Synergistic Inhibition of β_2-adrenergic Receptor–mediated Alveolar Epithelial Fluid Transport by Interleukin-8 and Transforming Growth Factor-β

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ABSTRACT

Background: Patients with acute respiratory distress syndrome who retain maximal alveolar fluid clearance (AFC) have better clinical outcomes. The release of endogenous catecholamines associated with shock or the administration of β_2-adrenergic receptor (β_2AR) agonists enhances AFC *via* a 3′-5′-cyclic adenosine monophosphate–dependent mechanism. The authors have previously reported that transforming growth factor-β1 (TGF-β1) and interleukin-8 (IL-8), two major mediators of alveolar inflammation associated with the early phase of acute respiratory distress syndrome, inhibit AFC upregulation by β_2AR agonists *via* a phosphoinositol-3-kinase (PI3K)–dependent mechanism. However, whether TGF-β1 and IL-8 cause an additive or synergistic inhibition of AFC is unclear. Thus, the central hypothesis of the study was to determine whether they synergistically inhibit the β_2AR-stimulated AFC by activating two different isoforms of PI3K.

Methods: The effects of TGF-β1 or IL-8 on β_2AR agonist–stimulated net alveolar fluid transport were studied using short-circuit current studies. Molecular pathways of inhibition were confirmed by pharmacologic inhibitors and Western blotting of p-Akt, G-protein–coupled receptor kinase 2, protein kinase C-ζ, and phospho-β_2AR. Finally, our observations were confirmed by an *in vivo* model of AFC.

Results: Combined exposure to TGF-β1 and IL-8/cytokine-induced neutrophil chemoattractant-1 caused synergistic inhibition of β_2AR agonist–stimulated vectorial Cl^- across alveolar epithelial type II cells (n = 12 in each group). This effect was explained by activation of different isoforms of PI3K by TGF-β1 and IL-8/cytokine-induced neutrophil chemoattractant-1 (n = 12 in each group). Furthermore, the inhibitory effect of TGF-β1 on 3′-5′-cyclic adenosine monophosphate–stimulated alveolar epithelial fluid transport required the presence of IL-8/cytokine-induced neutrophil chemoattractant-1 (n = 12 in each group). Inhibition of cytokine-induced neutrophil chemoattractant-1 prevented TGF-β1–mediated heterologous β_2AR downregulation and restored physiologic β_2AR agonist–stimulated AFC in rats (n = 6 in each group).

Conclusions: TGF-β1 and IL-8 have a synergistic inhibitory effect on β_2AR-mediated stimulation of pulmonary edema removal by the alveolar epithelium. This result may, in part, explain why a large proportion of the patients with acute respiratory distress syndrome have impaired AFC. **(Anesthesiology 2015; 122:1084-92)**

ACUTE respiratory distress syndrome (ARDS) is a clinical syndrome manifested by the rapid onset of respiratory failure associated with high mortality.[1] ARDS is characterized by increased permeability of the alveolar-capillary barrier, decreased surfactant function, and impaired alveolar fluid clearance (AFC).[2] Importantly, a minority of patients with ARDS who retain maximal AFC have better clinical outcomes.[3] Endogenous and exogenously administered β_2-adrenergic receptor (β_2AR) agonists have been shown to enhance alveolar epithelial fluid transport under physiological conditions[4–7] and removal of pulmonary edema in experimental models of lung injury,[8–10] as well as in one prospective study of extravascular lung water in ARDS patients.[11] However, two recent phase III multicenter trials that tested the effect of β_2-adrenergic agonist therapy to increase AFC in patients with ARDS were stopped for futility.[12,13]

What We Already Know about This Topic

- Alveolar fluid clearance is enhanced by β_2-adrenoceptor activation, associated with better outcomes in patients with acute respiratory distress syndrome
- Inflammatory mediators interleukin-8 and transforming growth factor-β1 inhibit this enhancement, but whether they interact additively or synergistically is unknown

What This Article Tells Us That Is New

- In human and rat alveolar epithelial cells, combined exposure to interleukin-8 and transforming growth factor-β1 synergistically inhibited β_2-adrenergic agonist–mediated Cl^- transport, important to fluid removal

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Although the reasons for the lack of success of these phase III clinical trials are likely multifactorial, we have recently reported that two inflammatory mediators, interleukin-8 (IL-8) (that is also called chemokine [C-X-C motif] ligand 1 [Cxcl1] in the new National Center for Biotechnology Information database) and transforming growth factor-β1 (TGF-β1) that play an important role in the pathogenesis of the early phase of ARDS,[14–22] inhibit epinephrine-dependent and cyclic adenosine monophosphate (cAMP)–mediated net fluid transport across rat alveolar epithelial type II (ATII) cell monolayers, as well as epinephrine-dependent AFC in a rat model of hemorrhagic shock *via* a phosphoinositol-3-kinase (PI3K)–dependent mechanism.[23,24] However, whether TGF-β1 and IL-8 would cause an additive or synergistic inhibition of AFC are still unclear. Thus, the central hypothesis of the study was to determine whether both mediators could synergistically inhibit the β_2AR-stimulated AFC by activating two different isoforms of PI3K. If confirmed, these results could, in part, explain why a large proportion of the patients with ARDS have impaired AFC.

