Protein Kinase C- ζ Mediates Lung Injury Induced by Diesel Exhaust Particles

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Recently, we reported that diesel exhaust particles (DEPs) disrupt tight junctions (TJs) in alveolar epithelial cells (AECs) via an increase in reactive oxygen species (ROS). In this study, we investigated the role of protein kinase C (PKC)- ζ activation in DEP-induced lung injury. C57/bl6 mice were instilled intratracheally with 50 μ l of saline containing 100 μ g of DEPs or titanium dioxide (TiO₂). Twenty-four hours later, bronchoalveolar lavage was performed to assess neutrophil counts and protein concentrations. In addition, *in vitro* experiments were performed in primary rat and human AECs exposed to DEPs (50 μ g/cm²) for 3 hours. Transepithelial electrical conductance was measured, and TJ protein association was analyzed by immunoprecipitation. To determine whether the overexpression of antioxidants prevented DEP-induced lung injury, AECs and mice were infected with adenoviruses containing catalase and manganese superoxide dismutase (MnSOD) plasmids. *In vivo*, the overexpression of catalase and MnSOD prevented DEP-induced neutrophil recruitment. The inhibition of PKC- ζ activation also prevented DEP-induced neutrophil recruitment *in vivo*. *In vitro*, DEPs activated PKC- ζ in AECs, but not in alveolar macrophages. Using a specific myristolated PKC- ζ pseudosubstrate peptide (PKC- ζ ps), we showed that PKC- ζ mediated the DEP-induced dissociation of occludin and zonula occludin-1 (ZO1) in rat and human AECs. In addition, the overexpression of constitutively active PKC- ζ induced the dissociation of occludin and ZO1 in AECs. DEP-induced TJ disruption occurs via PKC- ζ . TJ disruption seems to be in part responsible for DEP-induced lung injury.

Keywords: diesel exhaust particles; PKC- ζ ; occludin; ZO1; ROS

Numerous pollutants exert detrimental effects on human health (1, 2). Diesel exhaust particles (DEPs), which are among the most abundant pollutants (3), comprise a component of particulate matter (PM) of less than 2.5 μ m (PM_{2.5}) in size. The smallest particles are the most numerous, and can penetrate deepest into the lung, reaching the alveolar space (4). PM varies not only in size but in composition, which can include water-soluble and water-insoluble fine cores, with reactive and nonreactive surfaces. Soluble components of PM are the most bioavailable fraction of constituents playing a potential key role in the induction of adverse health effects, both at the site of their deposition in the lung, and in remote tissues and organ systems (5, 6). In addition, organic species can constitute

CLINICAL RELEVANCE

Air pollution is recognized as a detrimental agent that increases pulmonary and cardiovascular disease. The results of this study further our knowledge of the mechanisms by which air pollution, specifically diesel exhaust particle, disrupts alveolar barrier in human cells and the role of PKC- ζ as a mediator of this effect.

a large portion of motor vehicle exhaust particles, and are considered among the most significant causative constituents of ambient PM. The short-term inhalation of DEPs produces systemic and pulmonary inflammation, enhances bronchial hyperresponsiveness in patients with asthma, and increases sensitization to airborne allergens (7–9). Furthermore, inhaling DEPs increases the number of inflammatory cells in the airway, and up-regulates inflammatory mediators (10).

Lung injury is associated with a dysfunctional epithelial barrier allowing the development of alveolar edema and the easy migration of inflammatory cells to the alveolar space (10). Barrier integrity is regulated mainly by adhesion proteins. These include E-cadherin and tight junctions (TJs). TJs constitute the most apical junction, and delineate the apical and basolateral surfaces of epithelial cells. They are formed by transmembrane proteins, including claudins and occludin, that bind to the actin cytoskeleton directly or through the scaffolding proteins zonula occludens (ZO). We previously reported that DEPs disrupt the organization of TJs in alveolar epithelial cells (AECs) (11). DEPs induce occludin endocytosis and dissociation from ZO1, with an increase transepithelial electrical conductance (G_T) in AECs. These effects are prevented by the overexpression of antioxidant enzymes such as catalase and manganese superoxide dismutase (MnSOD). However, other signaling events are probably involved in the DEP-induced disruption of TJs.

