20 mRNA expression was not induced in 16HBE14o- cells treated with Dex, prior to LPS stimulation (*Fig 3A*).

Furthermore, analyses of p65 mRNA expression showed a significant induction of p65 by LPS alone ($p < 0.001$, 4 h vs. Medium Control, $n = 5-9$, *Fig 3B*) and GA₃ pre-treated 16HBE14o- show a significant reduction of LPS-induced p65 ($p < 0.001$, 4 h vs. Medium Control, $n = 5-9$, *Fig 3B*), suggesting that GA₃ negatively affects the NF-κB pathway. Pre-treatment of 16HBE14o- cells with Dex also attenuated LPS-induced p65 expression; however, this effect was not significant (*Fig 3B*).

To investigate if the observed induction of A20 and the subsequent reduction in p65 by GA₃ is responsible for the anti-inflammatory effects outlined in *Fig 1B* and *2B-1*,

16HBE14o- cells were treated with siRNA targeting A20. *Fig 3C* shows that when A20 is silenced in 16HBE14o- cells, the anti-inflammatory effect of GA₃ on LPS-induced IL-8 release is lost ($p < 0.001$, $n = 5$, *Fig 3C*). These experiments suggest that GA₃ acts in an A20-dependent manner.

GA₃ induces A20 mRNA and protein in PNECs

To investigate the effect of GA₃ on A20 in PNECs, cells were pre-treated with GA₃ (30 μ M) for 1 h before the addition of LPS to the cultures and incubated for a further 24 h. In non-stimulated cells, A20 mRNA levels remained unchanged over the duration of the experiment (data not shown). When cells were stimulated with LPS for up to 24 h, A20 mRNA levels increased significantly over culture medium control ($p < 0.01$, $n = 7$) with a peak expression at 4 h (*Fig 4A*). GA₃ exposure alone induced A20 mRNA in PNECs in a time-dependent manner ($p < 0.05$, 24 h vs. culture medium control, $n = 7$, *Fig 4A*). In GA₃ pre-treated cells, LPS (10 μ g/ml) increased A20 mRNA levels significantly over medium control ($n = 7$ with $p < 0.001$ at 4 h and $p < 0.01$ at 24 h). GA₃ pre-treated and LPS stimulated PNECs also showed a significant increase in A20 mRNA compared to LPS alone at 4 h ($p < 0.05$, $n = 7$, *Fig 4A*). In Dex pre-treated and LPS stimulates 16HBE14o- no significant induction of A20 mRNA was observed ($n = 5$). A20 protein expression (24 h after LPS challenge) was determined by Western Blotting and quantified. In accordance with mRNA levels, A20 protein was induced by GA₃ in PNECs challenged with LPS ($p < 0.05$, compared to GA₃, $n = 6$, *Fig 4B*).

Using an antibody against the C-terminal end of A20 that has been previously used to identify A20 cleavage products (<http://www.novusbio.com/primary-antibodies/tnfaip3>). We show that GA₃-dependent induction of A20 in PNECs results in an up-regulation of the whole A20 protein: The representative blot and the quantification in *Fig 4C* ($n = 4$) shows C-terminal A20 protein bands with a molecular weight of the expected 80 kD (whole protein) and those with approximately 60 kD and 50 kD corresponding to C-terminal A20 fractions containing zinc finger 1-2 (~50 kD) and zinc finger 1-3 (~60 kD) (Klinkenberg *et al.* 2001). The antibody also detects a C-terminal A20 fraction of around 40 kD, although bands of this size were not detected in this study. Incubation with GA₃ in the presence or absence of LPS leads to an increase of all protein bands in similar proportions (*Fig 4C*). Only in GA₃ and LPS treated cells, the 60 kD band, corresponding to zinc finger 1 to 3, was significantly increased compared to control cells ($p < 0.05$, $n = 4$, *Fig 4C*).