Materials and Methods

In the following section, there are some methods that are abbreviated for simplicity. A more complete description of these methods can be found in Supplemental Digital Content 1, http://links.lww.com/ALN/B132.

Reagents

All cell culture media were prepared in the Pittet Laboratory at the University of Alabama at Birmingham using deionized water and analytical grade reagents. (–) [125I] iodocyanopindolol was purchased from PerkinElmer (Waltham, MA). The PI3K isoform inhibitors, sw-14, PW12, and AS-605240, were provided by Ben Houseman, M.D., Ph.D. (University of California, San Francisco, San Francisco, California). IL-8 and cytokine-induced neutrophil chemoattractant-1 (CINC-1) enzyme-linked immunosorbent assay were purchased from R&D Systems (Minneapolis, MN). Human recombinant TGF-β1 was obtained from R&D Systems. Antibodies and phosphoantibodies for the β_2AR and protein kinase C-zeta (PKC-ζ) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies and phosphoantibodies for Akt were purchased from Calbiochem (San Diego, CA). Goat anti-mouse and goat anti-rabbit IRDye®-conjugated secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE). Protein concentration of cell lysates was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Soluble chimeric TGF-β type II receptor was a generous gift from Gerald Horan, Ph.D. (Biogen Idec, Cambridge, MA). CINC-1 blocking antibody and immunoglobulin G isotype control antibody were purchased from R&D Systems. All other reagents were obtained from Sigma (St. Louis, MO).

Cell Culture

Primary cultures of rat and human alveolar epithelial cells were used for the *in vitro* studies. ATII cells were isolated as previously described.[25,26]

Following approval of the University of California, San Francisco Committee on Human Research, human alveolar epithelial type II cells were isolated using a modification of methods previously described.[27]

Short-circuit Current Studies of Rat and Human ATII Cell Monolayers

Short-circuit current studies were performed as described previously.[24]

Isolation of Plasma Membrane-enriched Fraction

Isolation of plasma membrane-enriched fraction was performed as described previously.[24]

Saturation Binding Experiments

Saturation binding experiments were performed as described previously.[24]

Western Blot Analyses

Western blot analyses from cells homogenates were performed as described previously.[24]

CINC-1 Enzyme-linked Immunosorbent Assay

CINC-1 levels in cell culture supernatant from ATII cell monolayers were measured by an enzyme-linked immunosorbent assay purchased from R&D Systems following the manufacturer's instructions.

Cell Viability

The cell viability after exposure to different experimental conditions was measured by the Alamar Blue assay (Invitrogen, Grand Island, NY).[28] The cell media were replaced by medium containing 10% Alamar Blue and placed at 37°C in the cell incubator for 2 to 3 h. The medium was then collected and read on a plate reader at 570 nm.

Rat Studies

The protocol for the measurement of AFC in rats was approved by the University of California, San Francisco, Committee on Animal Research, and was performed as previously described.[24,29] Sample sizes were chosen based on previous experience, and randomization and blinding methods were not feasible for these experiments.

Statistical Analysis

All normal data are summarized as mean ± SD. Nonparametric data were summarized as mean ± interquartile range. For the statistical analysis, we used StatView 5.0® (SAS Inc., Cary, NC) and MedCalc® 7.2.0.2 (MedCalc Software Inc., Ostend, Belgium). The normal distribution was verified using the Agostino–Pearson test. For normally distributed

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data, one-way ANOVA followed by a Dunnett test was used to compare three or more experimental groups and a Student *t* test to compare two experimental groups. Bonferroni correction, controlling for false-positive error rate, was used to adjust for multiple comparisons. Nonparametric data were compared with a Kruskal–Wallis test followed by a Tukey *post hoc* test. A *P* value of less than 0.05 was considered statistically significant. Saturation binding experiments were analyzed by nonlinear regression. The maximal number of iodocyanopindolol-binding sites (Bmax) and the equilibrium dissociation constant (K_D) were calculated from saturation binding curves by nonlinear least squares curve fittings for one binding site. The goodness of fit was determined by the F ratio test. All statistical comparison of means was bilateral (two-tailed tests).