TJs are regulated mainly by kinases that localize at the plasma membrane and interact directly with TJ proteins. Protein kinase C- ζ (PKC- ζ) is an atypical PKC, very abundant in alveolar epithelium, and it is activated by reactive oxygen species (ROS) in AECs (12). PKC- ζ was reported to regulate TJ integrity and to mediate changes in the localization of TJ proteins (13, 14). This threonine-serine kinase translocates to the plasma membrane when activated (15), and localizes at the TJs to form a complex with proteins such as ZO1 and occludin (16). Therefore, we hypothesized that DEP-induced TJ disruption occurred via PKC- ζ . In addition, because the integrity of the epithelial barrier modulates the inflammatory response in several animal models (17, 18), we hypothesized that blocking the activation of PKC- ζ via a specific myristolated PKC- ζ pseudosubstrate peptide (PKC- ζ ps) will prevent DEP-induced lung injury.

MATERIALS AND METHODS

A more detailed explanation of our methods is available in the online supplement.

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Particles and Reagents

All reagents were from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. DEPs were provided by Dr. Ian Gilmour (United States Environmental Protection Agency, Washington, DC) (19). We appreciate the gift of carbon black from Dr. Martha Monick at the University of Iowa (Iowa City, IA), originally acquired from Degussa, GmbH (Düsseldorf, Germany). The physical characteristics of particles are summarized in Table E1 in the online supplement. In addition, Figure E3 depicts a representative transmission electron microscopy image of titanium dioxide and diesel exhaust particles.

In Vivo Experiments

Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Iowa. Six-week-old C57/bl6 mice from Jackson Laboratories (Bar Harbor, ME) were used. Mice were instilled intratracheally with 50 μ l of PBS containing 100 μ g of DEPs or titanium dioxide (TiO₂), using a precision Fortec Vaporizer (Cyprane, Keighley, UK). After 24 hours, the mice were killed, and bronchoalveolar lavage (BAL) was performed.

Cells

Primary rat alveolar Type II epithelial cells were isolated from Sprague Dawley male rats (Harlan Laboratories, Madison, WI), as previously described (20). BAL fluid from rats was centrifuged at 600 $\times$ g, and cell pellets were resuspended in Dulbecco's Modified Eagle's Medium/10% FBS to isolate alveolar macrophages. Human alveolar epithelial cells (A549) were obtained from the American Type Culture Collection (Manassas, VA).

Transepithelial Electrical Conductance

Transepithelial electrical resistance (R_t) was measured with a Millicell Electrical Resistance System (Millipore Corporation, Bedford, MA), and transepithelial electrical conductance (G_t) was calculated as the reciprocal of R_t .

Cell-Surface Biotinylation

After treatments, cell-surface proteins were labeled with 1 mg/ml EZ-link NHS-SS-Biotin (Pierce Chemical, Rockford, IL). One hundred micrograms of cell lysate proteins were rotated overnight at 4°C with streptavidin beads. The beads were washed, and biotinylated proteins were eluted and subjected to SDS-PAGE analysis.

Adenovirus Infection

Cells were infected with 20 plaque-forming units (pfu)/cell of adenovirus, either empty or containing the plasmid of interest (e.g., MnSOD [Ad5CMVMnSOD] and catalase [Ad5CMVCat], gifts from Dr. J. Engelhardt through the Gene Transfer Vector Core at the University of Iowa).

