PKC Activation Induces Inflammatory Response and Cell Death in Human Bronchial Epithelial Cells

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Abstract
A variety of airborne pathogens can induce inflammatory responses in airway epithelial cells, which is a crucial component of host defense. However, excessive inflammatory responses and chronic inflammation also contribute to different diseases of the respiratory system. We hypothesized that the activation of protein kinase C (PKC) is one of the essential mechanisms of inflammatory response in airway epithelial cells. In the present study, we stimulated human bronchial lung epithelial (BEAS-2B) cells with the phorbol ester Phorbol 12, 13-dibutyrate (PDBu), and examined gene expression profile using microarrays. Microarray analysis suggests that PKC activation induced dramatic changes in gene expression related to multiple cellular functions. The top two interaction networks generated from these changes were centered on NFκB and TNF-α, which are two commonly known pathways for cell death and inflammation. Subsequent tests confirmed the decrease in cell viability and an increase in the production of various cytokines. Interestingly, each of the increased cytokines was differentially regulated at mRNA and/or protein levels by different sub-classes of PKC isozymes. We conclude that pathological cell death and cytokine production in airway epithelial cells in various situations may be mediated through PKC related signaling pathways. These findings suggest that PKCs can be new targets for treatment of lung diseases.

Introduction
Airway epithelial cells are in the front line of host defence against various airborne pathogens, allergens, and irritants [1]. Airway epithelial functions as a physical barrier with secretion and clearance functions. In addition, airway epithelial cells can produce cytokines and chemokines to initiate local inflammatory and immune response. After airway injury, epithelial cell migration occurs as an early mechanism of repair, and this is mediated by cytokines and growth factors. Subsequent cell proliferation and differentiation restore the damaged epithelium [2]. Malfunction of cellular responses may lead to various chronic inflammatory diseases, such as asthma and chronic obstructive pulmonary disease [1].

The inflammatory responses of airway epithelial cells are an important component in innate immunity. However, excessive inflammatory responses can lead to cell death and tissue damage, and ultimately, chronic inflammation may contribute to the pathogenesis of airway diseases. Thus, inflammatory responses require precise regulation mediated by multiple intracellular signal transduction pathways [3].

Protein Kinase C (PKC) is a well-known family of homologous serine threonine kinases with a prominent role in many cellular functions. A total of 15 isozymes of PKC have been identified, and they are subsequently classified into 3 general subfamilies depending on their specific mode of activation: classical, novel, and atypical [4]. Pulmonary insult by various harmful substances can lead to activation of multiple PKC subtypes in airway epithelial cells. For example, in pulmonary epithelial cells exposed to asbestos, a carcinogen, PKCα is activated and translocated to the nucleus [5]. One study suggests that cell death induced by asbestos is PKCα-dependent [6]. PKC activation can induce dramatic morphological changes of human bronchial epithelial cells that lead to podosome formation, which is followed by secretion of matrix metalloproteases and alteration in cell motility [7,8]. Cigarette smoking induces IL-8 production and inhibition of ciliary motility in airway epithelial cells via PKC activation [9,10]. PKCα expression is noticeably high in the airway of COPD patients [11]. In the case of asthma, β-adrenergic receptor expression is increased by IL-1β stimulation and this is mediated by PKC [12].

These experimental and clinical observations suggest that PKC activation could be one of the essential mechanisms regulating the response of airway epithelial cells when they are stimulated by a variety of injurious factors. In this study, we hypothesized that PKC activation has profound effects on human airway epithelial cells through gene expression, cell proliferation, cell survival, motility, and especially the activation of inflammatory responses. We examined the effect of direct PKC activation in airway epithelial cells on gene expression using microarray, and further studied its impacts on the cell death and production of inflammatory mediators at both gene and protein levels.
Figure 1. PKC activation-induced gene expression profiles. (A) PCA analysis clearly separates the three groups: control (red), 0.5 hours of PDBu stimulation (blue), and 4 hours of PDBu stimulation (green). (B) Venn diagram shows the differentially regulated genes between the two time points. (C) Hierarchical clustering analysis of differentially expressed genes automatically segregated the three groups, and most of the genes were up-regulated by PKC activation.

