Protein Kinase Cα and ζ Differentially Regulate Death-Inducing Signaling Complex Formation in Cigarette Smoke Extract-Induced Apoptosis

Jeong-Woong Park, Hong Pyo Kim, Seon-Jin Lee, Xue Wang, Yong Wang, Emeka Ifedigbo, Simon C. Watkins, Motoi Ohba, Stefan W. Ryter, Yatin M. Vyas and Augustine M. K. Choi

*J Immunol* 2008; 180:4668-4678; doi: 10.4049/jimmunol.180.7.4668
http://www.jimmunol.org/content/180/7/4668

References
This article cites 60 articles, 27 of which you can access for free at:
http://www.jimmunol.org/content/180/7/4668.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Protein Kinase C\(\alpha\) and \(\zeta\) Differentially Regulate Death-Inducing Signaling Complex Formation in Cigarette Smoke Extract-Induced Apoptosis

Jeong-Woong Park,*† Hong Pyo Kim,† Seon-Jin Lee,† Xue Wang,† Yong Wang,† Emeka Ifedigo,† Simon C. Watkins,‡ Motoi Ohba,§ Stefan W. Ryter,† Yatin M. Vyas,§ and Augustine M. K. Choi‡

Cigarette smoke, a major risk factor in emphysema, causes cell death by incompletely understood mechanisms. Death-inducing signaling complex (DISC) formation is an initial event in Fas-mediated apoptosis. We demonstrate that cigarette smoke extract (CSE) induces DISC formation in human lung fibroblasts (MRC-5) and promotes DISC trafficking from the Golgi complex to membrane lipid rafts. We demonstrate a novel role of protein kinase C (PKC) in the regulation of DISC formation and trafficking. The PKC isoforms, PKC\(\alpha\), PKC\(\zeta\), PKC\(\epsilon\), and PKC\(\eta\), were activated by CSE exposure. Overexpression of wild-type PKC\(\alpha\) inhibited, while PKC\(\zeta\) promoted, CSE-induced cell death. Dominant-negative (dn)PKC\(\eta\) protected against CSE-induced cell death by suppressing DISC formation and caspase-3 activation, while dnPKC\(\zeta\) enhanced cell death by promoting these events. DISC formation was augmented by wortmannin, an inhibitor of PI3K. CSE-induced Akt phosphorylation was reduced by dnPKC\(\alpha\), but it was increased by dnPKC\(\zeta\). Expression of PKC\(\alpha\) in vivo inhibited DISC formation, caspase-3/8 activation, lung injury, and cell death after prolonged cigarette smoke exposure, whereas expression of PKC\(\zeta\) promoted caspase-3 activation. In conclusion, CSE-induced DISC formation is differentially regulated by PKC\(\alpha\) and PKC\(\zeta\) via the PI3K/Akt pathway. These results suggest that modulation of PKC may have therapeutic potential in the prevention of smoke-related lung injury. *The Journal of Immunology, 2008, 180: 4668–4678.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Address correspondence and reprint requests to Dr. Augustine M. K. Choi, Pulmonary and Critical Care Medicine, Brigham and Women’s Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115. E-mail address: amchoi@rics.bwh.harvard.edu or Dr. Yatin M. Vyas, Division of Pediatric Hematology-Oncology, Children’s Hospital of Pittsburgh, 3705 Fifth Avenue, Pittsburgh, PA 15213. E-mail address: yatin.vyas@chp.edu.

2 Abbreviations used in this paper: COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; CSE, cigarette smoke extract; DISC, death-inducing signaling complex; dn, dominant negative; FADD, Fas-associated protein with death domain; FasL, Fas ligand; LDH, lactate dehydrogenase; PKC, protein kinase C.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

www.jimmunol.org
Golgi complex and then translocated to the cell surface in response to specific stimuli, including p53 activation and IFN-γ stimulation in vascular smooth muscle cells (18, 19) or in mesenchymal cells (18). Furthermore, we have previously demonstrated that both Fas and caspase-8 associate with the Golgi complex-associated protein, GRASP65, in mouse lung endothelial cells subjected to hypoxia/reoxygenation stress (20). However, nothing is known to date about CSE-induced DISC formation, its regulation, or subcellular trafficking.

The protein kinase C (PKC) family is responsible for transducing many cellular signals during cell death. The PKC family consists of at least 12 broadly expressed serine/threonine kinase isoforms that have been divided into three subgroups based on their regulation: the conventional PKCs (α, βI, βII, and γ), the novel PKCs (δ, ε, θ, µ, and η), and the atypical PKCs (ζ, λ, and η) (21). Each PKC isoform is expressed by an individual gene, except for βI and βII. PKCs are expressed in a tissue-specific manner and respond to activation by distinct stimuli (22). Differential roles of PKC in apoptosis have been reported depending on specific isoforms, cell types, and/or stimuli. For example, PKCδ potentiated Fas-mediated apoptosis in T cells (23). Thrombin-induced resistance to apoptosis in normal lung fibroblasts involved PKCe but not PKCα (24). At present, little is known about the role(s) of PKC in CSE-induced apoptosis and in the regulation of DISC formation. We hypothesized that DISC formation represents a critical event in the CSE-induced apoptotic pathway, and that CSE-induced DISC formation may be regulated by specific PKC isoforms.