Constitutively Active PKC- ζ Plasmid Transfection

The mammalian expression vector for a C-terminal HA epitope tag (pHACE) plasmid encoding the gene for the catalytic domain of PKC- ζ (pHACE-PKCzeta-CAT) was provided by Dr. Jae-Won Soh (Department of Chemistry, Inha University, Incheon, South Korea) (21). The expression plasmid was transfected in A549 cells, using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Immunofluorescence

After treatment, cells were fixed and labeled with monoclonal anti-occludin and polyclonal anti-ZO1 (Invitrogen). Secondary antibodies, namely, Cy3-coupled goat anti-rabbit and FITC-coupled goat anti-mouse (Invitrogen), were used and visualized under a confocal fluorescence microscope.

Endotoxin Analysis

Endotoxin was measured using the kinetic chromogenic limulus amoebocyte lysate (LAL) (22, 23).

Statistical Analysis

An unpaired Student *t* test was used to determine significance between experimental groups. Statistical significance was set as $P < 0.05$. The data are presented as means $\pm$ SEs.

RESULTS

PKC- ζ Inhibition Prevents DEP-Induced Lung Injury

DEP inhalation has been shown to cause lung inflammation *in vivo* and to increase neutrophil recruitment (24), in addition to disrupting the alveolar epithelial barrier (11). Several authors have reported that PKC- ζ is involved in lung inflammatory processes induced by cigarette smoke, LPS, and bacterial infections (25, 26). Moreover, we have reported that hypoxia induces the reorganization of TJs in AECs through a PKC- ζ -mediated pathway (14). Therefore, we hypothesized that PKC- ζ inhibition will prevent DEP-induced lung injury *in vivo*. Mice were divided into four groups, and 100 μ g of TiO₂ or DEP were instilled intratracheally, together with DMSO or 1 μ g of PKC- ζ pseudosubstrate (PKC- ζ ps), suspended in 50 μ l of PBS. PKC- ζ ps is a myristolated peptide that binds specifically to the activation site of the PKC- ζ protein and blocks its activation. TiO₂ is an inert particle with a size of less than 0.1 μ m, and it was used as control for the presence of particles in the airway. A secondary control, carbon black, with a composition more similar to that of DEPs, was also used, and the results are summarized in Figure E1 in the online supplement.

Twenty-four hours after instillation, BAL was performed and neutrophil counts were determined. As shown in Figure 1A, DEPs increased neutrophil counts in BAL fluid ($P = 0.0147$). The DEP and TiO₂ samples used in these studies were assayed for endotoxin, and endotoxin was determined to be below the detection limit of 0.00048 EU/mg (< 0.048 pg/mg). Thus, the observed effects cannot be attributed to an inadvertent endotoxin coexposure.

Treatment with DEPs and PKC- ζ ps blocked the DEP-induced increase in neutrophils ($P = 0.2372$). In addition, protein concentrations were measured as an indicator of alveolar barrier integrity. As shown in Figure 1B, DEP instillation induced an increase of protein concentrations in BAL fluid, whereas this increase was not present in PKC- ζ ps-treated animals. Furthermore, to determine that the effects of DEPs *in vivo* are not related to a nonspecific particle effect, we tested carbon particles *in vivo*, which carry many of the properties of DEPs, but without the complex organic surface adsorbents. As shown in Figures E1A and E1B, carbon black did not increase the cell counts or protein concentrations in murine BALF.

We previously described that the overexpression of antioxidant enzymes prevents DEP-induced alveolar epithelial disruption (11). We set out to determine whether the overexpression of antioxidant enzymes in AECs prevents DEP-induced lung inflammation *in vivo*. Mice were instilled with 10⁹ pfu of adenovirus in 50 μ l of PBS. These adenoviruses contained the genes for catalase or MnSOD, or a combination of both (10^{4.5} of catalase plus 10^{4.5} of MnSOD). Empty adenovirus was used as control. Twenty-four hours later, mice were instilled with 100 μ g of DEPs or TiO₂ suspended in 50 μ l of PBS. BAL was performed 24 hours after DEP instillation. As shown in Figure 1, neutrophil counts in the BAL fluid increased from 6.6% in TiO₂-treated mice to 31.4% in mice treated with DEPs. The overexpression of catalase and MnSOD (Figures 1C and 1D) antioxidant enzymes partly blocked this effect. The combination of both viruses further blocked DEP-induced inflammation (Figure 1E) ($P = 0.0302$).