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Table 1. The top 20 genes that are significantly changed by 0.5 h PDBu treatment.

| gene name                                         | Gene Symbol | RefSeq   | p-value | Fold-Change |
|---------------------------------------------------|-------------|----------|---------|-------------|
| early growth response 1                           | EGR1        | NM_001964| 0.0003  | 12.768      |
| early growth response 3                           | EGR3        | NM_004430| 0.0065  | 8.316       |
| early growth response 2                           | EGR2        | NM_000399| 0.0006  | 8.212       |
| murine osteosarcoma viral oncogene homolog        | FOS         | NM_005252| 0.0020  | 6.765       |
| prostaglandin-endoperoxide synthase 2             | PTGS2       | NM_000963| 0.0006  | 5.153       |
| zinc finger protein 36, C3H type, homolog         | ZFP36       | NM_003407| 0.0003  | 4.031       |
| chemokine (C-X-C motif) ligand 2                  | CXCL2       | NM_002089| 0.0056  | 3.461       |
| interleukin 8                                     | IL8         | NM_000584| 0.0044  | 3.352       |
| dual specificity phosphatase 1                    | DUSP1       | NM_004417| 0.0044  | 3.193       |
| interleukin 6 (interferon, beta 2)                | IL6         | NM_006000| 0.0069  | 3.151       |
| hairy and enhancer of split 1                     | HES1        | NM_005524| 0.0011  | 3.143       |
| immediate early response 2                        | IER2        | NM_004907| 0.0069  | 3.130       |
| transmembrane protein 88                          | TMEM88      | NM_003411| 0.0034  | 3.104       |
| jun B proto-oncogene                              | JUNB        | NM_002229| 0.0003  | 3.099       |
| dual specificity phosphatase 5                    | DUSP5       | NM_004419| 0.0001  | 2.967       |
| pentraxin-related gene                            | PTX3        | NM_002852| 0.0011  | 2.960       |
| nuclear factor of kappa light polypeptide gene enhancer | NFKBIZ   | NM_031419| 0.0020  | 2.906       |
| chemokine (C-X-C motif) ligand 1                  | CXCL1       | NM_001511| 0.0069  | 2.860       |
| FBJ murine osteosarcoma viral oncogene homolog B  | FOSB        | NM_006732| 0.0087  | 2.821       |
| dual specificity phosphatase 6                    | DUSP6       | NM_001946| 0.0072  | 2.593       |

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PKC Activation and Inflammatory Responses

Downregulated

ANKRD1

BDNF

FIGN

PIK3C2B

RAB30

Upregulated

IL-1a

IL-1b

IL-8

EREG

MMP-9
Materials and Methods

Cell line and reagents
Human bronchial epithelial cell line (BEAS-2B) was obtained from ATCC (Manassas, VA) [7,8,13]. Cells are cultured in low-glucose Dulbecco's modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA) [14]. Cells were grown at 37°C with 5% CO2. Phorbol 12,13-dibutyrate (PDBu), a PKC activator, was purchased from Sigma Aldrich (St. Louis, MO). Bisindolylmaleimide I (BIM-1), Ro-31-8220, GO6976, and rottlerin were purchased from EMD Bioscience (Darmstadt, Germany) [7].

SiRNA
PKCβ1, PKCβ2, and scramble siRNA (Santa Cruz Biotechnologies, Santa Cruz, CA) were transfected into BEAS-2B cells using Oligofectamine (Invitrogen, Carlsbad, CA), as we previously described [7,13]. Cells were plated in 6 well plates at concentration of 150,000 cells per well. Each of the wells had 50 nM of siRNA and 10 μM of Oligofectamine, diluted in optiMEM. After the transfection, cells were incubated for 24 hours, washed and incubated again in DMEM with 10% fetal bovine serum for another 48 hours before PDBu stimulation.

Microarray
The mRNA expression profile in BEAS-2B cells was investigated with microarray, as we previously described [15]. Three groups were prepared: control, 0.5 hour of PDBu stimulation, and 4 hours of PDBu stimulation. Each group consisted of three biological replicates. RNeasy kit (Qiagen, Valencia, CA) was used to extract total RNA. High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, CA) and PTC-100™ Programmable Thermal controller (MJ Research Inc., Watertown, MA) were used to synthesize cDNA. The RNA Integrity Number (RIN) was determined by Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA). The microarray was Human Gene ST 1.0, containing 28,132 probe sets from Affymetrix (Santa Clara, CA). Affymetrix CEL files were imported and analyzed using Partek software (Partek Inc., St. Louis, MO). Microarray data were pre-processed using the robust multi-array average (RMA) technique. Principle Component Analysis (PCA), Hierarchical cluster analysis, and differential expression analysis (ANOVA) were performed in Partek. Benjamini-Hochberg false discovery rate adjustment was used to correct for multiple testing. Interaction networks were generated using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Inc., Redwood City, CA). Gene-annotation enrichment analysis was performed using DAVID Functional Annotation bioinformatics analysis (National Institute of Allergy and Infectious Diseases, NIH). The microarray data has been deposited in the Gene Expression Omnibus (GEO) repository (accession number GSE44747).