GA₃ reduces LPS induced cytokine release via reduction of NF-κB (p65)

We and others have previously shown that IL-8 production in epithelial cells is largely dependent on p65 activation (Dommisch *et al.* 2010, Kelly *et al.* 2013b). To investigate if GA₃ affects NF-κB signalling, p65 mRNA was analysed 1, 4 and 24 h after LPS challenge in GA₃ pre-treated PNECs. LPS significantly induced p65 mRNA expression compared to culture medium control at 1 h and 4h ($p < 0.05$ and < 0.001 , $n = 7$). However, p65 mRNA was significantly reduced in GA₃ treated PNECs 4 h after LPS challenge ($p < 0.05$, $n = 7$) (*Fig 5A*). Dex pre-treatment of PNECs and subsequent LPS stimulation, similar to pre-treatment with GA₃, resulted in a significant reduction of p65 mRNA 4h after stimulation ($p < 0.05$, $n = 5$) (*Fig 5A*).

LPS induced NF-κB (p65) activation starts with the posttranslational modification e.g. by phosphorylation of p65 (phosphorylation on serine 536), in the cytosol and is then translocated into the nucleus (Hall *et al.* 2006). Having shown a reduction of p65 mRNA by GA₃ treatment, we further confirmed that GA₃ exerts its anti-inflammatory action via modulation of NF-κB activation. Firstly, the level of nuclear activated NF-κB (p65) was significantly induced by stimulation with LPS at 15 min returning to culture medium control levels at 24h ($p < 0.001$ and $p < 0.01$, $n = 5$, *Fig 6A*). GA₃ pre-treated and LPS stimulated 16HBE14o- showed significantly reduced nuclear active p65 at 15 min compared to LPS stimulation alone ($p < 0.05$, $n = 5$, *Fig 6A*). Dex pre-treatment also reduced p65 activity 15 min after LPS stimulation ($p < 0.01$, $n = 5$, *Fig 6A*).

Secondly, phosphorylation of p65 on serine 536 was determined in whole cells (cytosolic and nuclear) extracts in 16HBE14o- by Western Blotting. Our results indicate a reduction of the phosphorylation on serine 535 in GA₃ pre-treated cells for up to 8 h after LPS challenge (*Fig 6B*).

Finally, we investigated key events in the pathway upstream of p65 in 16HBE14o- cells. LPS-mediated activation of NF-κB is reliant on the activation and subsequent degradation of IκBα. We determined cytosolic IκBα protein expression in PNECs and show that LPS treatment significantly reduces cytosolic IκBα ($p < 0.05$), while treatment with GA₃ restored in part IκBα protein levels ($p = 0.05$) (all $n = 6$, *Fig 6C*).

Overall these results confirm an anti-inflammatory effect of GA₃ in LPS stimulated airway epithelial cells. This appears to be mediated, in part at least, by the induction of A20 (mRNA and protein) and subsequent inhibition of key stages in the NF-κB pathway.

DISCUSSION

The cytoplasmic zinc finger protein A20 is a central negative regulator of NF-κB that governs multiple intracellular pathways. Here, we report that pre-incubation of LPS-stimulated airway epithelial cells (16HBE14o- cells and PNECs) with GA₃, significantly induces A20 mRNA and protein. Although GA₃ alone also induced A20 mRNA, this did not result in a detectable increase in protein, suggesting that GA₃ mainly enhances LPS-induced A20 expression. Maximal induction of mRNA was slightly delayed in PNECs compared to 16HBE14o- cells (1 h vs. 4 h), which could be explained by the differences in the cell types (16HBE14o- is a SV immortalized cell line (Kelly *et al.* 2013c), whereas PNECs are primary cells cultured for up to 3 passages). Furthermore, the induction of A20 was accompanied by a significant increase in IκBα levels and subsequent reduction in NF-κB expression and IL-6 and IL-8 release in both cell lines and primary epithelial cells. Silencing of A20 confirmed that the reduction in IL-8 release was A20 dependent.