Results

CINC-1 and TGF-β1 Act Synergistically to Decrease β_2AR Agonist–stimulated Cl^- Transport across Primary Rat ATII Cell Monolayers

Low doses (1 ng/ml) of CINC-1 (the rat homolog of IL-8) and TGF-β1, individually, did not inhibit epinephrine-dependent Cl^- transport across primary rat ATII cell monolayers. However, when CINC-1 and TGF-β1 (1 ng/ml) were added concomitantly to the cell medium, there was a statistically significant decrease in epinephrine-dependent Cl^- transport across primary rat ATII cell monolayers (fig. 1A). In contrast, using larger doses (10 ng/ml) of CINC-1 or TGF-β1 that we have previously shown to individually cause the maximal inhibitory effect of these inflammatory mediators on epinephrine-dependent Cl^- transport across primary rat ATII cell monolayers[23,24] did not result in an additive or synergistic inhibitory effect of epinephrine-dependent Cl^- transport (fig. 1B). Cell viability measured by Alamar Blue was not decreased by exposure to IL-8/CINC-1 or TGF-β1 (data not shown). These results indicate that there is a synergistic inhibitory effect of IL-8/CINC-1 and TGF-β1 on epinephrine-dependent Cl^- transport across primary rat ATII cell monolayers. However, these data also suggest that these inflammatory mediators may activate the same cell signaling pathway(s) to inhibit the β_2AR agonist–stimulated Cl^- transport across these cell monolayers.

CINC-1 and TGF-β1 Decrease β_2AR Agonist–stimulated Cl^- Transport across Primary Rat ATII Cell Monolayers via Different Isoforms of PI3K

In our previous studies,[23,24] we demonstrated that CINC-1– and TGF-β1–mediated decrease in β_2AR agonist–stimulated Cl^- transport occurred *via* activation of PI3K by using a pharmacologic inhibitor that blocked all isoforms of PI3K (PIK-90). To determine whether these two mediators could activate different isoforms of PI3K, we exposed ATII cell monolayers to PW12 and sw-14 (or AS-605240 which had to be used after our stock of sw-14 was depleted) to inhibit PI3Kα and PI3Kγ, respectively, before treating them with TGF-β1 or CINC-1, respectively. sw-14, but not PW12, inhibited Akt phosphorylation by CINC-1, a protein phosphorylated by the activation of the PI3K signaling pathway (fig. 2A), whereas PW12, but not sw-14, inhibited Akt phosphorylation by TGF-β1 (fig. 2B), indicating differential activation of PI3K isoforms by the respective mediators. Furthermore, AS-605240, but not PW12, reversed CINC-1–mediated decrease in epinephrine-dependent Cl^- transport (fig. 2C) and PW12, but not sw-14, rescued TGF-β1–mediated inhibition of epinephrine-dependent Cl^- transport (fig. 2D). Taken together, these results indicate that CINC-1–mediated inhibition of Cl^- transport is PI3Kγ-dependent, whereas TGF-β1–mediated inhibition is PI3Kα-dependent.

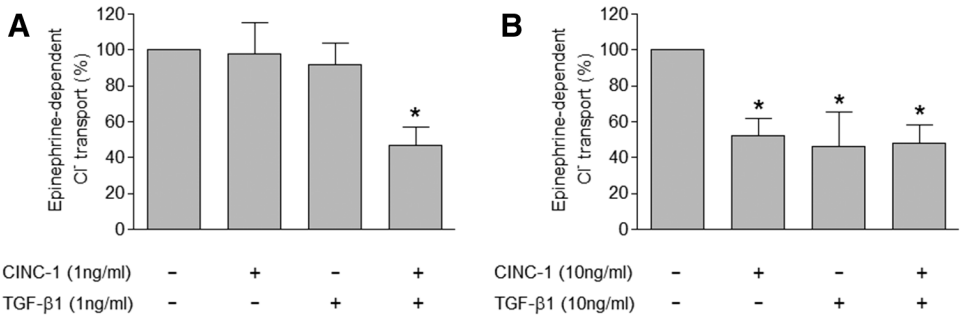

Fig. 1. Cytokine-induced neutrophil chemokine (CINC)-1 and transforming growth factor (TGF)-β1 cause a synergistic inhibition of β_2AR agonist–stimulated cystic fibrosis transmembrane conductance regulator (CFTR)-specific Cl^- transport across rat type II alveolar (ATII) cell monolayers. (*A*) Coexposure of rat ATII cell monolayers to a small dose (1 ng/ml for 6 h) of CINC-1 and TGF-β1 causes a synergistic inhibition of the epinephrine-stimulated CFTR-specific Cl^- absorption across the apical membrane of these cells while single exposure to 1 ng/ml of CINC-1 or TGF-β1 has no effect. (*B*) Coexposure of rat ATII cell monolayers to a dose (10 ng/ml for 6 h) of CINC-1 and TGF-β1 does not increase the inhibitory effect of single exposure of the same dose of CINC-1 and TGF-β1 on the epinephrine-stimulated CFTR-specific Cl^- absorption across the apical membrane of rat ATII cell monolayers. For all experiments, mean basal short-circuit current was −7 ± 1.8 μA, mean epinephrine-treated short-circuit current was −24 ± 2.4 μA. The results are means ± SD (n = 12); **P* < 0.05 from controls.