Cell viability assay
BEAS-2B cells were cultured in a 6-well plate at 300,000 cells per well for 24 hours. The cells were then treated with or without PDBu, trypsinized and resuspended in the culture medium. Viable cells were counted with a Sceptor 2.0 Handheld Automated Cell Counter (EMD Millipore, Billerica, MA).

Quantitative RT-PCR
The primers used for quantitative reverse transcription were as shown in Table S1. The total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA). qRT-PCR was performed with 2xQuantiTect SYBR Green PCR kit (Qiagen, Mississauga, Canada) on LightCycler480 (Roche, Mannheim, Germany) as previously described [15–17]. Each assay had a standard curve of five serial dilutions and a no-template negative control.

Measurement of soluble cytokines and chemokines in culture medium
After cells were stimulated with PDBu for 0.5 or 4 hours, culture medium was collected, centrifuged at 10,000 rpm, and the supernatant was stored at −80°C. Cytokines, chemokines and growth factors in the culture medium were quantified using MILLIPLEX MAP Human Cytokine/Chemokine - Premixed 42 Plex (Category number: MPMXHCYT060KMX42). Four replicates were tested in each of the groups. Concentrations of IL-6, IL-8, G-CSF, and GRO-α in culture medium were also quantified with DuoSet ELISA systems (R&D Systems, Minneapolis, MN). The medium was diluted either by 1:5 or by 1:10 to fit the standard curve of the kit. The assay was performed following the manufacturer’s instructions.

Western Blots and PKC Translocation assays
These experiments were performed according to the standard protocols as we previously described [7,14,18].

Statistical tests
Student-t test and analysis of variance (ANOVA) with linear contrasts, followed by post hoc analysis (Tukey’s range test), were used to compare the means of two or more groups. The significance cutoff was set to p≤0.05. The values are denoted as mean ± S.D. GraphPad Prism 5 software was used to calculate statistics and produce graphs.

Results
PKC activation results in significant changes in gene expression profile
To determine the overall effects of PKC activation on human bronchial epithelial cells, we first stimulated BEAS-2B cells with PDBu (500 nM, a dose selected from our previous studies) [7,8]. PDBu treatment induced translocation of PKCα from cytosol to membrane fractions, and increased phosphorylation of other PKC isoforms in a time-dependent manner (Figure S1). Overall gene expression changes by PKC activation were examined using microarray. We analyzed differences between three groups: control (no PDBu stimulation), 0.5 hours and 4 hours of PDBu stimulation. Principle Component Analysis (PCA) demonstrated that each of the groups is clearly distinct from each other (Figure 1A). We used ANOVA with linear contrasts for analysis of differential gene expression. We defined genes as significantly changed by false discovery rate (FDR) less than or equal to 5% and fold-change greater than 1.3. Upon 0.5 hours of PKC activation, 48 genes were significantly up-regulated, and they are mainly early response genes (e.g. Early growth response 1, 2, and 3, IER2, Fos,
JunB, FosB), and cytokines (e.g. CXCL2, IL-8, IL-6, PTX3, CXCL1) (Table 1). Furthermore, 532 genes were significantly changed after 4 hours of the PKC activation. The Venn-diagram shown in Figure 1B indicates that 34 of the 48 changed genes after 0.5 hours remained significantly changed after 4 hours. Hierarchical cluster analysis further demonstrates that PKC activation has a significant impact on the gene expression profile in a time-dependent manner (Figure 1C). Most of the genes modulated after 4 hours of PDBu stimulation were also up-regulated, as shown in the heat map (Figure 1C). Five significantly up-regulated and down-regulated genes indicated by microarray were validated with RT-PCR. All of them showed similar changes between RT-PCR and microarray data (Figure 2). The top 20 most significantly changed genes after 4 hours of PDBu stimulation are listed in Table 2. Overall, PKC activation induced significant changes in the gene expression profile.

