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Original article:

4-HYDROXY-2-NONENAL INDUCES ENDOTHELIAL CELL INJURY VIA PKCδ AND BIPHASIC JNK ACTIVATION

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ABSTRACT

4-Hydroxy-2-nonenal (4-HNE), a major product generated during oxidative stress, exhibits cytotoxic effects; however, the mechanisms of 4-HNE-induced endothelial cell injury are not well defined. To explore this issue, we examined how 4-HNE damages human umbilical vein endothelial cells (HUVECs) and found that 4-HNE induced biphasic activation of c-Jun N-terminal kinase (JNK). Both pre- and post-treatment of HUVECs with SP600125, a specific JNK inhibitor, significantly suppressed the cytotoxic effects of 4-HNE. Inhibition of protein kinase Cδ (PKCδ), which was also phosphorylated by 4-HNE, reduced endothelial cell injury as well as late-phase JNK phosphorylation elicited by 4-HNE. Inversely, pre-treatment of HUVECs with SP600125 suppressed PKCδ activation. Taken together, these results support the concept that 4-HNE induces vascular endothelial cell injury by the interaction between biphasic JNK activation and the PKCδ pathway.

Keywords: oxidative stress; cell injury, 4-Hydroxy-2-nonenal; endothelial cell; protein kinase Cδ; c-Jun N-terminal kinase

INTRODUCTION

A consequence of oxidative stress is peroxidation of membrane lipids, resulting in the generation of several highly reactive aldehydes which react with and alter the function of proteins and nucleic acids. Among aldehydes, the α,β-unsaturated aldehyde 4-hydroxy-2-nonenal (4-HNE) is a major product of the lipid peroxidation process (Petersen and Doorn, 2004) that exhibits a wide array of biological activities, including signal transduction, gene expression and modulation of cell proliferation and survival (Leonarduzzi et al., 2004).

4-HNE is detected in various lung and cardiovascular diseases, including chronic obstructive pulmonary disease (COPD) (Rahman et al., 2002), acute respiratory distress syndrome (ARDS) (Quinlan et al., 1994), atherosclerosis (Napoli et al., 1997), and cerebral ischemia (Petersen and Doorn, 2004). Vascular endothelial injury is recognized as one of the most critical steps in the development of these diseases. 4-HNE exerts cytotoxic effects on a variety of cells from different origins (Liu et al., 2000), including several endothelial cells (Usatyuk and Natarajan, 2004); however, the precise mechanisms of 4-HNE-induced endothelial cell injury have not been adequately elucidated.

Mitogen-activated protein kinases (MAPKs), including JNK, p38-MAPK, and extracellular signal-regulated kinase (ERK), are activated in response to various stimuli and are involved in a variety of biological events, including cell proliferation, differentiation, and cell death (Johnson and Lapadat, 2002). MAPK pathways appear to be sensitive to 4-HNE (Leonarduzzi et al., 2004). While the ERK pathway is implicated in growth-associated responses, p38 and JNK pathways are generally involved in pro-apoptotic responses (Johnson and Lapadat, 2002). We have recently demonstrated that JNK is requisite for endothelial cell death induced by cigarette smoke extracts (Hoshino et al., 2005). Likewise, JNK has been described as involved in 4-HNE-induced cell death (Petersen and Doorn, 2004).

Protein kinase C (PKC) is a family of serine/threonine kinases that plays key regulatory roles in cell function, such as gene expression, and cell proliferation and differentiation. It is generally accepted that modulation of PKC activity by 4-HNE has crucial consequences on signal cascades (Leonarduzzi et al., 2004). Recently, the novel PKC subfamily PKCδ has been cloned and identified as a pro-apoptotic isoform in various kinds of cells (Brodie and Blumberg, 2003); however, the roles of PKCδ in 4-HNE-treated endothelial cells have not been elucidated.

In this study, we investigated whether the JNK pathway is involved in the cytotoxic effects of 4-HNE on HUVECs, and whether PKCδ is requisite for 4-HNE-induced endothelial cell damage. We also examined the interaction between JNK and PKCδ pathways in the mechanisms of the cytotoxic effects of 4-HNE on HUVECs.