In the present study, using human lung fibroblasts (MRC-5), we investigated the assembly and subcellular trafficking of the DISC in response to CSE exposure. To elucidate the proximal events in Fas signaling, we examined how specific PKC isoforms regulate CSE-induced DISC formation. In MRC-5 cells exposed to CSE, we demonstrate that the DISC formation occurs initially in the Golgi complex, which precedes its accumulation in the plasma membrane. We show for the first time that distinct PKC isoforms, PKCe and PKCζ, differentially regulate CSE-induced DISC formation. Materials and Methods

Reagents

Abs against Fas, caspase-3 PKCs, and GRASP65 were from Santa Cruz Biotechnology. Anti-caspase-8 Ab and anti-human CD95 (anti-Fas) mAb CH11 were from BD Pharmingen. For fluorescent images, affinity-purified secondary Abs and species-absorbed conjugates (FITC488, Cy3, and Cy5) for multiple labeling were from Chemicon International. All other chemicals were from Sigma-Aldrich. Adenovirus-mediated gene transfer

Replication-deficient Ad5-type adenovirus vectors containing the cDNA of both wild-type and kinase-negative mutants of rabbit PKCα and mouse PKCζ were constructed as described previously (25).

Preparation of CSE

Kentucky 1R3F research-reference filtered cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, KY) were smoked using a peristaltic pump (VWR International). Before the experiments, the filters were cut from the cigarettes. Each cigarette was smoked in 6 min with a 17-mm butt remaining. Four cigarettes were bubbled through 40 ml of cell growth medium, and this solution, regarded as 100% strength CSE, was adjusted to a pH of 7.45 and used within 15 min after preparation.

Cell culture and treatments

The MRC-5 were cultured in DMEM containing 10% FBS, in humidified incubators at 37°C. For adenoviral transfections, cells were grown to 30% confluence and changed to serum-free medium containing 10⁶ CPU/ml of an adenoviral vector inserted with wild-type or dominant negative (dn)PKCe, PKCζ, or LacZ. Infected cells were incubated for 3 h, then restored to normal DMEM medium containing 10% FBS for an additional 2 days incubation. For CSE treatment, the MRC-5 were grown to 90% confluence and restored to fresh medium. MRC-5 were also treated with 100 ng/ml anti-human CD95 (anti-Fas) mAb as a positive control. Fas+/− fibroblasts were harvested from the lungs of MRL-Fas lpr3 mice (stock no. 000480) (The Jackson Laboratory), according to previously described protocols (26).

Cytotoxicity and viability assays

Lactate dehydrogenase (LDH) release was measured using a cytotoxicity detection kit (Roche Molecular Biochemicals), according to the manufacturer’s protocol. After gentle agitation, 200 μl of culture medium was removed at various times for the assay. For colorimetric MTT assay, MRC-5 cultured in DMEM containing 10% FBS were treated with CSE after adenoviral infection. After incubation for the indicated time, the culture medium was removed and 20 μl of 5 mg/ml MTT was added. Four hours later, the supernatant was discarded and 100 μl DMSO was added to each well. The mixture was shaken and measured at 595 nm using an ELX800 universal microplate reader (BioTek Instruments).

Cell fractionation

Total membrane fraction was isolated as described previously (27). MRC-5 were harvested in MB buffer (20 mM HEPES (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (trichloroetho, 250 mM sucrose) containing protease inhibitors and homogenized in a 1.5-ml Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation for 10 min at 500 × g, and the supernatants were centrifuged at 100,000 × g for 60 min at 4°C. The resulting pellet contained total cellular membranes.

The lipid rafts fraction was isolated as described previously (28). MRC-5 were harvested and homogenized in MBS (25 mM N-morpholinoethanesulfonic acid (pH 6.5), 0.15 M NaCl) containing 1% Triton X-100. Homogenates were adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube. A 5–30% density gradient of sucrose was used. The gradient was overlaid with 1.2, 1.0, and 0.8 M of sucrose in G buffer. Samples were centrifuged at 95,000 g centrifugation for 2.5 h. Two bands from the top, representing 0.8/1.0 and 1.0/1.2 M interfaces, were carefully removed and diluted with 0.8 M sucrose (4 ml of 30% sucrose, both in MBS lacking detergent) and centrifuged at 39,000 rpm for 18 h in a SW41 rotor (Beckman Instruments). A band at the interface of 5 and 30% sucrose was collected and used for immunoprecipitation and Western blot.

The Golgi complex was isolated using sucrose density gradient centrifugation, as described previously (20). After washing with PBS, the cells were harvested in G buffer (10 mM Tris-HCl, 0.25 M sucrose, 2 mM MgCl₂ (pH 7.4)) containing 10 mM CaCl₂, and protease inhibitors. The cells were disrupted with 20 strokes in a Potter-type homogenizer. The homogenate was centrifuged at 2500 × g for 10 min and the pellet was discarded. The resulting postnuclear supernatant was harvested and the sucrose concentration adjusted to 1.4 M final concentration. This suspension was loaded onto the bottom of an ultracentrifuge tube and overlaid in sucrose with 1.2, 1.0, and 0.8 M of sucrose in G buffer. Samples were then centrifuged at 95,000 × g for 2.5 h. Two bands from the top, representing 0.8/1.0 and 1.0/1.2 M interfaces, were carefully removed and diluted with G buffer without sucrose, collected by centrifugation at 80,000 × g for 30 min, and used for the experiments.