PKC activation modulates genes involved in pathways related to multiple functions, especially inflammatory response and cell death

We then performed DAVID Functional Annotation bioinformatics analysis on genes regulated by 4 hours of PDBu stimulation. The top ten functional terms are shown in Table 3. The data was further investigated via Ingenuity Pathway Analysis (IPA). The top five biologically functional networks included (1) cell death, cellular growth, and proliferation, (2) gene expression, (3) cell cycle

### Table 2. The top 20 genes that are significantly changed by 4 h PDBu treatment.

| Gene name                 | Gene Symbol | RefSeq    | p-value     | Fold-Change |
|---------------------------|-------------|-----------|-------------|-------------|
| Epiregulin                | EREG        | NM_001432 | 3.96E-06    | 14.33       |
| matrix metallopeptidase 9 | MMP9        | NM_004994 | 6.72E-06    | 12.97       |
| solute carrier family 16, member 6 | SLC16A6   | NM_004694 | 2.24E-05    | 10.47       |
| prostaglandin-endoperoxide synthase 2 | PTGS2      | NM_000963 | 2.28E-05    | 10.18       |
| leukemia inhibitory factor | LIF         | NM_002309 | 3.31E-06    | 10.00       |
| dual specificity phosphatase 6 | DUSP6      | NM_001946 | 4.24E-05    | 9.36        |
| glutamine-fructose-6-phosphate transaminase 2 | GFPT2      | NM_005110 | 1.67E-05    | 8.76        |
| MST131                    | MST131      | ENST00000423322 | 4.74E-05 | 8.18       |
| interleukin 1, alpha      | IL1A        | NM_000575 | 6.95E-06    | 8.14        |
| interleukin 1, beta       | IL1B        | NM_000576 | 1.27E-05    | 8.07        |
| interleukin 8              | IL8         | NM_000584 | 7.06E-05    | 8.00        |
| chemokine (C-C motif) ligand 2 | CCL2       | NM_002982 | 3.58E-04    | 7.68        |
| serpin peptidase inhibitor, clade B | SERPINB2 | NM_00143818 | 2.97E-06 | 7.61       |
| amphiregulin               | AREG        | NM_001657 | 9.60E-05    | 7.38        |
| inhibit, beta A            | INHBA       | NM_002192 | 1.25E-05    | 6.88        |
| metastasis suppressor 1    | MTSS1       | NM_014751 | 3.20E-05    | 6.86        |
| ankyrin repeat domain 1    | ANKRD1      | NM_014391 | 1.59E-04    | -6.65       |
| endothelial cell-specific molecule 1 | ESM1 | NM_007036 | 3.20E-05 | 6.51       |
| colony stimulating factor 2 | (granulocyte-macrophage) | CSF2 | NM_000758 | 1.02E-03 | 6.48 |
| early growth response 1    | EGR1        | NM_001964 | 5.31E-05    | 6.45        |

### Table 3. DAVID Annotation showing top 10 enriched functional terms associated with PKC activation.

| Term                                      | Count | p value (Benjamini) |
|-------------------------------------------|-------|---------------------|
| regulation of apoptosis                   | 62    | 3.69E-12            |
| response to wounding                      | 49    | 1.99E-12            |
| regulation of cell proliferation          | 58    | 5.53E-11            |
| regulation of phosphate metabolic process | 43    | 3.79E-10            |
| regulation of cytokine production         | 26    | 8.18E-10            |
| positive regulation of cellular biosynthetic process | 51    | 1.06E-09            |
| regulation of phosphorylation             | 41    | 1.23E-09            |
| positive regulation of biosynthetic process | 51    | 1.44E-09            |
| anti-apoptosis                            | 27    | 1.54E-09            |
| positive regulation of developmental process | 31    | 1.89E-09            |
PKC activation and Inflammatory Responses

PKC activation induces cell death in human lung epithelial cells

Both functional annotation enrichment and interaction networks suggest that activation of PKC leads to the up-regulation of pathways related to inflammatory response and cell death. We further analyzed whether PKC activation can induce cell death in BEAS-2B cells, as inferred by the microarray data analysis. When the cells were treated with PDBu for 4 hours, the level of cleaved caspase-3, activated effector caspase, was significantly increased (Figure 4A). We further treated cells with PDBu and counted viable cells using an automatic cell counter. While PDBu treatment did not affect cell viability within 4 hours, it induced a significant reduction in cell viability after 6 hours (Figure 4B).