Figure 1. Neutrophil count (A) and protein concentration (B) of bronchoalveolar lavage obtained from mice 24 hours after being treated with 100 mg of Titanium dioxide (TiO₂) or diesel exhaust particles (DEPs) in the presence or absence of 1 mg of protein kinase C- ζ pseudosubstrate (PKC- ζ ps). Mice were also pretreated with empty adenovirus or adenovirus containing the gene for catalase (C), manganese superoxide dismutase (MnSOD) (D), or both (E). Adv, adenovirus. ns, no significance, $P > 0.05$. * $P < 0.01$. ** $P < 0.005$. *** $P < 0.001$.

DEP Activates PKC- ζ in Alveolar Epithelial Cells, but Not in Alveolar Macrophages

After reaching the alveolar space, DEPs will encounter AECs and alveolar macrophages. Because DEPs increase ROS in AECs and alveolar macrophages (27), we set out to determine whether DEPs activate PKC- ζ in primary alveolar macrophages and primary rat AECs. Rats were anesthetized, and BAL was performed to isolate alveolar macrophages. Lungs were then extracted and processed to isolate alveolar epithelial cells (see MATERIALS AND METHODS). In culture, cells were treated with 50 $\mu\text{g}/\text{cm}^2$ of DEPs. The activation of PKC- ζ was determined by harvesting cells at different time points and assessing the phosphorylation state of PKC- ζ threonine residue at position 410 by Western blotting (WB). As shown in Figure 2A, DEPs increased PKC- ζ p410 abundance in AECs after 30 minutes of DEP exposure. In contrast, no activation of PKC- ζ was found in alveolar macrophages after 2 hours of exposure (Figure 2B). In addition, we preincubated AECs with 100 ng/ml of PKC- ζ ps for 30 minutes, cells were then treated with 50 $\mu\text{g}/\text{cm}^2$ of DEPs for 30 minutes, and active PKC- ζ abundance was analyzed by WB. As shown in Figure 2C, PKC- ζ ps blocked the DEP-induced activation of PKC- ζ in AECs. To determine whether ROS mediated DEP-induced PKC- ζ activation, we overexpressed antioxidant enzymes, as already described. The overexpression of both antioxidant enzymes prevented the activation of PKC- ζ after DEP exposure for 30 minutes (Figure 2D).

Inhibition of PKC- ζ Prevents DEP-Induced Barrier Disruption in Primary AECs

We previously reported that DEPs disrupt AEC barrier integrity, mainly affecting the occludin abundance at the

plasma membrane and its association with ZO1 (11). Because DEPs activate PKC- ζ in AECs and not in alveolar macrophages, we hypothesized that PKC- ζ ps prevents the DEP-induced disruption of TJs in AECs, thus maintaining the integrity of the alveolar epithelial barrier and preventing neutrophil recruitment. To test this hypothesis, we pretreated primary rat AECs with PKC- ζ ps for 30 minutes, followed by treatment with 50 $\mu\text{g}/\text{cm}^2$ of DEPs for 3 hours. We measured G_r as an indicator of barrier integrity, as well as occludin abundance at the plasma membrane by WB and occludin-ZO1 distribution as an indication of TJ integrity (28).