Immunoprecipitation and Western blot analysis

Proteins were isolated from the culture of MRC-5 with radiomunoprecipitation assay (RIPA) buffer (1× PBS, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.1 mg/ml PMSF, 30 μl/mg aprotinin, 1 mM sodium orthovanadate). For immunoprecipitation, 1 μg of anti-Fas Ab was added to 500 μg of total protein in 500 μl, rotated for 2 h at 4°C, then incubated with 20 μl of protein A-seaure membranes (Santa Cruz Biotechnology) for another 2 h, spun down at 500 × g, and washed three times with RIPA buffer. Next, 20 μl of loading buffer (100 mM Tris-HCl, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol) was added. SDS-PAGE and Western Immunoblot analysis were then performed.

Caspase activity assays

Caspase-3 fluorogenic substrate, Ac-DEVD-afc, was from BD Biosciences. Caspase activity in cell lysates was determined according to the manufacturer’s instructions, using an Aminco-Bowman series-2 spectrophotometer (440/500-nm excitation/ emission), and expressed as fold increase of caspase-3 activity over control.
**Immunofluorescent cell imaging**

Based on the Nomarsky images, MRC-5 cells were selected for fluorescent analysis in the same focal plane. Immunofluorescent-labeled cells were analyzed after fixing at the indicated time points, as previously described (29). In all experiments, a Zeiss upright research microscope with Everest digital microscopy workstation, which uses the SlideBook image acquisition and processing software (Intelligent Imaging Innovations), was used. Images were obtained in two-dimension (x-y axis) and processed using Photoshop 7.0 software (Adobe Systems).

**Immunohistochemistry**

The smoked mouse lung sections were fixed in 10% formaldehyde for 24 h and processed for paraffin embedding. To perform the immunohistochemistry, paraffin-embedded tissues were deparaffinized in xylene, rehydrated, retrieved, and immunostained with anti-Fas and anti-caspase-8 (Santa Cruz Biotechnology). The positively staining area, brown in color, was determined against the negatively staining region, shown relatively as blue in color.

**In vivo cigarette smoke exposure**

Animals were housed according to guidelines from the American Association for Laboratory Animal Care and Research Protocols and were approved by the Animal Care and Use Committee (University of Pittsburgh School of Medicine). Male, age-matched C57BL/6 strain mice (The Jackson Laboratory) were exposed to CS or filtered air under identical conditions beginning at 8 wk of age. Total-body CS exposure was performed in a stainless steel chamber (71 x 61 x 61 cm) using a smoking machine (Model TE-10, Teague Enterprises), similar to that reported by others (30). The smoking machine puffs each 1R3F cigarette for 2 s, for a total of 9 puffs before ejection, at a flow rate of 1.05 L/min, providing a standard puff of CSE (0–40%) for 1 h, and DISC was analyzed. Total Fas (A) or IgG bands (A and B) were used as loading controls. C, Immunofluorescence images of MRC-5 double-labeled with indicated Abs (blue and green) are shown. The cyan pseudocolor (arrow, top panel) indicates a colocalization of Fas and caspase-8. The same images with either the green or blue color removed are shown for clarity (middle and bottom panels). Data in this figure are representative of 20–49 cells analyzed for each time point. All panels are the same scale.

**Statistical analysis**

All values are expressed as means ± SE. Statistical significance was determined by Student’s t test, and a value of p < 0.05 was considered significant.

**Results**

CSE induces MRC-5 cell death through the extrinsic apoptotic pathway

DISC formation represents the most proximal event in the Fas-mediated extrinsic apoptotic pathway (15). We evaluated the DISC formation at both the population and single-cell levels by biochemical and fluorescent imaging analysis. First, we examined the kinetics of DISC formation as a function of CSE exposure in MRC-5 cells. MRC-5 cells were treated with 20% CSE for 0, 1, 3, and 6 h. Cell lysates were immunoprecipitated with anti-Fas and immunoblotted with anti-caspase-8 (p43/41) to detect DISC formation. The DISC formed maximally at 1 h and then declined to background levels by 6 h (Fig. 1A). The total level of Fas was unaltered by CSE treatment at the various exposure times (0–6 h), and therefore it was used as a loading control along with IgG (Fig. 1A). In a dose-response experiment, maximum DISC formation was observed after 1 h exposure to CSE concentrations of 10–20%, but it was diminished at higher concentrations of CSE (>30%) (Fig. 1B). Next, we examined DISC formation in single cells using immunofluorescence microscopy. MRC-5 cells were double-labeled with a rabbit anti-Fas polyclonal Ab (blue) and mouse anti-caspase-8 mAb (green) after exposure to 20% CSE for 0, 15, 30, 60, and 180 min (Fig. 1C). The images show a time-dependent increase in CSE-induced DISC formation in individual cells, as evidenced by the cyan pseudocolor resulting from the colocalization of Fas (blue) and caspase-8 (green). CSE treatment of MRC-5 resulted in the time-dependent increase in DISC-positive cells. Consistent with the above biochemical studies, the single-cell image studies demonstrated maximum DISC formation at 1 h CSE exposure with 59% (n = 49) of cells displaying DISC formation at this time (Fig. 1C).
Next, we investigated the effect of CSE on MRC-5 cell death. We monitored CSE-induced toxicity by assaying LDH release in the media as a measure of cell death, and we also used the MTT assay as a measure of cell viability. For LDH release assay, MRC-5 cells were exposed to 20% CSE for 0, 10, 24, and 48 h. As shown in Fig. 2A, LDH release following CSE exposure was significantly increased in a time-dependent manner relative to untreated cells. CSE decreased cell viability after 24 h exposure in a dose-dependent manner, with the most significant reduction in cell viability (43%) occurring at 20% concentration (Fig. 2B). Thus, subsequent observations in this study were made at the CSE dose and kinetic combinations that preceded the appearance of significant LDH release or loss of viability.