PKC activation increases the gene expression and secretion of multiple cytokines in human lung epithelial cells

Microarray analysis suggested that the activation of inflammatory response and cytokine production occurs by PKC activation. Thus, we investigated the changes in production of chemokines and cytokines in BEAS-2B cells after PDBu treatment. To determine the overall cytokine profile, the cell-culture medium was screened with cytometric bead array (CBA) that simultaneously detects 42 human cytokines. Among them, 23 cytokines were detected in the culture medium of BEAS-2B cells under control condition (Figure 5A). PDBu stimulation increased production of cytokines at 0.5 hours, and many of them further increased after 4 hours. Fifteen of the 23 cytokines were significantly increased after 4 hours (Figure 5B).

We then compared the microarray analysis with the cytokine profile data from CBA, to determine whether the cytokines and chemokine were modulated at the mRNA and/or protein levels. We found that G-CSF, GM-CSF, GRO-1, IL-6, IL-8, TGFβ, TGFB, TNFα, TNFβ, VEGF, MIP-1β, and PDGF-αα had increased both in mRNA and in protein levels in the culture medium after 4 hours of PDBu stimulation. This suggests that the cytokines were both synthesized and released following PKC activation. In contrast, Fractalkine, IFNγ2, IFNγ, and IL-7 were increased in protein level, but not in mRNA level, suggesting that these cytokines were released in response to the PKC activation, but their gene expression was not increased upon PKC activation. Finally, FGF, IL-1α, IL-1β, and MIP-2 were significantly up-regulated in mRNA level, but were not elevated at protein levels. This suggests that the gene expression of these cytokines is up-regulated by PKC activation, but the protein synthesis and/or release may be PKC-independent. These finding are summarized in Figure 5C.

PKC activation induced production of cytokines may be mediated by different sub-class of PKC isozymes

Both classical and novel PKC isozymes can be activated upon PDBu stimulation. To further dissect which subgroups of the isozymes are responsible for cytokine release, we incubated cells with PKC inhibitors 1 hour prior to PDBu stimulation. Bisindoyl-maleimide-1 (Bim-1, 1 μM) and Ro31-8220 (1 μM) are pan PKC inhibitors, while GÖ6986 (1 μM) and rottlerin (1 μM) are specific classical and novel PKC inhibitors, respectively.

All of the cytokines whose production is increased after PDBu stimulation were effectively inhibited by pan PKC inhibitors. G-CSF was significantly reduced by GÖ6986, suggesting that classical PKC isozymes are involved in regulating its production (Figure 6A). GRO-1α and IL-7 were significantly blocked by rottlerin, suggesting that their production is regulated by novel PKCs (Figure 6B). IFNγ2 and IL-6 were effectively inhibited by either classical or novel inhibitor, suggesting both of the PKC types are involved in the regulation of their production in a

Figure 3. Top two IPA interaction networks suggested by microarray are NFκB and TNF-α. Top two interaction networks generated by Ingenuity Pathway Analysis from significantly regulated genes after 4 hours of PDBu stimulation. The networks were centered on NFκB (A) and TNF-α (B). Both of these networks are closely related to inflammation and cell death.

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Figure 4. PKC activation induces cell death in lung epithelial cells. (A) The cleaved form of Caspase-3 is significantly increased after 4 hours of PDBu stimulation. (B) The number of live cells was decreased significantly after 6 hours.

