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## Resolvin E1 maintains macrophage function under cigarette smoke-induced oxidative stress



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### ABSTRACT

**Cigarette smoke (CS) induces oxidative stress, which disables macrophage function. In this study, we examined whether Resolvin E1 (RvE1), a pro-resolving mediator known to enhance macrophage functions, attenuates the damage of macrophages by CS extract (CSE) induced oxidative stress. RvE1 blocked p47phox translocation to plasma membrane induced by CSE in a macrophage cell line, RAW264.7 cells, resulting in suppression of superoxide production. Furthermore, pretreatment of RAW264.7 cells with RvE1 restored the phagocytic activity and reduced cell death induced by treatment of CSE. These results suggest that RvE1 plays important roles in preserving macrophage function under CS-induced oxidative stress.**

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### Introduction

Chronic obstructive pulmonary disease (COPD), comprised of small airway diseases and pulmonary emphysema, is currently the fourth leading cause of death globally, with mortality rates continuing to increase [1]. Cigarette smoke (CS), a complex mixture of over 4700 components including reactive oxygen species (ROS), is a major risk factor for the development of COPD [1–4], and an imbalance between oxidants and anti-oxidants plays an essential role in its pathogenesis [5]. In smokers, ROS generation is also enhanced in alveolar macrophages, a condition that is related to tissue injury and cell death in COPD [6].

On the other hand, alveolar macrophages play a key role in the host defense to lung infections which is detrimental to the prognosis of patients with COPD [7]. We, as well as other investigators, have shown that, when macrophages are exposed to CSE, their ability to phagocytose apoptotic cells [8–10], microorganism [11], and polystyrene beads [12] is decreased, compared to several type of macrophages

that were not exposed, and served as a cell culture model. It has also been demonstrated that a relationship exists between the phagocytic activity of macrophages, the resolution of acute inflammation [13]; decreased clearance of apoptotic neutrophils (efferocytosis) in macrophages from CS extract (CSE)-exposed mice, as we previously demonstrated [8]. Collectively, this results in the sustained inflammation of and further destruction of the lungs. Hence, a drug that can restore the protective functions of macrophages might be useful for the treatment of COPD.

It has been demonstrated that an increased intake of omega-3 polyunsaturated fatty acids is associated with a decreased risk of COPD and emphysema [14] and with an improvement in symptoms and exercise capacity [15,16]. Resolvin E1 (RvE1) is one of the bioactive lipid mediators synthesized from omega-3 polyunsaturated fatty acids, which not only suppresses acute inflammation by decreasing neutrophil infiltration, but also actively promotes the resolution of inflammation by enhancing the clearance of apoptotic neutrophils by macrophages [17–21]. In addition, RvE1 has been shown to block TNF- $\alpha$ - or FMLP-induced superoxide generation in human neutrophils [22]. Furthermore, we recently showed that RvE1 attenuates lung inflammation without compromising host defense in a murine model of aspiration pneumonia [23].

The objective of this study was to determine whether RvE1 attenuates CSE-induced oxidative stress and cell death in a macrophage cell line, RAW264.7, and at the same time, increases phagocytic functions against *E. coli*.

**Abbreviations:** CS, cigarette smoke; CSE, cigarette smoke extract; COPD, chronic obstructive pulmonary disease; NOX2, NADPH oxidase2; RvE1, Resolvin E1; ROS, reactive oxygen species

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## Materials and methods

### Preparation of CSE

Research grade 3R4F cigarettes (Kentucky Tobacco Research Institute) were used to prepare the CSE. CSE was prepared fresh for each experiment by bubbling the smoke from one cigarette through 10 ml of medium in a 15 ml conical tube followed by passing through a 0.22  $\mu$  m filter to remove large particles and maintain sterility. This solution was designated as 100% CSE [8].

### Cell culture and CSE exposure *in vitro*

A murine macrophage cell line, RAW264.7 cells ( $5 \times 10^4$  cells/well DMEM containing 10% FBS), were grown in 6-well plates for 24 h followed by serum-starvation for 2 h prior to the stimulation by CSE. RAW264.7 cells were incubated with or without RvE1 (1–100 nM, Cayman Chemical) for 30 min, and then CSE (1% or 10%) or acrolein (10 or 100  $\mu$ M, Tokyo Chemical Industry, Japan) was added into the indicated wells.