El-Mofty *et al.* suggested that GA₃ had carcinogenic effects and lead to tumour formation in Swiss Albino mice (El-Mofty *et al.* 1994). However, we tested GA₃ at a range of concentrations from 0-300 μM and found no evidence of cellular toxicity or increased proliferation even at 3 or 30 μM (Figure S1, Supplement). The proliferation assay was conducted over 72 h to ensure at least one complete cell cycle had taken place. In our hands the doubling time of 16HBE14o- cells is approximately 28 h.

Our results are in line with data by Kasamatsu *et al.* (2012), who showed that incubating adipose-derived stem cells with GA₃ (1 mM) did not alter cell morphology or viability. Early work by Miklussák *et al.* (1980) suggested that administration significantly improves metabolic functions in patients with lung cancer. More recently, Zhang *et al.* (2012) showed that the gibberellin derivative 3-chlorine-3,15-dioxy-gibberellic acid methyl ester (GA-13315), possessed potent antitumor and antiangiogenic activity *in vitro* and *in vivo*.

LPS activation of the NF- κ B pathway is dependent on the ubiquitination and subsequent proteasomal degradation of TRAF6 (Mabilleau *et al.* 2011). This process frees bound signalling molecules for subsequent downstream events including the activation of NEMO, degradation of cytosolic I κ B and translocation of NF- κ B subunits to the nucleus. NF- κ B is sequestered in the cytoplasm, bound by members of the I κ B family of inhibitor proteins, which includes I κ B α . IKK phosphorylates I κ B α , resulting in its K48-linked ubiquitination and subsequent proteasomal degradation. This then allows translocation of the NF- κ B subunits p65/p50 to the nucleus to activate target genes. To address where GA₃ acts in the NF- κ B pathway, we show a significant reduction in cytosolic I κ B α protein in LPS stimulated cells, suggesting that it has been degraded to release the NF- κ B subunits to the nucleus. GA₃ treatment inhibits the LPS induced degradation of I κ B α , which may explain the anti-inflammatory effect seen (reduced IL-8 and IL-6 release and p65 mRNA). Furthermore, we show that GA₃ treatment leads to reduced p65 nuclear activity (*Fig 6A*) and this is associated with decreased phosphorylation of p65 on serine 536 (*Fig 6B*).

Information about the induction of A20 is still controversial. A20 has been described as being NF- κ B dependent, and da Silva *et al.* (2012a) showed that this activation of the ‘anti-inflammatory arm of NF- κ B’ is associated with reduced expression of phosphorylated (ser536) p65 compared to the induction of phosphorylated (ser536) p65 by TNF α alone. Similarly, we show here that in bronchial epithelial cells the induction of A20 by GA₃ pre-treatment leads to a reduction in the expression of phosphorylated (ser536) p65 compared to LPS stimulated cells (*Fig. 6B*) suggesting that the phosphorylation of p65 – e.g. by IKK kinases (Hall *et al.* 2006) – could be a target of A20. Our data on p65 nuclear activity (*Fig. 6A*) may suggest that GA₃ itself induces low levels of NF- κ B (although not statistically significant), but further phosphorylation states or DNA binding sites of p65 would need to be investigated.

Finally, the A20 protein consists of an N-terminal OTU (Ovarian tumour) domain and a C-terminal domain that consists of 7 Cys-Cys zinc fingers. Work by Evans *et al.* (2004) assigned the DUB (deubiquitination) activity to the OTU domain whereas the anti-inflammatory activity was in the zinc finger domain (especially zinc finger 4 and 7 have been identified to exhibit the anti-inflammatory effect of A20 (O’Reilly and Moynagh 2003, Dommisch *et al.* 2010, Tokunaga *et al.* 2012). Furthermore, De Valck *et al.* (1996) showed