We next evaluated the role of the Fas-dependent apoptotic pathway in MRC-5 cell death. MRC-5 cells treated with anti-Fas agonistic Ab (anti-CD95) sustained significant loss of cell viability, which validates a role for the Fas-dependent signaling pathway in fibroblast cell death (Fig. 2C). Furthermore, lung fibroblasts...
derived from Fas−/− mice were protected against cell death induced by CSE (20%) in vitro, relative to wild-type fibroblasts that displayed significant cell death under these conditions (Fig. 2D), indicating a major role for Fas-dependent pathways in fibroblast cell death induced by CSE.

We also evaluated the activation of caspase-8 as a function of CSE exposure, because DISC formation triggers the proteolytic autoactivation of caspase-8, which in turn activates downstream caspase-1 and caspase-3. After exposing MRC-5 to 20% CSE for 0, 1, 3, 6, and 12 h, cell lysates were immunoblotted with anti-caspase-8. There was a clear increase in the appearance of the cleaved caspase-8 subunit p18 after 6 h of CSE exposure (Fig. 2E). Caspase-3 is an executioner caspase, the activation of which represents a distal event in apoptosis-signaling pathways. Caspase-3 is normally present in an inactive proenzyme form, but it can be activated by proteolytic processing of its inactive zymogen into its cleaved p17 and p19 forms. There was a clear expression of the cleaved caspase-3 subunit p19 after 6 h of CSE exposure (Fig. 2E). Collectively, these results indicate that CSE exposure induced MRC-5 cell death in a time- and dose-dependent manner, and that this cell death is dependent on the activation of the Fas/caspase-8 signaling pathway.

We also examined Fas and caspase-8 expression in the lung sections from mice that were exposed to CS, 5 days per week for 24 wk with each exposure for an average of 3 h. The smoke treatment conditions were designed to mimic passive smoke exposure of humans in whole-body exposure chambers. As shown in Fig. 2F, immunohistochemical staining of lung tissue sections of CS-exposed mice displayed increased inflammatory cell content, including alveolar macrophages, emphysematous dilatation, and increased expression of Fas and caspase-8 in alveolar macrophages and septal wall cells, relative to lungs from sham-treated mice. These results support the notion that Fas-dependent cell death may occur as a component of tissue injury during in vivo CS exposure.

Subcellular trafficking of DISC in CSE-induced apoptosis

We examined the subcellular localization of the DISC in MRC-5 cells in response to CSE. MRC-5 cells were fractionated to isolate Golgi complex, or membrane lipid rafts and immunoprecipitation studies were performed to identify the DISC. As shown in Fig. 3A, the DISC formed as early as 30 min after treatment with 20% CSE in the Golgi fractions. The level of Golgi-associated DISC declined thereafter in a time-dependent manner. In contrast to that of the Golgi complex, the level of DISC formation in the lipid rafts increased in a time-dependent manner following CSE exposure (Fig. 3A). We next analyzed DISC formation in response to 20% CSE exposure at the single-cell level using immunofluorescence microscopy. MRC-5 cells were triple-labeled with Fas, caspase-8, and GRASP65, a marker of Golgi (31) as shown in Fig. 3B. In the steady state, Fas and caspase-8 were present in both the Golgi and extra-Golgi subcellular compartments, predominantly demonstrating a nonoverlapping distribution (Fig. 3B). After 3 h, although Fas and caspase-8 were still detected inside the Golgi, the DISC, identified by the yellow pseudocolor from the overlap of red and green, was formed outside the Golgi complex in 40% of CSE-treated cells (n = 35) (Fig. 3B, top panels). The DISC as indicated by cyan pseudocolor (Fig. 3B, bottom panels) was associated with the lipid rafts in 42% of cells examined (n = 41). The results show that the DISC is preformed in the Golgi complex and translocated to the plasma membrane during CSE exposure.