### Measurement and visualization of ROS generation

Intracellular ROS generation was detected with 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes), a membrane-permeable precursor that yields fluorogenic dichlorofluorescein (DCF) through oxidation [24]. In a typical run, the cells were incubated with 10  $\mu$ M DCFH-DA for 30 min at 37 °C in DMEM, and washed twice with PBS, and then analyzed using a FACScan flow cytometer (Beckton Dickinson).

### Measurement of p47-phox translocation.

RAW264.7 cells were pelleted and resuspended in fractionation buffer (10 mM HEPES, pH 7.4, 0.25 M sucrose, 5 mM EGTA, 2.5 mM MgCl<sub>2</sub>) and sonicated. Unbroken cells and nuclei were pelleted by centrifugation (13,000  $\times$  g, 10 min, 4 °C), and the supernatants were separated into membrane and cytosol fractions by ultracentrifugation (100,000  $\times$  g, 60 min, 4 °C) [25]. The membranes were solubilized in cell lysis buffer (Cell Signaling Technology) containing protease inhibitor (Roche).

### Western blot analysis

Cells were lysed in cell lysis buffer (Cell Signaling Technology) with complete protease inhibitors (Roche). Protein extracts were subjected to SDS-PAGE (Bio-Rad), transferred to PVDF membranes (Bio-Rad), and then probed with an anti-Akt antibody (1:1000; Cell Signaling Technology), an anti-HO-1 antibody (1:1000; Stressgen Biotechnologies), an anti-p47-phox antibody (1:1000; Millipore), or an anti-actin antibody (1:1000; Sigma-Aldrich). After incubation with a peroxidase-conjugated secondary antibody, the immunoreactive bands were visualized using an enhanced chemiluminescence kit (GE Health Care).

### Phagocytic function assay

The phagocytic function of RAW264.7 cells was assessed using a Vybrant phagocytosis kit (Molecular Probes) with the following modifications. RAW264.7 cells ( $5 \times 10^4$  cells/well) were grown in 96-well microplates for 12 h prior to stimulation. After incubation with RvE1 for 30 min, the cells were treated with CSE or acrolein for 2 h, after which, 20  $\mu$ l of fluorescein-labeled *E. coli* bioparticles (5 mg/ml) was added. Two hours later, the fluorescence intensity of each well was determined with a fluorescence plate reader (Wallac ARVO SX), using 480 and 520 nm for excitation and emission, respectively. Phagocytic

index (PI) is presented as the relative fluorescence intensity in the experimental samples compared to the intensity in the non-treated cells.

### Confocal laser scanning microscopy

RAW264.7 cells were grown on glass-bottom dishes and fixed by treatment with 4% paraformaldehyde for 10 min on ice. Cells were incubated with 100 ng/ml of tetramethyl rhodamine isothiocyanate (TRITC)-labeled phalloidin (Sigma-Aldrich) and then analyzed using confocal laser microscopy (Leica). To identify the size of the cells, they were measured under a microscope, and the image was processed digitally using an eight-bit image analyzer (NIH Image software).

### Cell death analysis

To determine the extent of damage to cells by CSE in the presence or absence of RvE1 (10 nM), RAW 264.7 cells were stained with a 0.4% trypan blue solution at room temperature for 3 min and the cells were then counted in a hemocytometer. The cells were then stained cells observed under a microscope. For statistical analysis, four different digital images of cells under the microscope (each covering 50–100 cells to be counted) were acquired and the stained cells were counted [26].

### Assessment of cell viability

Cell viability was assessed by MTT cell proliferation (Cayman Chemical) following the manufacturer's instructions.

### Statistical analysis

For a comparison between more than two groups, and multiple comparisons, an ANOVA test, which followed by the Bonferroni/Dunn multiple comparison test, was used. The data were analyzed using the Graphpad Prism4 (GraphPad Software). Statistical significance was accepted at *P* value of less than 0.05. The number of samples per group (*n*) is specified in the figure legends.