that A20 zinc fingers have the ability to self-assemble to build the whole A20 zinc finger domain. To investigate if GA₃ can induce the whole A20 protein we used two antibodies against full length A20 and the C-terminal domain specifically (O'Reilly and Moynagh 2003, Dommisch *et al.* 2010). Our data suggest that in PNECs, incubation with GA₃ in the presence or absence of LPS leads to an increase of all protein bands in similar proportions representing the whole protein and the zinc finger domain of the A20 protein. However, in cells treated with GA₃ and LPS the 60 kD fraction appeared to be increased compared to unstimulated cells. The 60 kD band has been described as zinc finger (ZF) 1-3 (~60 kD) (Klinkenberg *et al.* 2001) and our findings are in accordance with the description for this commercial A20 antibody in THP1 cells (<http://www.novusbio.com/primary-antibodies/tnfaip3>). ZF 4 has been shown to facilitate the NF- κ B reducing anti-inflammatory action of A20 (Wertz *et al.* 2004; Lu *et al.* 2013), but mice with a non-functional ZF4 showed that this domain might not be needed for the anti-inflammatory function of A20. In T cells MALT1 cleaves A20 into a 37 kD and a 50 kD fraction (McAllister-Lucas and Lucas 2008; Malinverni *et al.* 2010), but to date this 60 kD band has not been further identified.

The current study has some limitations: Firstly, the effect of GA₃ on inflammatory mediators and the mechanism of its anti-inflammatory action have been investigated in cell culture, which may also impose several limitations. In most experiments we used an immortalised cell line (16HBE14o-). A characteristic of these cells is that they release IL-8 and IL-6 throughout proliferation. This may explain why cytokine levels are already relatively high in untreated cells. Furthermore, compared to macrophages or monocytic cell lines, relatively high amounts of LPS are needed to stimulate airway epithelial cells and this is true for cell lines and primary cells alike. Another limitation of our study is that the effects of GA₃ on p65 and I κ B α – although associated with A20 induction – have not been further validated using siRNA experiments. However, we do show that the anti-inflammatory effect of GA₃ is directly dependent on the induction of A20, as GA₃ has no effect on the release of inflammatory mediators in cells lacking A20 (knock down of A20 via siRNA, *Fig. 3C*). In PNECs, the number of experiments is limited by the low cell number available from nasal brushings and the fact that PNECs can only be expanded up to passage 3. Due to the lack of PNECs our investigation of induction of zinc finger fractions was only performed four times, which may lead to an imperfect statistical estimation. Many products are known to contain trace amounts of LPS and small amounts of LPS may desensitize to a subsequent challenge.

The GA₃ preparation used in this study passes the tests for plant cell culture applications (Sigma), but has not been specifically tested for its LPS content. However, determination of TLR4 mRNA in GA₃ treated cells shows no change in the expression of TLR4 while in LPS stimulated cells TLR4 shows a time-dependent upregulation (*Fig. S2*, Supplement). Overall this may suggest that the GA₃ preparation lacks TLR4 stimulating endotoxins. Finally, future work is required to determine if GA₃ reduces pro-inflammatory cytokine secretion when cells are treated after LPS challenge.

In summary, we describe that pre-incubation of airway epithelial cell lines and primary nasal epithelial cells with GA₃ (single bolus of 30 µM) induces the NF-κB regulator A20 at mRNA and protein level thereby exerting an anti-inflammatory effect upon subsequent LPS stimulation. Our work provides molecular evidence (i) for A20 as a therapeutic target in inflammatory diseases and (ii) for the plant diterpenoid gibberellin in reducing NF-κB driven inflammatory responses. Gibberellin or gibberellin-derived derivatives could be developed into anti-inflammatory drugs for the potential treatment of chronic inflammatory diseases associated with A20 dysfunction such as salivary gland degeneration (Kasamatsu *et al.* 2012), liver degeneration (da Silva *et al.* 2012b), bone resorption associated with inflammatory diseases (Shimada *et al.* 2008, Mabileau *et al.* 2011) and psoriasis (Gon *et al.* 2004, Kelly *et al.* 2012b). Furthermore, the concept of pharmacological induction of A20 presents A20 as a potential drug target to reduce or normalise the inflammatory response in chronic inflammatory airway diseases such as CF, asthma and COPD. Further work will investigate GA₃ in primary cells from patients with chronic airways disease.