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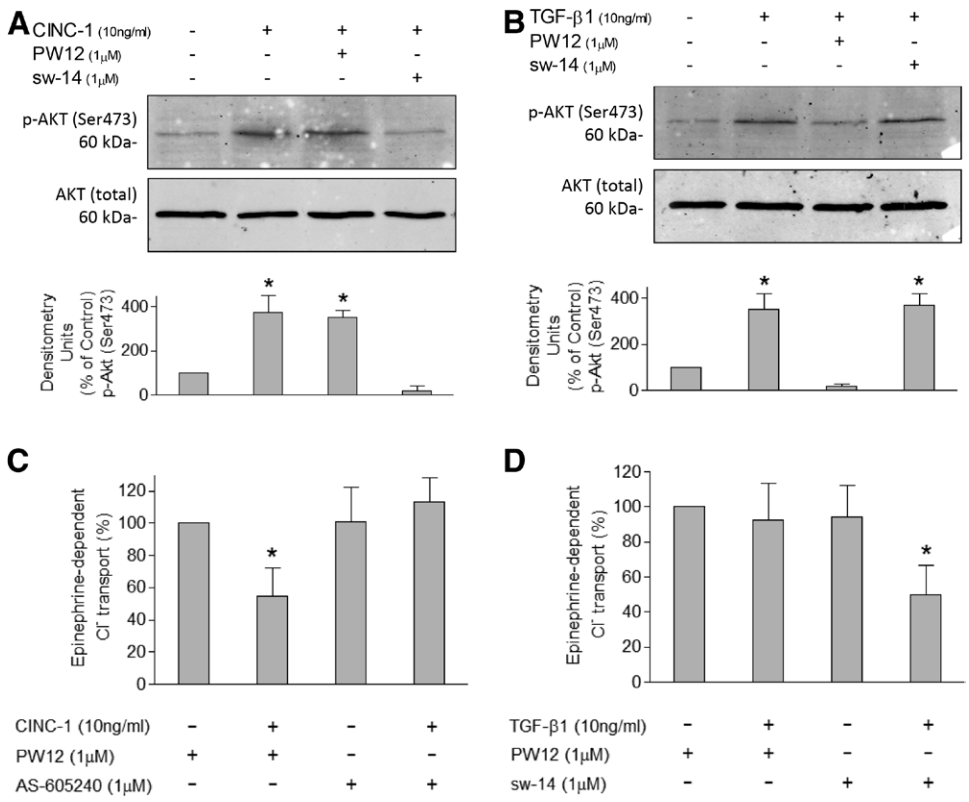

Fig. 2. Cytokine-induced neutrophil chemokine (CINC)-1 and transforming growth factor (TGF)-β1 decrease β_2-adrenergic receptor agonist–stimulated Cl^- transport across rat type II alveolar (ATII) cells *via* the activation of different phosphoinositide 3 (PI3)-kinase isoforms. (*A*, *B*) Short exposure to CINC-1 or TGF-β1 induces phosphorylation of Akt in rat ATII cells *via* the activation of different PI3K isoforms (PI3Kα for TGF-β1 and PI3Kγ for CINC-1). Densitometry analysis results are means ± SEM (n = 4); **P* < 0.05 from controls. (*C*, *D*) Pretreatment with specific inhibitors of different PI3-kinase isoforms (PI3Kα for TGF-β1 and PI3Kγ for CINC-1) prevents CINC-1– or TGF-β1–induced inhibition of the epinephrine-stimulated Cl^- transport across the apical membrane of rat ATII cells. For all experiments, the results are means ± SD (n = 12); **P* < 0.05 from controls.

Inhibition of Endogenous CINC-1 Prevents TGF-β1–mediated Inhibition of β_2AR Agonist–stimulated Cl^- Transport across Primary Rat and Human ATII Cell Monolayers and AFC in an In Vivo *Model of AFC in Rat*

IL-8/CINC-1 is a signaling molecule that is secreted by numerous cell types.[30,31] We found that it was indeed present in the supernatant of primary rat ATII cells and was not increased by stimulation of cell monolayers with TGF-β1 (fig. 3). We further found that the amount of secreted CINC-1 was sufficient to act synergistically with TGF-β1 to inhibit β_2AR signaling in ATII cells. Indeed, pretreatment with CINC-1– or IL-8–blocking antibodies in rat and human ATII cells, respectively, prevented TGF-β1–mediated inhibition of epinephrine-dependent Cl^- transport across rat ATII cell monolayers (fig. 4, A and B). In contrast, inhibition of endogenous TGF-β1 by soluble chimeric TGF-β type II receptor did not prevent the CINC-1–mediated inhibition of epinephrine-dependent Cl^- transport across rat ATII cell monolayers (data not shown). Finally, we also found that pretreatment with CINC-1–blocking antibody reversed TGF-β1–mediated inhibition of epinephrine-dependent AFC in rat (fig. 5). Taken together, these data indicate that the release of endogenous IL-8/CINC-1 by ATII cells or other cell types is required for the TGF-β1–mediated inhibition of epinephrine-dependent net alveolar fluid transport.