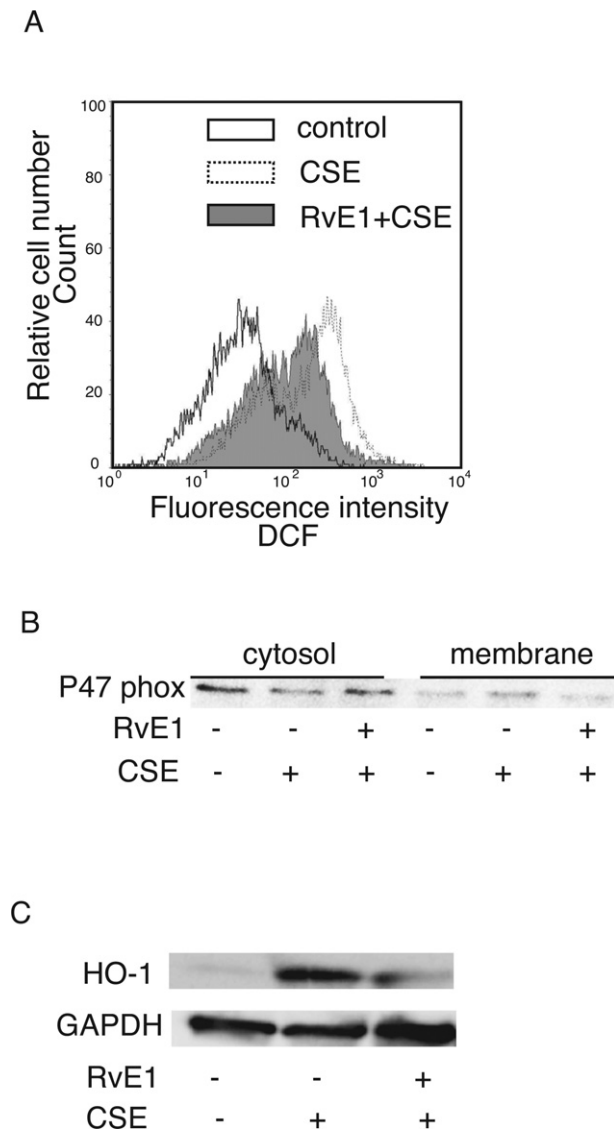
## Results

### RvE1 blocks CSE induced ROS production

We measured intracellular ROS levels in RAW264.7 cells using a DCFH-DA probe, and the results indicated that ROS levels were elevated after a 4 h exposure to 10% CSE (Fig. 1(A)). RvE1 at a concentration of 10 nM reduced the elevation in intracellular ROS level induced by CSE in RAW 264.7 cells (Fig. 1(A)). In order to determine whether RvE1 suppressed ROS production by inhibiting the activation of NADPH oxidase 2 (NOX2), we checked the translocation of p47phox from the cytosol to the cell membrane by Western blot analysis. CSE exposure (10%, 4 h) induced a shift in p47phox immunoreactivity to the plasma membrane in RAW264.7 cells, as previously reported [27], and this translocation was blocked by treatment with 10 nM RvE1 (Fig. 1(B)). Exposure to CSE (10%, 4 h) induced the expression heme oxygenase (HO)-1, a sensitive marker for oxidative stress [28], the levels of which were also reduced in the cells that had been treated with CSE and RvE1 (Fig. 1(C)). These results suggest that RvE1 may suppress the CSE-induced p47phox translocation to the plasma membrane, resulting in the inhibition of NOX2 and a reduction in ROS production in RAW264.7 cells.