CSE induces PKC activation in MRC-5 cells

Because PKC has emerged as a potent regulator of apoptotic pathways, we evaluated the effect of PKC on the extrinsic apoptotic pathway induced by CSE, mainly focusing on DISC formation. First, we investigated whether CSE can alter the expression or activation of PKC isoforms in MRC-5 cells by Western immunoblotting. PKCγ and PKCξ was not altered in response to exposure to 20% CSE (Fig. 4A). The expression level increased in PKCα and decreased in PKCζ in a time-dependent manner, whereas it remained unchanged in the remainder of PKC isoforms tested (data not shown). To evaluate PKC activation, MRC-5 cells treated with 20% CSE for 0, 0.5, and 1 h were fractionated to isolate membrane and cytosolic fractions, as described in Materials and Methods. Both fractions were immunoblotted with antisera against various PKC isoforms. After 30 min of CSE exposure, the membrane fraction of MRC-5 exhibited increased PKCα and ζ (Fig. 4B) as well as PKCε and η (data not shown).
showed), indicating activation of these PKC isoforms by CSE exposure. The remainder of PKC isoforms tested did not show expression in the membrane fraction (data not shown).

PKCs regulate CSE-induced cell death in MRC-5

Next, we investigated the relative role of PKCα and PKCζ on DISC formation in the CSE-induced extrinsic apoptotic pathway. MRC-5 at 30% confluence were infected with adenovirus containing cDNAs for LacZ, or dnPKCα for 48 h, and exposed to 20% CSE for 0, 1, 3, and 6 h. The DISC formed in both dnPKCα and LacZ-infected cells subjected to CSE, with a maximum at 1 h (Fig. 5A). However, DISC formation in response to CSE was increased in cells infected with dnPKCα relative to LacZ-infected cells (Fig. 5A). To obtain fluorescent images at a single cell level, MRC-5 cells triple-labeled with Fas, caspase-8, and GRASP65 were analyzed after 1 h CSE treatment. The cyan pseudocolor in the merged images indicates a colocalization of Fas and caspase-8, showing a...
stronger fluorescent signal in the cells infected with dnPKCα compared with LacZ-infected cells (Fig. 5B), consistent with the biochemical observations (Fig. 5A). DISC-positive cells were increased in dnPKCα-infected cells (73%) compared with LacZ-infected cells (45%) (n = 45–55). We assessed the effect of PKCα on CSE-induced cell death, using the MTT assay. MRC-5 infected with dnPKCα had lower viability after CSE challenge relative to the LacZ-infected cells (Fig. 5C). Consistent with the observation in Fig. 5A, DISC formation was markedly diminished in PKCα-infected cells relative to that observed in LacZ-infected cells (Fig. 5D). Consequently, PKCα-infected cells were more resistant to CSE exposure (Fig. 5E). The efficiency of adenoviral infection was determined in LacZ-infected cells by monitoring β-galactosidase activity (Fig. 5F). To investigate the role of PKCζ on CSE-induced apoptosis, MRC-5 cells were infected with dnPKCζ, and its effect on CSE-induced DISC formation was assessed. The DISC formation in MRC-5 infected with dnPKCζ was decreased relative to LacZ-infected cells (Fig. 6A). Single-cell fluorescent imaging of CSE-treated MRC-5 cells triple-labeled with anti-GRASP65, anti-caspase-8, and anti-Fas demonstrated the formation of DISC (i.e., colocalized distribution of caspase-8 and Fas) in only 13% of dnPKCζ-expressing cells compared with 46% in the LacZ control (n = 47 for dnPKCζ, n = 52 for LacZ control) (Fig. 6B). We also

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** PKCζ promotes CSE-induced cell death in MRC-5. A, MRC-5 cultured at 30% confluence were infected with 10⁶ CPU/ml dnPKCζ and LacZ in serum-free medium. Cells were incubated for 3 h and then restored to normal DMEM medium containing 10% FCS for an additional 2 days of incubation. Cells were exposed to 20% CSE for the indicated times, and cell lysates were subjected to immunoprecipitation with anti-Fas Ab, followed by immunoblotting with caspase-8. B, Representative fluorescent images of each of DISC in dnPKCζ- or LacZ-infected MRC-5 cells. Cells triple-labeled with indicated reagents (green, blue, and red) are shown as an overlay of Nomarski and fluorescent images (left column) or of corresponding fluorescent images (right column). The cyan pseudocolor (boxed area) in each panel indicates DISC. Data in this figure are representative of ~47–52 cells analyzed for each condition, from two to three independent experiments. C, MTT assay was used to evaluate the viability after 24-h incubation with 20% CSE. Data from dnPKCζ-infected cells were compared with LacZ-infected cells at 0 and 20% CSE concentrations. D, MRC-5 infected with PKCζ or LacZ were exposed to the different concentrations (0–40%) for 48 h, and cell cytotoxicity was assessed by lactate dehydrogenase assay. E, The caspase-3 activity was assessed in dnPKCα- and ζ-infected cells, and compared with LacZ at each time point. Data in C–E were analyzed using Student’s t test. *, p < 0.05; **, p < 0.01.