MATERIALS AND METHODS

Reagents. 4-HNE was purchased from Cayman Chemical Co. (Ann Arbor, MI). JNK inhibitors (SP600125, AS601245), p38 inhibitor (SB203580), MEK1/ERK1/2 inhibitor (PD98059), and PKCδ inhibitor (Rottlerin) were from CALBIOCHEM (Darmstadt, Germany). Super oxide dismutase (SOD) was from MP Biomedicals (Eschwege, Germany). N-acetyl-L-cystein (NAC) and catalase were from Wako Pure Chemical Industries (Osaka, Japan).

Cell culture. Human umbilical vein endothelial cells (HUVECs), purchased from the Japan Health Sciences Foundation (Osaka, Japan), were cultured in Ham's F12K medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10 % fetal bovine serum (FBS), 10 ng/ml basic fibroblast growth factor (PEPROTECH EC, London, UK), 100 µg/ml heparin (Wako, Osaka, Japan) 1460 mg/l, streptomycin and 100 U/ml penicillin. HUVECs between passages 4 to 8 were used in the experiments.

WST-8 assay. The HUVECs were seeded onto a 96-well collagen-coated plate at a density of $1x10^4$ cells per well and allowed to attach for 24 hr. The cells were then cultured overnight in a medium containing 2 % FBS for quiescence and exposed to 4-HNE. In most experiments, the cells were preincubated with various reagents for 30 min and exposed to 4-HNE for 6 hr. Cell viability was determined using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. In brief, 10 µl reactive solution of the assay kit was added to each well, and after incubation for 1 hr, the absorbance of the sample was measured at 450 nm with a microplate reader at a reference wavelength of 630 nm.

LDH assay. Cell injury was determined using a LDH Cytotoxicity Detection kit (TaKaRa Bio Laboratories, Tokyo, Japan). In brief, after exposing the cells to 4-HNE for 16 hr, 100 µl supernatant of the cells was collected, added to 100 µl assay solution, and the mixture was incubated for 30 min at room temperature in the dark. The absorbance of the sample was then measured at 490 nm with a microplate reader at a reference wavelength of 630 nm.

Immunoblot analysis. The cells were lysed with RIPA buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM

EDTA, 1 % Nonidet P-40, 1 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 0.04 TIU/μl aprotinin, 1 mM sodium orthovanadate, and 50 mM sodium fluoride. The lysates were subjected to SDS–PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were then incubated with rabbit primary antibodies against phospho-JNK, phospho-p38, phospho-ERK, and phospho-PKCδ (Thr505) (Cell Signaling, Beverly, MA) at a dilution of 1:250 for 1 h at room temperature. Bands were visualized with donkey anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Amersham, Piscataway, NJ) and a chemiluminescence reagent (Amersham, Piscataway, NJ). To determine the total amounts of JNK, p38, ERK, and PKCδ, antibodies against JNK1, p38, the ERK1 and ERK2 mixture (Santa Cruz Biotechnology, Santa Cruz, CA), PKCδ, (Cell Signaling, Beverly, MA), and actin (MP Biomedicals, Eschwege, Germany) were used at a dilution of 1:1000.

Statistics. All values are expressed as means ± SD. The statistical significance of the differences was evaluated by ANOVA, and Bonferroni's tests were used for post hoc analysis. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Involvement of oxidative stress on 4-HNE-induced endothelial cell injury

First, our determination of the cytotoxic effects of 4-HNE on HUVECs revealed that 4-HNE reduced cell viability and increased LDH release, indicating that 4-HNE induced endothelial cell injury (Fig.1A and B).