### RvE1 attenuates CSE-induced damage of phagocytic function

The exposure of RAW264.7 cells to 1% or 10% CSE for 4 h resulted in a decrease in phagocytic activities in a dose-dependent manner

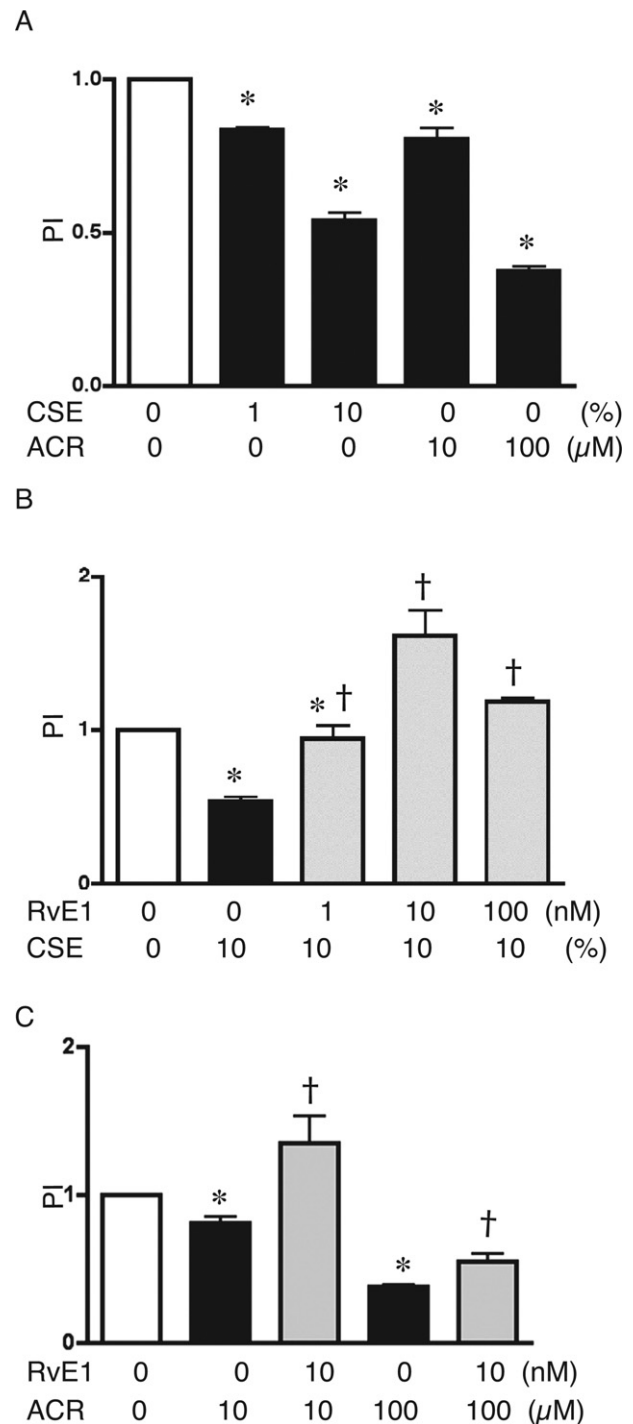


**Fig. 1.** RvE1 inhibited the intracellular ROS production induced by CSE in RAW264.7 cells. After incubation with 10 nM RvE1 for 30 min, RAW264.7 cells were exposed to CSE (10%) for 4–6 h. (A) Relative ROS concentrations in cells were quantified by flow cytometric analysis of DCF fluorescence. Data show data from three representative experiments. (B) Localization of p47phox in the cytosolic and membrane fractions examined by immunoblot analysis. Data are representative of three experiments. (C) Induction of HO-1 expression examined by Western blot analysis. Data are representative of two experiments.

without evidence of impairing cell viability (Fig. 2(A) and Fig. S1) [8]. The repressed phagocytic function in these cells was reversed by the pretreatment with RvE1 at concentrations of 1–100 nM (Fig. 2(B)). We then examined the effects of RvE1 (10 nM) on the acrolein-induced phagocytic defects. Acrolein is a low molecular weight,  $\alpha,\beta$ -unsaturated aldehyde that is present in high concentrations (> 4.0 parts per million) in cigarette smoke [29,30]. As shown in Fig. 2(C), acrolein also suppressed phagocytic function, which was ameliorated by the pretreatment with RvE1.