![FIGURE 7](http://www.jimmunol.org/)

**FIGURE 7.** PKCα and ζ differently modulate Akt phosphorylation on Ser473. A, MRC-5 infected with dnPKCα, dnPKCζ, or LacZ were exposed to 20% CSE for 1 h. Expression of phospho-Akt was analyzed by immunoblotting using total cell lysates. B, Effect of the PI3K inhibitor, wortmannin, on DISC formation. Wortmannin (10 μM) was used to treat the MRC-5 for 1 h before the treatment with 20% CSE for different time periods (0–1 h). Fas was immunoprecipitated with anti-Fas Ab in total cell lysates, followed by immunoblotting with anti-caspase-8 Ab.
assessed the effect of PKCζ on CSE-induced loss of cell viability using the MTT assay. MRC-5 infected with dnPKCζ displayed increased cell survival relative to the LacZ-infected cells after CSE exposure (Fig. 6C). In contrast, cell death was increased in PKCζ-infected cells compared with LacZ-infected cells (Fig. 6D). Additionally, the activation of caspase-3 during CSE treatment of MRC-5 was increased in dnPKCα-infected cells, but it decreased in dnPKCζ-infected cells, compared with LacZ-infected cells (Fig. 6E).

These data indicate that PKCs differentially regulate the DISC formation and caspase-3 activation, and they consequently affect cell death during CSE-induced apoptosis in an isoform-specific fashion.

PKCα and PKCζ differentially modulate Akt phosphorylation at Ser473

The PI3K/Akt pathway is an important determinant of apoptosis and survival in many cell types. Phosphorylation of Akt at Ser473 is critical for preventing activation-induced apoptosis (32). Because our studies showed differential effects of PKCα and PKCζ on CSE-induced DISC formation and apoptotic cell death, we hypothesized that the two PKC isoforms would also differentially regulate activation of Akt. To evaluate the effect of PKCα and PKCζ on Akt phosphorylation, MRC-5 were infected with dnPKCα, dnPKCζ, or LacZ, exposed to 20% CSE for 1 h, and followed by immunoblotting analysis for Akt activation as shown in Fig. 7A. Akt phosphorylation on Ser473 was reduced in

FIGURE 8. PKCα protects mouse lung against cigarette smoke-induced apoptosis in vivo. A, Mice were subjected to intratracheal administration of adenovirus containing either PKCα or LacZ and then exposed to CS or sham treatment for 4 wk as described in Materials and Methods. Lung tissue homogenates were evaluated for DISC formation, as detected by immunoprecipitation with anti-Fas Ab, followed by immunoblotting with anti-caspase-8 Ab. Total Fas served as the standard. B, Results were quantified by densitometry. A p value of <0.05 was considered a significant difference between groups (n = 3 mice). Inset, PKCα expression in lung tissue following adenoviral infection with either PKCα or LacZ was determined by Western immunoblot analysis (top panel), with total Fas as the standard. C, Lung homogenates were also evaluated for caspase-8 and caspase-3 cleavage by Western immunoblot analysis. D, Lung tissue sections were evaluated for apoptosis by TUNEL staining. Arrowheads indicate TUNEL-positive cells. Image is at ×10 magnification. E, Lung tissue sections were analyzed for tissue injury using H&E staining. F, Mice were subjected to intratracheal administration of adenovirus containing either PKCζ or LacZ and then exposed to CS or sham treatment for 4 wk as described in Materials and Methods (n = 5 mice for each group). PKCζ expression in lung tissue following adenoviral infection with either PKCζ or LacZ was determined by Western immunoblot analysis after 4 wk cigarette smoke exposure, with β-actin as the standard (top panel). Lung homogenates were evaluated for caspase-3 cleavage by Western immunoblot analysis (n = 5; p < 0.05).
PKCα and PKCζ regulate CSE-induced cell death in vivo

Finally, we evaluated the role of PKCα in an in vivo model of smoke exposure. Mice were infected with adenovirus containing PKCα or LacZ and exposed to CS for 4 wk. Mice infected with PKCα displayed diminished DISC formation in total lung tissue, relative to LacZ-infected mice, as detected by immunoprecipitation of lung homogenates with Fas followed by immunoblotting with caspase-8 (Fig. 8A and B). Additionally, diminished caspase-8 and caspase-3 activation were detected in lung tissue of the CS-exposed mice infected with PKCα relative to LacZ-infected controls (Fig. 8C). The antiapoptotic effect of PKCα in vivo was confirmed by TUNEL staining. Lung tissue from PKCα-infected mice displayed a diminished number of TUNEL-positive cells relative to LacZ-infected controls after 4 wk of CS exposure (Fig. 8D). H&E staining (Fig. 8E) clearly shows that PKCα-infected mice were protected against lung injury in response to 4 wk CSE. Lungs from PKCα-infected smoke-exposed mice showed similar histology as their sham-exposed counterparts or as LacZ-infected wild-type mice. In contrast, LacZ-infected mice showed clear histological indication of lung injury after 4 wk CSE.

Mice were also infected with PKCζ and exposed to cigarette smoke for 4 wk. As shown in Fig. 8F, PKCζ infection augmented the activation of caspase-3 in lung tissue in response to cigarette smoke exposure, relative to LacZ-infected mice.

Discussion

Although the role of apoptosis in CSE-induced cell death remains incompletely understood (33–34), we demonstrate that CSE exposure induces an extrinsic apoptotic pathway involving DISC formation and downstream activation of caspases-8/caspase-3 in MRC-5 cells (Figs. 1 and 2). Upon treatment with CSE, we observed a rapid time-dependent intracellular DISC formation within the Golgi complex (Fig. 3A). Both caspase-8 and Fas colocalized with the Golgi complex-associated protein, GRASP65 in MRC-5 cells under untreated conditions, although a portion of Fas and of caspase-8 were also separately distributed in the cytoplasm outside the Golgi complex (Fig. 3B). These observations are consistent with previous findings that cells can express Fas both intracellularly and at the cell surface after translation of Fas, and that cytoplasmic Fas predominantly localizes to the Golgi complex (35). Other DISC components, such as FADD and caspase-8, may also localize within the Golgi complex for processing and then transfer to the plasma membrane (36–37).