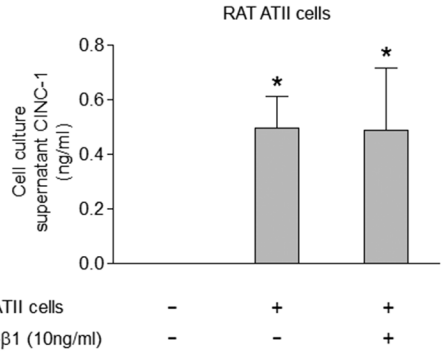

Fig. 3. Basal release of cytokine-induced neutrophil chemokine (CINC)-1 by rat type II alveolar (ATII) cell monolayers is not affected by prior exposure to transforming growth factor (TGF)-β1. There is a basal release of CINC-1 by rat ATII cell monolayers that is not affected by exposure to TGF-β1 (10 ng/ml for 6 h). CINC-1 was not detected in the cell medium containing 10% fetal bovine serum. The results are means ± SD (n = 8). **P* < 0.05 from controls.

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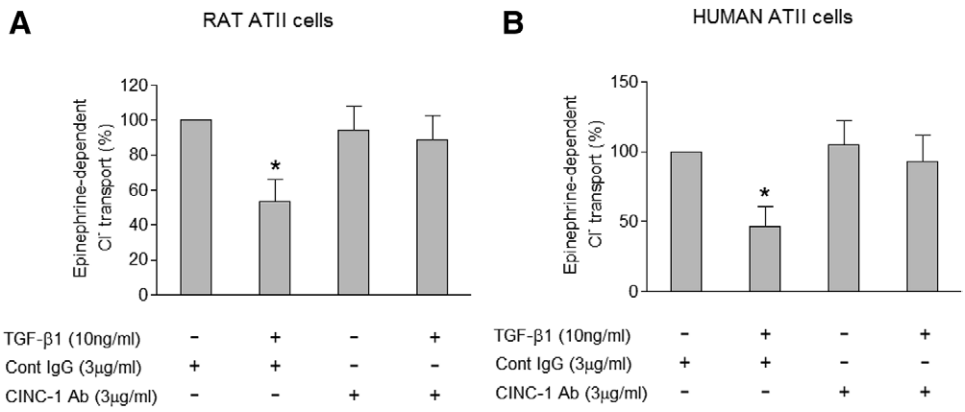

Fig. 4. Cytokine-induced neutrophil chemokine (CINC)-1 blockade prevents transforming growth factor (TGF)-β1–mediated inhibition of β_2-adrenergic receptor agonist–stimulated alveolar net fluid transport across primary rat (*A*) and human (*B*) type II alveolar (ATII) cell monolayers. Pretreatment with a blocking antibody to CINC-1 but not with its isotype control antibody prevents TGF-β1–induced inhibition of the epinephrine-stimulated Cl^- transport across the apical membrane of rat or human ATII cells. For all experiments, the results are means ± SD (n = 12); *$P < 0.05$ from controls.

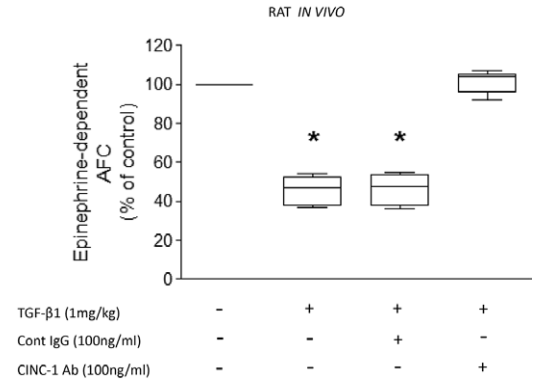

Fig. 5. Cytokine-induced neutrophil chemokine (CINC)-1 blockade prevents transforming growth factor (TGF)-β1–mediated inhibition of the epinephrine-stimulated alveolar fluid clearance (AFC) in rats. Pretreatment with a blocking antibody to CINC-1 but with its isotype control antibody prevents TGF-β1–induced inhibition of the epinephrine-stimulated AFC in rats (AFC control 7.1% ± 1.3/30 min, epinephrine-treated 14.2% ± 1.3/30 min). Results are means ± interquartile range (n = 6 in each group); *$P < 0.05$ from controls. Cont = control; IgG = immunoglobulin G.

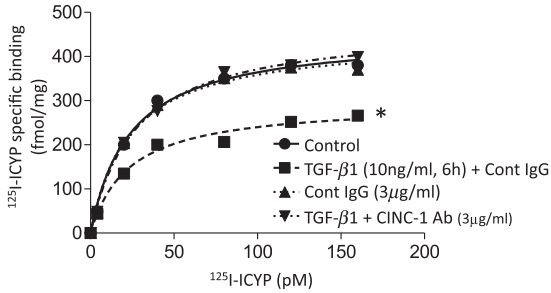

Fig. 6. Cytokine-induced neutrophil chemokine (CINC)-1 blockade prevents transforming growth factor (TGF)-β1–mediated downregulation of β_2-adrenergic receptor (β_2AR) on rat type II alveolar cells. Pretreatment with a blocking antibody to CINC-1 but not with its isotype control antibody prevents TGF-β1–mediated decrease in β_2AR cell membrane density, measured by saturation binding experiments ([125I] iodocyanopindolol, [125I]-ICYP) (n = 6). For all experiments, the results are means ± SD; *$P < 0.05$ from controls. Cont = control; IgG = immunoglobulin G.