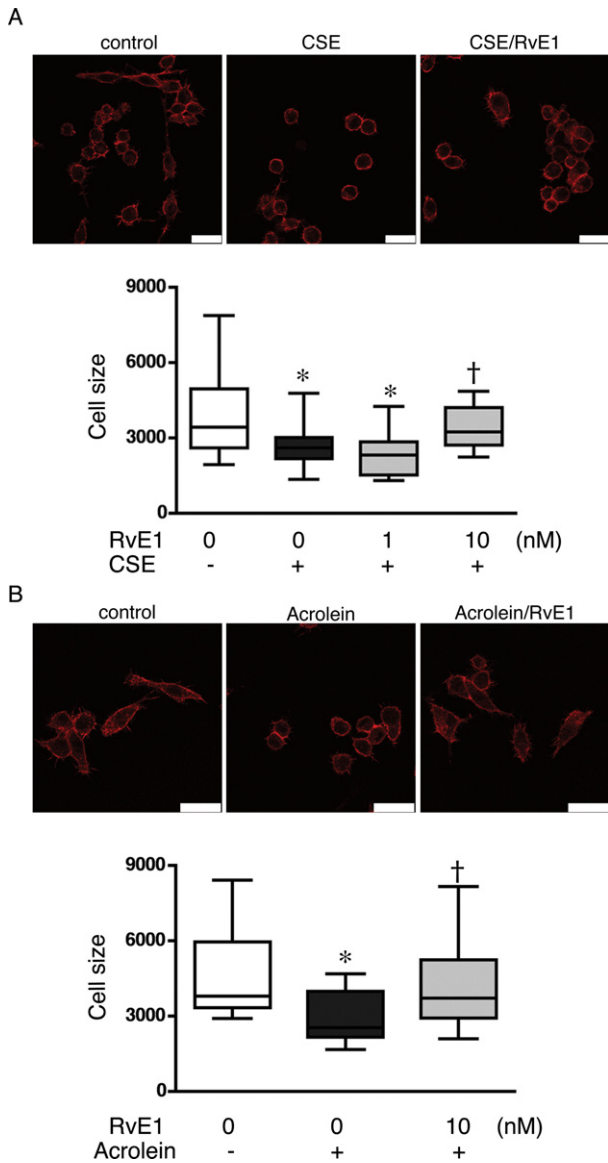
#### RvE1 represses CSE induced cytoskeletal changes

A recent study indicated that the polymerization of actin at the leading edge had greatly deteriorated after CSE treatment, resulting in an impaired phagocytic function in macrophages [8]. Therefore, the attenuation of CSE impaired-phagocytic function by RvE1 prompted



**Fig. 2.** RvE1 restored the phagocytic activity decreased by treatment of CSE or acrolein in RAW264.7 cells. RAW264.7 cells treated with CSE, acrolein, and/or RvE1 were incubated with FITC-labeled *E. coli*. Phagocytic index (PI) is presented as the relative fluorescence intensity in the experimental samples compared to the intensity in the non-treated cells, arbitrarily designated as 1.0. (A) Dose-response effects of CSE or acrolein (4 h) on phagocytic activity. \* $P < 0.05$  vs. control ( $n = 5$ ). Pretreatment with RvE1 enhanced phagocytic activity in the presence of CSE (B) or acrolein (C). \* $P < 0.05$  vs. control. † $P < 0.05$  vs. CSE or acrolein alone ( $n = 4$ ).

us to examine whether RvE1 was capable of restoring CSE induced-actin modification. Polymerized actin fibers were stained with phalloidin and analyzed by laser scanning confocal microscopy. As shown in Fig. 3(A), 4 h-CSE exposure evoked a retraction in the leading-edge that occurred simultaneously with the reduction of cell size. The mean size of the CSE-exposed RAW264.7 cells was  $66 \pm 17\%$  that