The inhibition of PKC- ζ prevented the DEP-induced increase in G_r (Figure 3A) and the reduction of occludin abundance at the plasma membrane (Figure 3B). Interestingly, occludin abundance at the plasma membrane decreased with the inhibition of PKC- ζ , regardless of DEP exposure. As shown in Figure 3C, the DEP-induced dissociation of ZO1/occludin was also prevented by pretreatment with PKC- ζ ps. Figure 3D shows the distribution of occludin and ZO1 in AECs according to immunofluorescence. Under control conditions, occludin was reduced and the colocalization with ZO1 was diminished with DEP treatment. Pretreatment with PKC- ζ ps increased occludin-ZO1 colocalization under control conditions, and prevented DEP-induced changes in occludin and ZO1. The inhibition of PKC- ζ prevented DEP-induced barrier disruption in primary human AECs. To confirm the relevance of this pathway further in humans, we isolated primary human AECs from three donors. Cells

Figure 2. Concentrations of active protein kinase C- ζ (PKC- ζ) according to Western blotting (WB) in alveolar epithelial cells (AECs) (A) and alveolar macrophages (B) after treatment with 50 $\mu\text{g}/\text{cm}^2$ of DEPs. (C) Concentration of active PKC- ζ according to WB in AECs pretreated with PKC- ζ ps. (D) Concentration of active PKC- ζ according to WB in AECs infected with adenovirus containing catalase or MnSOD gene. * $P < 0.05$. A.U. arbitrary units.

were exposed to DEPs and PKC- ζ ps in the same way that rat AECs were exposed. The G_i was measured, and active PKC- ζ abundance was assessed by WB. Immunoprecipitation was also performed to determine ZO1–occludin association. As shown in Figure 4A, DEPs disrupted the epithelial barrier, as evidenced by the increase of G_i 3 hours after DEP exposure. PKC- ζ ps treatment prevented the DEP-induced increase in G_i . Similar to rat AECs, DEPs induced the activation of PKC- ζ by 30 minutes after DEP exposure (Figure 4B), and induced the dissociation of ZO1 and occludin, disrupting TJ structure (Figure 4C). Pretreatment with PKC- ζ ps prevented the dissociation of ZO1–occludin induced by DEP exposure. In addition, to determine that the effect of DEPs on TJs is not related to a nonspecific particle effect, we tested carbon particles, which carry many of the properties of DEPs, without the complex organic surface adsorbents. As shown in Figure E1C, carbon black did not increase ZO1 and occludin dissociation. Taken together, these data support the hypothesis that PKC- ζ activation mediates DEP-induced alveolar epithelial barrier disruption, and that the inhibition of PKC- ζ activation blocks DEP-induced TJ disruption, preserving the integrity of the epithelial barrier.

PKC- ζ Is Necessary and Sufficient to Induce the Dissociation of Occludin–ZO1 in AECs

To determine whether PKC- ζ activation is sufficient to elicit the dissociation of occludin and ZO1 in AECs, we overexpressed a constitutively active form of PKC- ζ , using recombinant DNA technology (Figure E2A). Primary AECs are challenging to transfect, and therefore experiments were performed in A549 cells, which are human alveolar epithelial cells. We previously showed that the occludin–ZO1 association is affected in a fashion similar to that of primary AECs in response to DEPs (11). As shown in Figure 5A, occludin is reduced at the plasma membrane in response to DEP exposure, and this effect was prevented by pretreatment with PKC- ζ ps, similar to primary rat and human AECs. We then overexpressed active PKC- ζ in A549 cells by transfecting them with a plasmid containing a constitutively active form of PKC- ζ . Twenty-four hours after transfection, cells were harvested, and occludin was immunoprecipitated from the cell lysate. Figure 5B shows the association of occludin and ZO1 in A549 cells and in transfected A549 cells. The overexpression of constitutively active PKC- ζ induced a dissociation of occludin and ZO1, compared with nontransfected A549 cells.