Since 4-HNE is known to exert various effects on intracellular signaling cascades by inducing oxidative stress through the production of reactive oxygen species (ROS) production or the depletion of glutathione (GSH) (Uchida et al., 1999), we investigated whether oxidative stress participates in 4-HNE-induced endothelial cell injury. NAC significantly attenuated cell damage in a dose-dependent manner (Fig.1A and B); however, neither SOD nor catalase exerted any protective effects (Fig.1C and D). Furthermore, inhibition of intracellular ROS production, including NADPH oxidase, xanthine oxidase, or mitochondrial electron transport, did not affect the cytotoxicity (data not shown). Taken together, these data suggest that the 4-HNE-induced cell injury of HUVECs is not due to oxidative stress exerted by intracellular production of ROS.

Involvement of JNK in 4-HNE-induced endothelial cell injury

Since MAPK pathways play critical roles in signal transduction in response to various stimuli (Johnson and Lapadat, 2002), we investigated whether 4-HNE activates MAPKs in HUVECs. 4-HNE elicited biphasic activation of JNK: early-phase activation occurred within 5 min and subsequently disappeared within 15 min, whereas late-phase activation became obvious 150 min after 4-HNE stimulation and increased until 240 min (Fig. 2A). Activation of ERK1/2 was also biphasic with a time course almost similar to that of JNK. In contrast, p38 was phosphorylated as early as 5 min after exposure to 4-HNE and remained elevated until 240 min.

To examine the role of MAPKs in 4-HNE-induced endothelial injury, we tested the effects of MAPK cell pathway inhibitors. SP600125, a JNK inhibitor, significantly reduced 4-HNE-induced endothelial cell injury (Fig. 2B and C). AS601245, another JNK inhibitor, also ameliorated the cytotoxicity (data not shown). In contrast, neither inhibitor p38 nor inhibitor ERK affected cell viability or LDH release in 4-HNE-treated HUVECs (Fig. 2B and C). Remarkably, SP600125 effectively suppressed the cytotoxic effects even when added 60 min after exposure to 4-HNE (Fig. 2D).