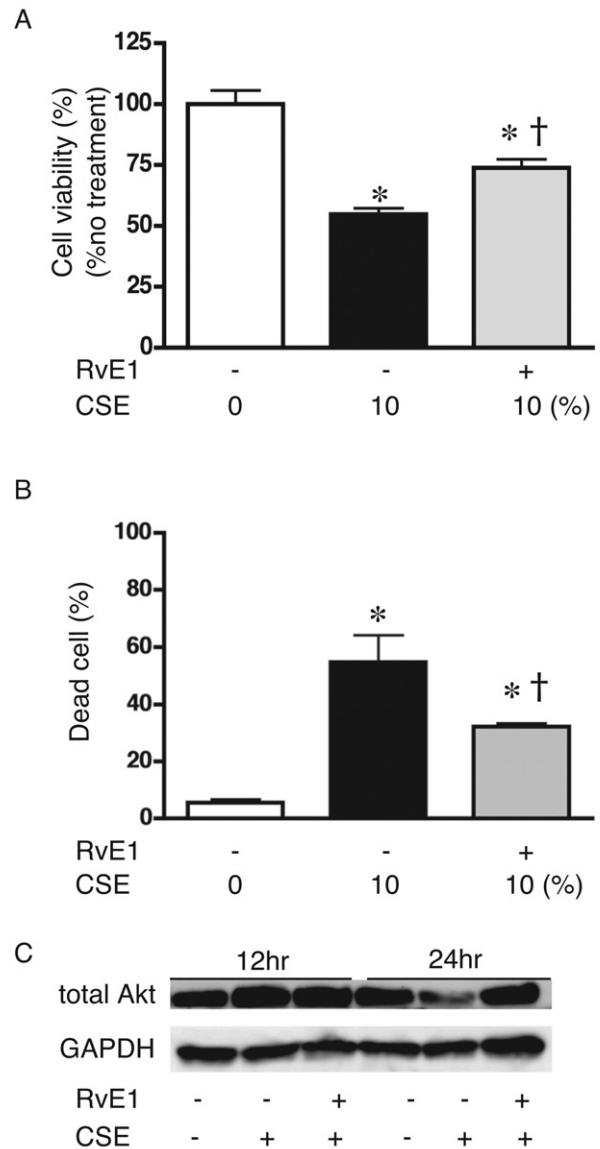


**Fig. 3.** RvE1 protected the disruption of cytoskeletal structure caused by CSE or acrolein in RAW264.7 cells. RAW264.7 cells were incubated with RvE1 or vehicle for 30 min, CSE (10%, A) or acrolein (10  $\mu$ M, B) was then added, followed by incubation for 4 h. Representative florescent images with TRITC-labeled phalloidin are shown. Cell size was measured under a microscope after collecting 30 cells from each culture. Open bar: vehicle-treated cells. Dark gray bar: CSE- or acrolein-treated cells. Light gray bar: acrolein-treated cells in the presence of RvE1 (10 nM). The line in each column indicates the mean value. \* $P < 0.05$  vs. control. † $P < 0.05$  vs. CSE or acrolein alone ( $n = 4$ ). Scale bar: 25  $\mu$ M.

of the control cells. When we treated RAW 264.7 cells with 10 nM RvE1 30 min prior to CSE-exposure, CSE-induced cytoskeletal disruption was recovered to  $87 \pm 21\%$  that of the control cells. Stabilizing actions of RvE1 were also observed when the stimulus was replaced with acrolein (Fig. 3(B)).

*RvE1 suppresses CSE-induced cell death and degradation of the Akt protein*

Although short-term (4 h) exposure to CSE had little effect on cell viability, a longer exposure (24 h) to 10% CSE in serum free medium resulted in a reduced cell viability and an increased the ratio of dead cells, compared with vehicle treated cells (Fig. 4(A) and (B)). Under this condition, pretreatment with 10 nM RvE1 before CSE exposure



**Fig. 4.** RvE1 rescued CSE-induced cell death in RAW264.7 cells. RAW264.7 cells were incubated for 24 h in serum-free DMEM medium including 10% CSE in the presence or absence of 10 nM RvE1. (A) Viability of cells was determined by MTT assays. \* $P < 0.05$  vs. control. † $P < 0.05$  vs. CSE alone ( $n = 3$ ). (B) Dead cells were determined by means of a trypan blue exclusion test. \* $P < 0.05$  vs. control. † $P < 0.05$  vs. CSE alone ( $n = 4$ ). (C) Expression of the Akt protein was determined by Western blot analysis. Representative data from two experiments.