Since the ligand for Fas (FasL) is extracellular, Fas membrane trafficking must occur for maximal signaling (16, 17, 38), a critical parameter in determining the ability of the cell to undergo apoptosis (39). Fas, however, can also be activated in a ligand-independent fashion (40), resulting in DISC formation and activation of caspase-8, which subsequently triggers a downstream signaling cascade that culminates in apoptosis. Previous studies reported that activation of membrane Fas leads to the recruitment of FADD and caspase-8 to lipid rafts in mouse thymocytes (41), human CD4+ T cells (27), and human lymphoblastoid CEM cells (28). In contrast, we demonstrated that intracellular assembly of the DISC occurs first within the Golgi complex before its translocation to the lipid rafts (Fig. 3). These results suggest that the Golgi-associated DISC forms independently of FasL and translocates to the plasma membrane where FasL-mediated apoptosis may be initiated and amplified. We previously observed similar Golgi-to-plasma membrane translocation of the DISC in mouse lung endothelial cells subjected to hypoxia-reoxygenation stress (20, 42).

The DISC, which represents the apical event in the extrinsic apoptotic pathway, is subject to multiple regulatory mechanisms, including transcription factors such as NF-κB, AP-1, Stat-3 (43–44), and the PI3K/Akt signaling pathway (45). Inhibition of JNK or PKC can abolish Fas/CD95-tyroisine phosphorylation, which in turn reduces Fas membrane trafficking and DISC formation (38). The protein-tyroisine phosphatase FAP-1 can suppress Fas trafficking to the cell surface (39, 46, 47), whereas dynamin-2 facilitates Fas translocation by protein–protein interaction (48), implicating that Fas-tyroisine phosphorylation is required for the DISC formation. We have previously shown that DISC formation during hypoxia-reoxygenation-induced endothelial cell apoptosis was inhibited by expression of the endogenous caspase-8 inhibitor FLIP (42) and of Bcl-XL (20). We also reported that DISC formation during hyperoxia-induced endothelial cell apoptosis depended on reactive oxygen species generation, and could be inhibited by carbon monoxide, as well as by inhibitors of ERK1/2 MAPK or NADPH oxidase-dependent pathways (49).

Previous studies have demonstrated that PKCs do not modulate the expression levels of Fas (50–51) or FADD (51). Furthermore, modulation of FADD phosphorylation state by PMA had no effect on the affinity of FADD for either the Fas DISC (52, 53) or the TRAIL DISC (54). The relative role of individual PKC isoforms in Fas-mediated apoptosis and DISC formation remains unclear. Little is known about the expression or activation state of PKCs in response to CSE exposure, or about their roles in CSE-induced apoptosis. CSE induced the plasma membrane translocation of PKC, which preferentially involved the PKCα, ζ (Fig. 4B), e, and η isoforms. Of these active forms, PKCα, a conventional PKC, has been implicated as a regulator of many processes associated with apoptosis in lung cells (42, 55). In our current study, we focused on the effects of PKCα and ζ in CSE-induced apoptosis. Further studies may be required to elucidate the roles of PKCe or PKCζ.

The results of the current study suggest that PKCα and PKCζ, which are both activated by cigarette smoke, exert opposing effects on apoptosis. Since apoptosis is induced by CSE in wild-type cells, we hypothesize that proapoptotic processes, including the influence of PKCζ, are dominant under these conditions. Consistent with this hypothesis, adenosinergic-mediated expression in vitro and in vivo of PKCα protected against cell death and lung injury in response to CSE, whereas expression of PKCζ promoted cell death and lung apoptosis (Figs. 5, 6, and 8). We showed that inhibition of PKCα with a dominant negative mutant augmented CSE-dependent DISC formation and cell death (Fig. 5C), whereas expression of PKCα inhibited DISC formation. This result is consistent with previous studies that PMA-induced PKC, mostly representing activation of conventional PKCs, protected against apoptosis. PMA treatment inhibited TRAIL-induced cytotoxicity by decreasing the recruitment of key obligatory death domain-containing adaptor proteins (54), and it also inhibited Fas binding to FADD in Jurkat cells (52). In contrast, we observed that PKCζ exerted the opposite effect of PKCα in CSE-induced DISC formation. This apparent proapoptotic effect of PKCζ in the current model contrasts with several previous studies. For example, PKCζ inhibited DISC formation in acute myeloid leukemia cells, possibly through phosphorylating FADD (56), as well as in Jurkat cells during FasL-induced apoptosis (51). In contrast, PKCζ exerted a proapoptotic signaling role in rat hepatocytes, whereby PKCζ mediated...
the ceramide-dependent activation of NADPH oxidase, leading to DISC formation, in response to FasL stimulation (40).