Figure 3. (A) Transepithelial conductance of AECs after 3-hour exposure to 50 µg/cm² DEPs in the presence or absence of PKC-ζ ps. (B) Concentrations of cell-surface occludin and E-cadherin according to WB of biotinylated proteins of AECs after 3-hour exposure to 50 µg/cm² DEPs in the presence or absence of PKC-ζ ps. (C) Zonula occludens (ZO)-1/occludin association, according to immunoprecipitation (IP) of occludin and WB analysis of associated ZO1 of AECs after 3-hour exposure to 50 µg/cm² DEPs in the presence or absence of PKC-ζ ps. (D) Immunofluorescence of occludin (green) and ZO1 (red) of AECs after 3-hour exposure to 50 µg/cm² DEPs in the presence or absence of PKC-ζ ps. ns, no significance, $P > 0.05$. ** $P < 0.005$.

To confirm further that PKC-ζ mediates DEP-induced ZO1-occludin dissociation, we knocked down PKC-ζ in A549 cells, using specific anti-PKC-ζ small interfering RNA (Figure E2B). As shown in Figure 5C, knocking down PKC-ζ in A549 cells prevented the DEP-induced occludin-ZO1 dissociation.

DISCUSSION

The alveolar epithelium represents an important barrier against inhaled xenobiotics from the environment, and its integrity is fundamental for survival. DEP exposure has been associated with an increase in asthma exacerbations (29), infections (30), and cardiovascular risk (2). All these adverse outcomes are favored by a leaky epithelial barrier that allows the penetration of allergens, bacteria, and particles to the bloodstream, as well as the migration of inflammatory cells from the bloodstream to the alveolar space.

People living in polluted cities inhale millions of DEPs with each breath. These particles can reach the deepest levels of the

airway tree and affect the 75 m² of alveolar epithelium surface area, where they accumulate during a lifetime. Moreover, subjects with chronic obstructive pulmonary disease (COPD) and smokers present even higher accumulations of particulate matter (PM) in lung tissue, contributing to lung inflammation (22).

Most of the data regarding the effects of diesel exhaust in humans come from epidemiological or inhalation studies, and therefore subjects are exposed not only to PM, but to other components including polycyclic aromatic hydrocarbons (PAHs) and other gases. Therefore, our results only address the effects of diesel exhaust particles, and not necessarily the effects of other components that interact with these particles and cause further detrimental effects in humans. In addition, a high degree of variation in particle and aggregate size is evident, which adds a variability to the particle-cell interaction surface that can be significantly different in our experiments compared with that in nature. Furthermore, TiO₂ and carbon black were used as controls in our experiments. They have very different characteristics

Figure 4. (A) Transepithelial conductance of human AECs after 3-hour exposure to 50 $\mu\text{g}/\text{cm}^2$ DEPs in the presence or absence of PKC- ζ ps. (B) Level of active PKC- ζ abundance according to WB of human AECs after exposure to 50 $\mu\text{g}/\text{cm}^2$ DEPs. (C) ZO1/occludin association according to IP of occludin, and WB analysis of associated ZO1 of human AECs, after 3-hour exposure to 50 $\mu\text{g}/\text{cm}^2$ DEPs in the presence or absence of PKC- ζ ps. ns, no significance, $P > 0.05$. ** $P < 0.005$. MM, molecular mass; R.U., relative units.

regarding size, surface area, and aggregation patterns. Despite these variables, they exert little effect in lung inflammation and TJ integrity, suggesting that the intrinsic components of DEPs are potentially responsible for the effects observed (among these components, PAHs and transition metals have been

suspected). Regardless of this limitation, our results confirm what other authors have reported in terms of DEP-induced lung inflammation (31). In addition, we have described a novel mechanism for DEP-induced lung injury. We demonstrate that PKC- ζ activation is necessary and sufficient to disrupt TJs and

Figure 5. (A) Level of cell-surface occludin and E-cadherin according to WB of biotinylated proteins of A549 cells after 3-hour exposure to 50 $\mu\text{g}/\text{cm}^2$ DEPs in the presence or absence of PKC- ζ ps. (B) IP of occludin and WB of associated ZO1 after 3-hour exposure to 50 $\mu\text{g}/\text{cm}^2$ DEPs in control (CT) A549 cells and A549 cells transfected with constitutively active PKC- ζ plasmid (Active PKC- ζ DNA). (C) IP of occludin and WB of associated ZO1 after 3-hour exposure to 50 $\mu\text{g}/\text{cm}^2$ DEPs in A549 cells transfected with scramble siRNA or anti-PKC ζ small interfering (si)RNA. ns, no significance, $P > 0.05$. * $P < 0.01$. ** $P < 0.005$.