increased cell viability and reduced the cell death (Fig. 4(A) and (B)). Acrolein also induced the reduction of cell viability, which was ameliorated by the pretreatment with RvE1 (Fig. S2). It has been reported that a long-term exposure to CSE induces the proteasomal degradation of Akt, and that this leads to the death of human lung fibroblasts [31]. We, therefore, examined whether RvE1 could affect Akt degradation caused by CSE. As shown in Fig. 4(C), the levels of total Akt were decreased after RAW264.7 cells were subjected to a 24 h-CSE exposure, whereas pretreatment with 10 nM RvE1 blocked CSE-induced Akt protein degradation.

**Discussion**

Highly ROS present in CS are thought to contribute to the development of COPD. Furthermore, they simulate macrophages to produce more ROS mainly through the activation of NOX [27]. The findings

reported herein indicated that RvE1 blocks the elevation in intracellular peroxide levels, p47phox translocation, and the induction of HO-1 induced by CSE (Fig. 1). These data suggest that RvE1 modulates CS-induced superoxide generation by suppressing NOX2 activation. Although ROS have been classically believed to be necessary for the killing of pathogens, recent studies have been shown that the deletion of NOX2 improves the resolution of lung viral infections [32,33]. Therefore, the paradoxical role of RvE1 to NOX2 may be important for the resolution of CS-induced inflammation by suppressing the excessive production of ROS in the lung.

Investigators have previously shown that alveolar macrophages from COPD patients exhibit a reduced phagocytic activity to *E. coli* [8,9]. Our data also demonstrate that *E. coli* clearance by macrophages was impaired by a 4 h-exposure to CSE or acrolein. As we previously reported [8], the CSE-induced suppression of phagocytosis in macrophages was associated with actin cytoskeletal disruption. These phenotypes may be mediated by oxidative stress, because (1) hydrogen peroxide causes actin oxidation resulting in cytoskeletal disruption [34,35], (2) acute suppression of efferocytosis by CS can be completely abrogated by the presence of superoxide dismutase, an anti-oxidant enzyme [9].

As discussed above, the findings reported here demonstrate that RvE1 has properties that permit it to downregulate CS-induced oxidative stress. Furthermore, a recent study in our laboratory has shown that RvE1 can increase the *in vivo* clearance of *E. coli* [21]. Therefore, we speculated that RvE1 could ameliorate the impaired phagocytosis in CSE-treated RAW264.7 cells. As we expected, RvE1 at a concentration of 10 nM concentration restored the phagocytic function of macrophages (Fig. 2(B) and (C)), and showed protective effects against the disruption of cytoskeletal structure caused by CSE (Fig. 3).

It has been shown that a long term-exposure to hydrogen peroxide induces the dephosphorylation and proteolysis of Akt [36], a serine/threonine kinase that mediates cell survival, growth, proliferation, and inflammation. In addition, CSE can also induce the proteasomal degradation of Akt in lung fibroblasts, which leads to cell death [31]. In the present study, we demonstrated that treatment with RvE1 was able to attenuate cell viability and death, and suppress Akt degradation in macrophages during long term-CSE exposure (Fig. 4). These results imply that the regulation of Akt is another drug target of interest in terms of the treatment of COPD [37].

In conclusion, our study has demonstrated that, at low concentrations, RvE1 can suppress ROS production, ameliorate phagocytic function, and reduced cell death, in macrophages that have been exposed to CSE. The combined effects of RvE1 on oxidative stress, host defense, and the enhanced resolution of inflammation have new potential therapeutic applications in COPD under stable conditions as well as during periods of exacerbation. Further studies should be conducted to investigate the *in vivo* effects of RvE1 on COPD.

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## Contributions

R.T. conceived and conducted of the experiments. R.T. and K.A. wrote the manuscript. F.K., M.A., N.M., and K.A. analyzed the data. F.K., M.A., J.M., H.S., N.M., and M.S. edited the manuscript. K.A. supervised the project.

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## Appendix A. Supplementary Material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2012.10.001.

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