The mechanism(s) by which PKC\(\alpha\) and \(\zeta\) regulate DISC formation in the CSE-induced apoptosis model remain unclear; however, the experiments (Fig. 7) strongly suggest a role for the prosurvival PI3K/Akt pathway. An interaction between PKC\(s\) and the Akt pathway was previously reported to occur during UV light-induced apoptosis, where PKC\(\alpha\) inhibited Akt function, whereas PKC\(\zeta\) enhanced the phosphorylation of Akt on Ser\(^{377}\) (57). In the present study, the introduction of dnPKC\(\alpha\) reduced, whereas dn PKC\(\zeta\) increased, Akt phosphorylation (Fig. 7A). Furthermore, DISC formation in response to CSE was significantly increased in wortmannin-treated cells (Fig. 7B), suggesting that the opposing effects of these PKCs may be at least in part attributable to Akt phosphorylation status.

Interestingly, the lipid-derived apoptosis mediator ceramide can regulate PKCs in several systems. Ceramide inhibited PKC\(s\) without altering PKC\(\alpha\) protein levels (58). In lung endothelial cells, CS induced a rapid, sustained ceramide up-regulation (59). Lung ceramide levels were also markedly higher in subjects with emphysema from chronic cigarette smoking (59). We speculate that CS may inhibit the antiapoptotic role of PKC\(s\) in vivo through ceramide generation. In contrast, PKC\(\zeta\) is activated in vitro by ceramide, as well as in National Institutes of Health 3T3 fibroblasts treated with sphingomyelinase (60). Given that relationships between ceramide generation (as observed in chronic smokers and in vitro) and PKC regulation have been observed, we hypothesized that both PKC\(\alpha\) and PKC\(\zeta\) may modulate apoptotic phenotypes in vivo in the context of chronic smoke exposure. Accordingly, we demonstrated that expression of PKC\(\alpha\) in vivo reduced lung cell apoptosis in response to prolonged cigarette smoke exposure. Specifically, PKC\(\alpha\) expression inhibited DISC formation, caspase-8/ caspase-3 activation, and histological indications of tissue injury and apoptosis in smoke-exposed lung, whereas PKC\(\zeta\) augmented caspase-3 activation in smoke-exposed lung.

The focus of the present paper is to characterize the apoptotic responses of lung fibroblasts in response to cigarette smoke. The lung is a heterogeneous organ consisting of multiple cell types including fibroblasts of the lung parenchyma, as well as bronchial, airway, and alveolar epithelial cells, endothelial and smooth muscle cells of the pulmonary vasculature, smooth muscles of the airway, alveolar macrophages, and other specialized cells (such as mast cells). We cannot exclude the possibility that apoptotic responses in other cell types may have an equal or perhaps greater contribution to the pathogenesis of pulmonary illnesses triggered by cigarette smoke. We chose in the present study to perform a detailed mechanistic study of apoptosis in one cell type (fibroblasts). The characterization of apoptotic responses and mechanisms in all of the cell types of the lung is beyond the scope of this paper. We have, however, conducted in vivo experiments to extrapolate the findings to total lung tissue (Fig. 8). We show that expression of PKC isoforms in vivo differentially modulates apoptosis and tissue injury in the lungs of chronic cigarette smoke-exposed mice.

We have observed that cigarette smoke induces DISC formation and caspase-8 activation in human bronchial epithelial cells (Beas-2B) (X. Wang, H. P. Kim, M. H. Huang, S. W. Ryter, and A. M. Choi, unpublished observations), which is consistent with the fibroblast results. Further experimentation is warranted to determine activation profiles of PKC isoforms in other cell types such as epithelial cells.

We also evaluated the expression of PKC isoforms in human clinical samples with COPD relative to that of normal control lung. The expression of PKC\(\alpha\) and PKC\(\zeta\) were elevated in COPD, with dominant expression of the proapoptotic isoform PKC\(\zeta\) at advanced stages of disease progression (data not shown).

In conclusion, our observations have several important implications for CSE-induced apoptosis. First, we demonstrate that CSE induces intracellular DISC formation in MRC-5 cells, involving the recruitment of procaspase-8 and FADD to Fas within the cytosolic Golgi compartment. This event appears to represent a prerequisite for translocation of the preformed DISC to the lipid rafts, and it likely occurs in a FasL-independent fashion. Thus, the appearance of DISC in the lipid raft may represent the initial event in a FasL-independent extrinsic apoptosis pathway triggered by CSE, although it may also serve to facilitate subsequent FasL-induced apoptosis. Second, our results indicate that PKC activation modulates DISC assembly at the level of recruitment of procaspase-8 to Fas, providing additional insight into the mechanisms underlying CSE-induced Fas-mediated apoptosis. Furthermore, our data support the view that distinct PKC isoforms may have differential effects on DISC formation (40, 48, 50–54, 56). The phosphorylation status of Akt may partly affect the DISC formation. We cannot exclude, however, that PKCs may regulate DISC formation by modulating the phosphorylation state of other DISC components. We have also shown that PKC isoforms can differentially modulate lung injury and cell death in vivo. Finally, our data suggest that PKC-modulating compounds may be useful tools for regulating apoptosis, and furthermore, that they may have potential applications in CS-related diseases that have limited therapeutic options at present.