Inhibition of Endogenous CINC-1 Reverses TGF-β1–mediated Heterologous Desensitization and Downregulation of the β_2AR at the Cell Membrane

We have previously reported that both CINC-1 and TGF-β1 are capable of mediating recruitment of G-protein–coupled receptor kinase 2 (GRK2) to the cell membrane and thus cause heterologous desensitization and downregulation of the β_2AR. To explain the mechanism by which the secretion of endogenous CINC-1 is required for the inhibitory effect of TGF-β1 on epinephrine-dependent net alveolar fluid transport, we first measured β_2AR density at the cell membrane of ATII cells by saturation binding experiments. We found that TGF-β1–mediated decrease in cell membrane β_2AR density was inhibited with pretreatment with a CINC-1–blocking antibody (fig. 6). Second, we tested the hypothesis that IL-8/CINC-1, but not TGF-β1, would induce the phosphorylation of PKC-ζ, a kinase that needs to be activated in order for GRK2 to form a complex with PI3K that then translocates to the cell membrane of ATII cells and inhibits β_2AR signaling in these cells.[23,32] We found that there was a statistically significant increase in phosphorylation of PKC-ζ above untreated cells in primary rat ATII cell monolayers exposed to CINC-1 (10 ng/ml, 30 min), but not to TGF-β1 (10 ng/ml, 30 min) (fig. 7, A and B). Taken together, these data indicate that IL-8/CINC-1, but not TGF-β1, causes the activation of PKC-ζ and may explain why the presence of this chemokine is required for the inhibitory effect of TGF-β1 on β_2AR agonist–mediated net AFC.

Discussion

In this study, we demonstrate (1) that there is a synergistic effect between low doses (1 ng/ml) of CINC-1 and TGF-β1 in inhibiting β_2AR agonist–stimulated, cAMP-mediated net

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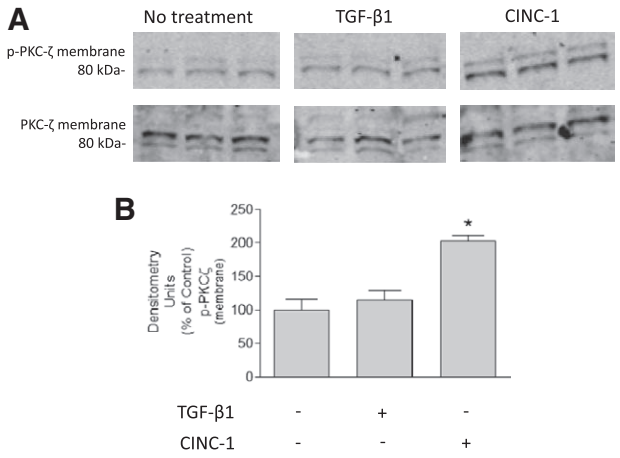

Fig. 7. Cytokine-induced neutrophil chemokine (CINC)-1 but not transforming growth factor (TGF)-β1 activates protein kinase C-zeta (PKC-ζ) in rat type II alveolar cell monolayers. (*A*) Treatment with CINC-1 (10 ng/ml for 30 min), but not TGF-β1 (10 ng/ml for 30 min), leads to increased phosphorylation of membrane-bound PKC-ζ. (*B*) Normalized phospho-PKC-ζ/PKC-ζ ratios determined by densitometry analysis from bands in part *A* (n = 3). For all experiments, the results are means ± SD; **P* < 0.05 from controls.

alveolar fluid transport across cell monolayers and AFC *in vivo* that is explained by the activation of two different PKC isoforms by these two mediators; (2) that IL-8/CINC-1, but not TGF-β1, causes the activation of PKC-ζ and GRK2 and may explain why the presence of this chemokine is required for the inhibitory effect of TGF-β1 on β_2AR agonist–mediated net AFC.

Stimulation of net alveolar fluid transport is necessary to clear edema in patients with ARDS.[3,33] Previous experimental and some clinical evidence indicate that cAMP-mediated stimulation of AFC by endogenous catecholamines or by the administration of β_2AR agonists is an important mechanism in the resolution of pulmonary edema.[4,6,10,11,33,34] Despite large increases in endogenous catecholamine release secondary to shock states, impaired AFC is present in roughly 80% of patients with ARDS.[3] In addition, two placebo-controlled phase III clinical trials using β_2-adrenergic agonists in patients with ARDS were stopped for futility. Unfortunately, β_2AR agonists did not reduce ventilator-free days or mortality in patients with ARDS.[12,13]