Figure 6. Schematic representation of proposed pathway. ROS, reactive oxygen species; TJ, tight junction.

epithelial barrier in AECs, favoring the recruitment of neutrophils and the development of DEP-induced lung injury.

PKC- ζ has been previously implicated in mediating alveolar epithelial disruption in response to hypoxia in alveolar epithelia (14) and the blood–brain barrier (13), as well as in the regulation of the endothelial barrier (32). This kinase also regulates occludin distribution and the assembly of TJs in Madin-Darby canine kidney and Caucasian colon adenocarcinoma (Caco-2) cells (33). Atypical PKCs, such as PKC- ζ , directly associate with the C-terminal domain of ZO1 and occludin (16). The phosphorylation of specific residues in the C-terminal domain of occludin determines its subcellular localization (33–36). This occludin phosphorylation may play opposing roles in distinct biological systems, or alternatively, the phosphorylation of different residues may have different consequences (37). As evidenced by our results, PKC- ζ seems to play a role in the maintenance of occludin at the plasma membrane in AECs, because the inhibition of PKC- ζ by itself is able to decrease the abundance of occludin at the plasma membrane of AECs (Figure 3B). Despite these changes in occludin distribution, the G_i of AECs was not affected by the PKC- ζ inhibitor. Furthermore, blocking PKC- ζ activation maintained the G_i and the ZO1–occludin association after exposure to DEPs, suggesting that this association is ultimately responsible for the integrity of TJs and the epithelial barrier. This result is consistent with previous reports showing that although occludin knockout mice have normal TJs and epithelial barrier phenotypes (38), TJs are not formed with the combined suppression of ZO1/ZO2 expression (39), demonstrating that ZO proteins are sufficient and necessary for TJ formation and epithelial barrier integrity. In addition, evidence exists that ZO proteins play not only a structural role but also a signaling role in the formation and integrity of TJs (40).

Although DEPs in our model did not activate PKC- ζ in alveolar macrophages, the interactions between alveolar macrophages, alveolar epithelia, and endothelia play a role in the development of inflammation in response to inhaled DEPs. PKC- ζ also activates the mitogen-activated protein kinase cascade in various cells (41, 42), and the activation of PKC- ζ by DEPs could activate other pathways linked to inflammation and cytokine release, such as NF- κ B (43). Furthermore, the activation of PKC- ζ mediates lung inflammatory response to LPS and cigarette smoke in mice (25), and PKC expression is elevated in chronic inflammatory processes of the lung, such as COPD (44). The maintenance of the epithelial barrier *in vitro* by PKC- ζ inhibition and a decrease in neutrophil recruitment *in vivo* after the instillation of DEPs indicate that the activation of PKC- ζ in AECs is necessary for DEP-induced lung injury. However, the activation of PKC- ζ in other cell populations that we did not study (such as endothelial cells and neutrophils) could exert an influence on the *in vivo* effects observed with PKC- ζ ps treatment.

The evidence presented here shows that DEPs affect the alveolar epithelial barrier through a PKC- ζ -mediated pathway, thus broadening our knowledge of the mechanisms by which DEPs affect the alveolar epithelium and the role of PKC- ζ in the development of lung injury (Figure 6). Furthermore, our results raise the possibility of developing pharmacological interventions to prevent lung injury by targeting PKC- ζ in the lung.

Author disclosures are available with the text of this article at www.atsjournals.org.

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