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CONFLICTS OF INTEREST STATEMENT

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1: Effect of GA₃ on LDH and cytokine release from the bronchial epithelial cell line 16HBE41o-

(A) 16HBE14o- cells were treated with GA₃ for 24 h and LDH release into the culture medium determined. At 0.03, 0.3, 3 and 30 μ M, GA₃ did not cause any significant LDH release, while 300 μ M caused a significantly higher LDH release (blue bars) when compared to vehicle controls (grey bar) ($p < 0.001$, $n = 6$). (B) 16HBE14o- bronchial epithelial cells were treated with GA₃ (0.03 – 300 μ M, 1 h), or Dexamethasone [Dex, 1 μ M, 1 h]. LPS [10 μ g/ml] was subsequently added to the cultures. Interleukin (IL)-8 release was determined by ELISA after 24 h. GA₃ (3-300 μ M) and Dex [1 μ M] pre-treatment in LPS stimulated 16HBE14o- caused a significant reduction in IL-8 release ($p < 0.01$, $n = 5-9$). (C) The maximal effective concentration to reduce IL-8 (by 47%) was 28.19 μ M, while a reduction of 40% was achieved at 2.82 μ M (all green squares). (D) 16HBE14o- cells were treated with GA₃ (0.03 - 30 μ M, 1 h), or Dexamethasone [Dex, 1 μ M, 1 h] prior to LPS exposure [10 μ g/ml]. IL-6 release was determined by ELISA after 24 h. GA₃ [30 μ M] and Dex [1 μ M] pre-treatment in LPS stimulated 16HBE14o- caused a significant reduction in IL-6 release ($p < 0.01$, $n = 5-8$). For figure B and D: medium control (grey), GA₃ (blue), LPS (red), GA₃+ LPS (purple), Dex (green)).

Figure 2: Effect of GA₃ on LDH and cytokine release from primary nasal epithelial cells (PNECs)

(A) PNECs were pre-treated with GA₃ (3 - 300 μ M for 1 h) before LPS (10 μ g/ml) was added to the cultures for 0-24h. Analysis of LDH in the supernatants showed no significant increase in LDH release (blue bars) above the vehicle control (grey bar) as a result of treatment with GA₃. (B-1) LPS treatment significantly increased IL-8 release ($p < 0.05$, $n = 8$). (B-2) GA₃ pre-treatment [30 μ M, 1h] significantly reduced LPS-induced IL-8 release in PNECs ($p < 0.05$, $n = 8$) at 24 h. Consistently, (C-1) LPS treatment significantly increased IL-6 release ($p < 0.05$, $n = 7$), while, (C-2) GA₃ [30 μ M, 1h] pre-treatment significantly reduced LPS-induced IL-6 release in PNECs ($p < 0.05$, $n = 7$) after 24h exposure. For figure B and C: medium control (grey), GA₃ (blue), LPS (red), GA₃+ LPS (purple)).

Figure 3: Anti-inflammatory effect of GA₃ in 16HBE14o- cells is A20 dependent.

(A) 16HBE14o- bronchial epithelial cells were pre-treated with LPS [10 µg/ml] alone for 24 h, or with GA₃ [30 µM, 1h] or Dexamethasone [Dex, 1 µM, 1 h] prior to the addition of LPS [10 µg/ml] for a further 24 h, and A20 mRNA was determined by qPCR. Both LPS and GA₃ treatment significantly induced A20 mRNA in 16HBE14o- cells ($p < 0.01 - 0.001$, $n = 5-9$). (B) p65 mRNA was significantly increased 4 h after LPS treatment (10 µg/ml, $p < 0.001$, $n = 5-9$). This effect was significantly attenuated when cells were pre-treated with GA₃ [30 µM, 1h] ($p < 0.001$, $n = 5-9$). For figure A and B: medium control (grey), GA₃ (blue), LPS (red), GA₃+LPS (purple), Dex (green)). (C) However, when A20 was silenced with siRNA, the anti-inflammatory effect of GA₃ (determined by IL-8 release of LPS stimulated cells (24 h)) is lost ($p < 0.001$, $n = 5$, Fig 3C).