Although the reasons for the lack of success of these two phase III clinical trials are likely multifactorial, a possible mechanism to explain inhibition of β_2AR-mediated AFC stimulation could be the alveolar release of inflammatory mediators during the acute phase of ARDS. Among these mediators, IL-8 and TGF-β1 have been shown to be critical mediators of the early phase of ARDS in humans. Indeed, IL-8 is increased in the bronchoalveolar lavage fluid and pulmonary edema fluid of patients with ARDS and is a strong predictor of morbidity and mortality.[14–18] Interestingly, impairment of AFC observed during respiratory syncytial virus lung infection in mice was caused by decreased response to β_2AR agonists. This decreased response was mediated by KC, the murine homolog of IL-8, and reversed *via* inhibition of either KC or its receptor CXCR2.[32] IL-8 blockade also significantly attenuates lung injury caused by ischemia-reperfusion injury, smoke inhalation, or acid aspiration.[35–37] Finally, we previously reported that the rate of AFC was inversely related to the levels of IL-8 in the undiluted pulmonary edema fluid obtained at the time of endotracheal intubation in ARDS patients, suggesting that IL-8 may play a role in reducing alveolar epithelial fluid transport in patients with ARDS.[23] TGF-β1, another inflammatory mediator that is released within the airspaces during the early phase of ARDS,[19–22] is known to inhibit β_2AR messenger ribonucleic acid expression and cAMP-mediated vectorial fluid transport.[38–42]

In previous studies from our laboratory, we found that the mechanisms leading to the inhibition of β_2AR agonist–mediated AFC by IL-8/CINC-1 and TGF-β1 include inhibition of β_2AR agonist–mediated net alveolar fluid transport by recruiting GRK2 to the cell membrane, inducing heterologous desensitization and downregulation of the β_2AR.[23,24] Both mechanisms are PI3K-dependent. In the present study, we tested the hypothesis that both inflammatory mediators added at the same time could have an additive or synergistic effect on the inhibition of the cAMP-stimulated alveolar fluid transport at concentrations that did not have any effect when used individually. We found indeed that there was a synergistic effect between low doses (1 ng/ml) of CINC-1 and TGF-β1 in inhibiting β_2AR agonist–stimulated, cAMP-mediated net alveolar fluid transport across cell monolayers and AFC *in vivo*. Interestingly, using higher doses (10 ng/ml) of both mediators that provide the maximal inhibitory effect for each mediator when used individually did not show any additional additive or synergistic inhibition of the β_2AR signaling in ATII cell monolayers. These results suggest that both mediators may activate similar cell signaling pathway(s) to inhibit β_2AR signaling in the alveolar epithelium, but possibly *via* different isoforms of the same protein(s), as suggested by previously published studies.[43,44] Thus, we examined the effect of these mediators on the activation of several isoforms of that kinase and found that IL-8 activates the gamma isoform of PI3K while TGF-β1 activates the alpha isoform of that kinase. These results may, in part, explain why a large percentage of patients with ARDS secondary to septic or hemorrhagic shock have a reduced AFC early after onset of the syndrome,[3,33] despite the release into the bloodstream of large amount of endogenous catecholamines that we and others have shown to increase AFC rate by several folds in animal models of septic or hemorrhagic shock.[10,45]

Because the heterologous desensitization and downregulation of the β_2AR by either IL-8/CINC-1 or TGF-β1 required the activation of both GRK2 and PI3K in ATII cells, the second aim of the study was to determine whether the presence of either IL-8/CINC-1 or TGF-β1 was required for the observed inhibition of the alveolar epithelial

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β_2AR cell signaling by the other inflammatory mediator. The results showed that CINC-1 was secreted at baseline by ATII cells and that TGF-β1–mediated inhibition of the *in vitro* epinephrine-dependent alveolar fluid transport and *in vivo* AFC was reversible with pretreatment with CINC-1–blocking antibody. Furthermore, TGF-β1–mediated downregulation of the β_2AR were prevented with pretreatment with a CINC-1–blocking antibody. In contrast, pretreatment of ATII cells with a chimeric soluble TGF-β1 type II receptor that we have previously shown to efficiently inhibit TGF-β1 signaling in ATII cells[24] did not prevent the inhibitory effect of IL-8/CINC-1 on β_2AR signaling in ATII cells.

Because we had shown that both IL-8/CINC-1 and TGF-β1 activate PI3K signaling *via* two different isoforms of this kinase, we then tested the hypothesis that IL-8/CINC-1, but not TGF-β1, would cause the phosphorylation of PKC-ζ, a kinase that needs to be activated in order for GRK2 to form a complex with PI3K that then translocates to the cell membrane of ATII cells and inhibits β_2AR signaling in these cells. Indeed, the data showed that CINC-1, but not TGF-β1, phosphorylated PKC-ζ in rat primary ATII cells. Thus, these results explain why CINC-1 blockade prevented the TGF-β1–mediated inhibition of the β_2AR signaling pathway, whereas the inhibition of TGF-β1 signaling did not prevent the effect of IL-8/CINC-1 on that signaling pathway. The schematic representation in figure 8 summarizes the findings of the present study by showing first that IL-8/CINC-1 and TGF-β1 activate two different isoforms of PI3K and second that IL-8/CINC-1, but not TGF-β1, activates both GRK2 and PI3K, an activation that is required for the inhibition of β_2AR agonist–stimulated, cAMP-mediated AFC.