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cooperative fashion (Figure 6C). In contrast, neither the classical
nor the novel PKC inhibitor alone significantly reduced Fractalkine
and IL-8 levels (Figure 6D), suggesting that both subclasses
may independently regulate their production, thus, complete
blockage requires blocking of both pathways. Figure 6E summa-
rizes these findings. To confirm the results from cytometric beads,
we then measured a few of the cytokines with ELISA.
Furthermore, PKC\textsubscript{a} and PKC\textsubscript{d} siRNA were used as examples
to verify the roles of classical and novel PKC isozymes. Each of the
siRNA specifically blocked the expression of their target PKC
isoforms (Figure 7A). PKC\textsubscript{d} but not PKC\textsubscript{a} siRNA reduced PDBu-
induced GRO-1\textalpha (Figure 7B). Both PKC\textsubscript{a} and PKC\textsubscript{d} siRNA,
when treated alone, significantly reduced PDBu-induced IL-6
(Figure 7C). Moreover, neither PKC\textsubscript{a} nor PKC\textsubscript{d} siRNA reduced
PDBu-induced IL-8 (Figure 7D). These results are in agreement
with the inhibitor data (Figure 6). However, PKC\textsubscript{a} downregula-
tion was unable to decrease the PDBu-induced production of G-
CSF, which contrasts with the inhibitor study (Figure 7E),
suggesting that the production of G-CSF is mediated by other
classical PKC isozymes.

Discussion

Although many previous studies have demonstrated that PKC
family can be activated by a variety of environmental substances
and inflammatory cytokines and is involved in inflammation in the
airway, the direct role of PKC in the inflammatory response is
largely unknown. In the present study, we addressed this question
by bioinformatics approach to determine the PKC-induced
cellular responses in human bronchial epithelial cells. We
examined the overall change in the gene and cytokine expression
profile. Our microarray result shows that PKC activation elicits
cell death and production of multiple cytokines. Furthermore, classical and novel PKCs
regulate the production of various biologically important cytokines
in a different fashion.

PKC activation effectively regulates gene expression in human
bronchial epithelial cells. Microarray analysis suggests that PKC
activation induces change in more than 500 genes. Most of the

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**Figure 5. PKC activation increases the secretion of various cytokines.** (A) The basal level of cytokines produced by BEAS-2B cells. Twenty-
three cytokines were detected by Cytometric Beads Assay. (B) The fold-change of cytokines after PDBu stimulation at two different time points: 0.5 h
and 4 h. (C) These cytokines are differentially regulated at gene and/or protein levels, as determined by comparing the microarray and the
cytometric beads data.
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A

G-CSF

pg/ml

IL-7

pg/ml

IFNα2

pg/ml

Fractalkine

pg/ml

D

control PDBu BIM-1 Ro31-8220 Go6976 Rottlerin

control PDBu BIM-1 Ro31-8220 Go6976 Rottlerin

E

| Effective Inhibitors                  | Cytokines inhibited                                      |
|---------------------------------------|--------------------------------------------------------|
| Pan PKC inhibitors                    | GM-CSF, IL-8, TNFα, Fractalkine, IFNγ, VEGF, and Eotaxin|
| Pan and classical PKC inhibitors      | G-CSF                                                  |
| Pan and PKCδ inhibitors               | GRO, IL-7                                              |
| Pan, classical and PKCδ inhibitors    | IFNα2, IL-6, and TGFα                                   |
Figure 6. PDBu-induced cytokines were selectively regulated by different PKC sub-class isozymes. (A) G-CSF was inhibited by pan or classical PKC inhibitors. (B) IL-7 and GRO-1α were inhibited by pan PKC or novel PKC inhibitors. (C) IFNα and IL-6 are inhibited by pan, classical PKC or novel PKC inhibitors. (D) Fractalkine and IL-8 are inhibited only by pan PKC inhibitors. (E) The table summarizes the selective regulation of cytokines by different PKC subclass isozymes.

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Figure 7. PKCα and PKCδ siRNA were utilized to confirm the inhibitor results. (A) The protein expressions of PKCα and PKCδ were specifically downregulated by their respective siRNAs. (B) PKCδ, but not PKCα siRNA, was able to inhibit the GRO-1α production. (C) Both PKCδ and PKCα siRNA inhibited the IL-6 production. (D) Neither PKCδ siRNA nor PKCα siRNA were able to inhibit the IL-8 production. (E) PKCα siRNA did not reduce the G-CSF production, suggesting that classical PKCs other than PKCα regulate the production of G-CSF.

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genes were up-regulated, indicating that the major effect of PKC activation is on gene transcription. Early response genes were up-regulated within the first 0.5 hours of PDBu stimulation. Proteins encoded by these genes are transcription factors that regulate other gene expression. Several genes encoding cytokines are also up-regulated immediately after the PKC activation, indicating that the immediate inflammatory response of airway epithelial cells can be turned on by PKC related mechanisms.