Figure 4: GA₃ induces A20 mRNA and protein in PNECs

PNECs were pre-treated with LPS alone [10 µg/ml], or pre-treated with GA₃ [30 µM, 1 h] or Dexamethasone [Dex, 1 µM, 1 h] prior to the addition of LPS [10 µg/ml] to the cultures for 0-24 h and A20 mRNA and protein expression investigated. GA₃ (blue bars) induced (A) A20 mRNA in PNECs in a time-dependent manner, with a significant increase at 24 h ($n = 7$, $p < 0.05$: 24h vs. Medium Control (grey bar)). When cells were stimulated with LPS [10 µg/ml] for up to 24 h (red bars), A20 mRNA levels increased significantly over Medium Control (grey bar) ($p < 0.05$ and $p < 0.01$, $n = 7$). In GA₃ [30 µM] pre-treated cells, LPS [10 µg/ml] increased A20 mRNA levels significantly (purple bar) over LPS alone (red bar) ($p < 0.05$, $n = 7$). Pre-treatment with Dex (green bar) did not significantly induce A20 mRNA ($n = 5$). (B) Western Blot analysis confirmed an increase in A20 protein expression when cells were pre-treated with GA₃ and then challenged with LPS (purple bar) ($p < 0.05$, $n = 6$, over GA₃ alone (blue bar)). (C) Representative blot and quantification of Western Blots after use of IMG-161A, an antibody against the C-terminal end of A20 that can identify several zinc fingers. Incubation with GA₃ in the presence or absence of LPS leads to an increase of all protein bands in similar proportions with the highest increase in the 50kD (zinc finger 1-2) band compared to medium control or LPS alone. Only in GA₃ pre-treated and LPS treated cells the 60 kD band, corresponding to zinc finger 1 to 3, was significantly increased compared to control cells ($p < 0.05$, $n = 4$, Fig 4C).

Figure 5: GA₃ suppresses p65 expression in PNECs

PNECs were treated with LPS alone [10 µg/ml], or pre-treated with GA₃ [30 µM, 1 h] or Dexamethasone [Dex, 1 µM, 1h] prior to the addition of LPS [10 µg/ml] for 1, 4 or 24 h. LPS-induced p65 expression (mRNA) was subsequently measured by qPCR and found to be significantly reduced in GA₃ treated PNECs 4 h after LPS challenge (p<0.05, n=5-7). Medium control (grey), GA₃ (blue), LPS (red), GA₃+ LPS (purple), Dex (green)).

Figure 6: Effect of GA₃ is associated with reduced p65 and increased cytosolic IκBα

(A) LPS induced activation of nuclear p65 was significantly reduced in GA₃ pre-treated PNECs 4 h after LPS challenge (p<0.05, n=5). Medium control (grey), GA₃ (blue), LPS (red), GA₃+ LPS (purple), Dex (green)). (B) Phosphorylation of p65 on serine 536 was determined in whole cells extracts in 16HBE14o- by Western Blotting. Results indicate a reduction of the phosphorylation on serine535 in GA₃ pre-treated cells for up to 8 h after LPS challenge. (C) Representative Blot and quantification of IκBα protein expression in GA₃ pre-treated PNECs. LPS treatment (24 h) significantly reduces cytosolic IκBα compared to untreated and GA₃ treated cells (p<0.05 and p<0.001, n=6). Pre-treatment with GA₃ (1h before addition of LPS for 24h) restores in part cytosolic IκBα protein levels (p<0.05, n=6). Medium control (grey), GA₃ (blue), LPS (red), GA₃+ LPS (purple)).