The present study has several limitations. First, our data are mostly from *in vitro* experiments that will require *in vivo* confirmation in humans, although we have reported preliminary data indicating that the rate of AFC is inversely related to the levels of IL-8 in the undiluted pulmonary edema fluid obtained during the early phase of ARDS.[23] Second, the timing and kinetics of the release of IL-8 and TGF-β1 within the airspaces of the lung after onset of ARDS are not fully understood and could play an important role regarding the synergistic AFC inhibition by these two inflammatory mediators. Finally, there are other potential explanations for the lack of success of the two phase III trial with β_2AR agonist treatment to ARDS patients.[12,13] Indeed, inadequate aerosol delivery of the β_2AR agonist, alveolar epithelial damage and agonist-induced downregulation of the β_2AR, could in part explain these negative results.[46,47]

Clinical Implications and Conclusion

The results presented in this study have some important clinical implications. Indeed, the majority (up to 80%) of the patients with ARDS have an impaired AFC, despite the release of large amount of endogenous catecholamines associated with septic and hemorrhagic shock or clinical illness.[3] The mechanisms of the AFC inhibition are not well understood but are of importance because patients with impaired AFC are at higher risk for poor outcome.[3] We provide in this study a new potential explanation for AFC inhibition during the early phase of ARDS, a synergistic inhibitory effect on the β_2AR by IL-8 and TGF-β1 that could prevented, in part, by PI3K inhibitors already in clinical use for cancer therapy, inflammation, and coronary heart disease[48] that would be beneficial in reversing the inhibitory effect of IL-8 or TGF-β1 on β_2AR agonist–stimulated, cAMP-mediated AFC without directly antagonizing their other beneficial effects.

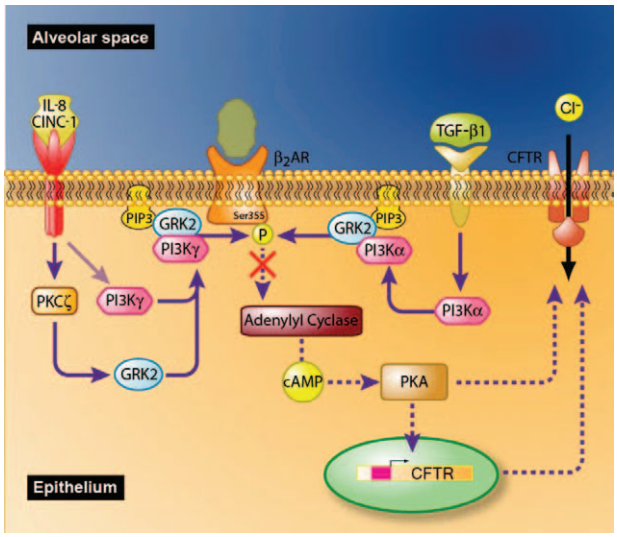

Fig. 8. Schematic representation of the mechanisms by which interleukin-8 (IL-8)/cytokine-induced neutrophil chemokine (CINC)-1 and transforming growth factor (TGF)-β1 have a synergistic inhibitory effect on the β_2-adrenergic receptor (β_2AR) signaling pathway in type II alveolar (ATII) cells. IL-8/CINC-1 and TGF-β1 cause the activation of different phosphoinositide 3-kinase (PI3K) isoforms. However, IL-8/CINC-1 but not TGF-β1 phosphorylates G-protein–coupled receptor kinase 2 (GRK2) *via* a protein kinase C-zeta (PKC-ζ)–dependent mechanism explaining why the blockade of IL-8/CINC-1 prevents the TGF-β1–mediated inhibition of the β_2AR signaling pathway in ATII cells. This results in the translocation of the protein complex GRK2 and PI3K to the cell membrane. This protein complex causes phosphorylation at the Ser355 heterologous desensitization and downregulation of the β_2AR in ATII cells. IL-8/CINC-1 and TGF-β1 then prevent the activation of 3′-5′-cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway that upregulates the vectorial fluid transport across the alveolar epithelium *via* phosphorylation and increased expression of cystic fibrosis transmembrane conductance regulator (CFTR) at the plasma membrane of ATII cells. The *solid lines* indicate the pathways stimulated by IL-8/CINC-1 and the *dashed lines* indicate the pathways inhibited by these mediators.

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Competing Interests

The planning, conduct, and reporting of the research were performed by Dr. Pittet and the members of his laboratory at the University of California San Francisco, San Francisco, California, and then at the University of Alabama at Birmingham, Birmingham, Alabama, with collaboration from the University of Nice-Sophia-Antipolis, Nice, France. There is no conflict of interest related to the conduct and reporting of the results by any of the authors of the present manuscript. The authors declare no competing interests.

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