Results from Functional Annotation and Ingenuity Pathway studies support our hypothesis that PKC activation is involved in multiple cellular functions, which include gene expression, wound healing, cell proliferation, metabolic process, cellular movements and immune cell trafficking. However, the most dominant effects of PKC activation in airway epithelial cells are cytokine production and apoptosis. The top IPA interaction network is around NFκB complex, a well-known transcription factor mediating inflammatory responses by controlling gene expression of various pro-inflammatory cytokines, adhesion molecules, and matrix metalloproteinases [19–22]. This finding supports other previous observations that various pathogen-induced inflammatory responses are mediated through PKC and NFκB. For example, chitinase is an enzyme produced by a variety of organisms such as insect, parasite, fungus, bacterium, plant and animal. Chitinase activates classical PKC, ERK and NFκB in sequence to synthesize and release IL-8, a pro-inflammatory chemokine, in lung epithelial cells [23]. Moraxella catarrhalis, a bacterial species that exacerbates chronic obstructive lung disease, can also induce IL-8 production by activating PKCα, ε, and θ to augment NFκB-regulated transcription [24]. TNF-α, another important molecule suggested by the interaction network, is a well-known cytokine that initiates inflammation, immune response and cell death [25–27]. It has been reported that TNF-α induced ICAM-1 expression in human airway epithelial cells is mediated through a pathway regulated by PKC and NFκB [28]. The functional annotations revealed that genes related to both apoptosis and cell proliferation are turned on by PKC activation. When cells are under stress, both death anti-death signals can be activated. The balance between these signals ultimately determines the fate of the cells. For instance, NFκB has been suggested to play a dichotomous role in apoptosis by either inducing cell death through up-regulating FasL production, or by inhibiting cell death through suppression of caspase-8 and regulating Akt pathways [29–31]. Also studies have suggested that TNFα may also play a role in cell proliferation and anti-apoptosis. Thus we further validated PKC activation modulation of cell survival via two separate tests that determines cell viability. Among different pathways of programmed cell death, Caspase mediated cell death is the most prevalent in many biological systems [32,33]. We found that cleaved caspase-3 increased significantly after 4 hours of PKC activation. The number of viable cells significantly reduced 6 hours after PKC activation. This confirms that PKC activation leads to programmed cell death as suggested by the microarray analysis.

As demonstrated by CBA, human bronchial epithelial cells can produce various cytokines, chemokines and growth factors. Most cytokines levels were elevated by PKC activation, and almost all of the increased cytokines are pro-inflammatory. All of the top elevated cytokines (i.e., IL-6, IL-8, GM-CSF, IFNγ) are related to innate immunity, indicating PKC activation is crucial for the initial defence mechanism in the airway against pathogens [34–35]. Notably, one of the growth factors that were significantly up-regulated after PKC activation is PDGF. PDGF has been reported to be secreted in response to thrombin and induces lung and airway remodelling [39]. We also found although most of cytokines can be increased by PKC activation at both mRNA and protein levels, some of the cytokines were elevated only at the mRNA level, while some others only at protein level. Inhibitor study suggests that these cytokines and chemokines are differentially regulated by sub-class PKC isozymes. Although these studies are preliminary, it shows the complexity of inflammatory responses, whereby each inflammatory cytokine is regulated by different pathways. It also demonstrates that the functional genomic studies are powerful tools allowing one to examine the overall mechanisms in a comprehensive approach.

In summary, PKC activation alone initiates gene expression related to multiple cellular functions, especially to cell death and inflammatory responses in airway epithelial cells. The fact that different PKC isozymes differentially regulate various cytokines at mRNA and/or protein levels shows the complexity of regulation on inflammatory responses. Further studies can elucidate these mechanisms and their specific downstream pathways to help develop new therapies for airway diseases related to inflammation induced by different pathogens or acute immune response.

Supporting Information

Figure S1 PDBe treatment (500 nM) rapidly induced translocation of PKCζ from cytosol to membrane fraction (top) and increased phosphorylation of various PKC isozymes (bottom). (TIF)

Table S1 The primers sequence used for qRT-PCR to validate microarray analysis. (TIF)

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Author Contributions

Conceived and designed the experiments: ML. Performed the experiments: HK XHB. Analyzed the data: HK RZ. Contributed reagents/materials/analysis tools: ML XHB. Wrote the paper: HK RZ ML.

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