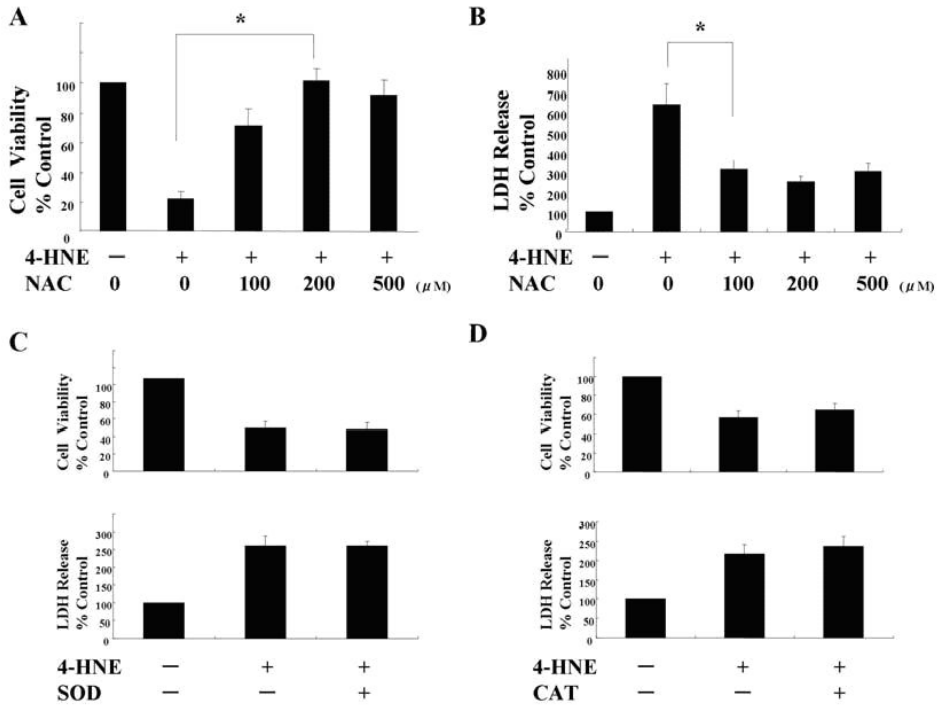

Figure 1: Effect of antioxidants on 4-HNE-induced endothelial cell injury. (A) WST-8 assay and (B) LDH assay of HUVECs treated with or without NAC and then exposed to 50 μM 4-HNE. Graphic results are expressed as a percentage of control value, mean ± SD (n=6). WST-8 assay and LDH assay of HUVECs treated with or without (C) 10 U/ml SOD or (D) 1000 U/ml catalase (CAT) and then exposed to 50 μM 4-HNE. Graphic results are expressed as a percentage of control value, meaṇ ± SD (n=6). The figure is representative of three separate experiments with similar results. *P < 0.01.

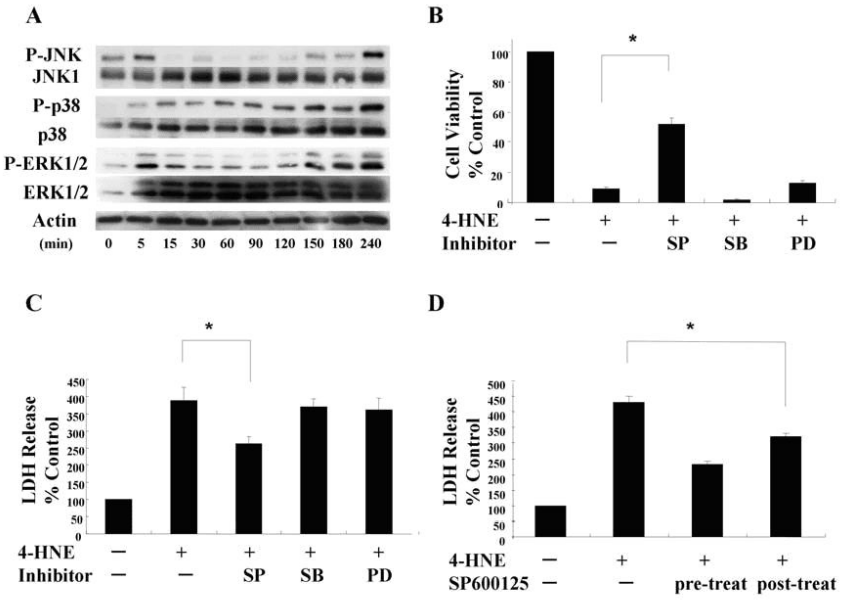

Figure 2: JNK involvement in 4-HNE-induced endothelial cell injury (A) The phosphorylation of MAPKs after exposure of HUVECs to 50 μM 4-HNE for the durations indicated. (Upper panels) phospho-MAPKs (Lower panels) total MAPKs and actin as loading controls. The results are representative of three separate experiments with similar results. (B) WST-8 assay and (C) LDH assay of HUVECs pretreated with 20 μM JNK inhibitor (SP), 20 μM p38 inhibitor (SB), or 20 μM MEK1/ERK1/2 inhibitor (PD) and then exposed to 50 μM 4-HNE. Graphic results are expressed as a percentage of control value, mean ± SD (n=6). The figure is representative of three separate experiments with similar results. (D) LDH assay of HUVECs pretreated or postreated with 20 μM JNK inhibitor. Graphic results are expressed as a percentage of control value, mean ± SD (n=6). The figure is representative of three separate experiments with similar results. *P < 0.01.

These results indicate that early-phase JNK activation is not sufficient to induce endothelial cell injury. Instead, late phase of JNK activation is, at least partly, requisite for the induction of the cytotoxic effects of 4-HNE.

Involvement of PKCδ in 4-HNE-induced endothelial cell injury

Activation of PKCδ by 4-HNE has been described as pro-apoptotic in several types of cells (Kutuk and Basaga, 2007). Our investigation of whether 4-HNE regulates the PKCδ pathway in HUVECs revealed rapid and transient phosphorylation of PKCδ 5 min after exposure to 4-HNE, with a gradual decrease until 30 min (Fig. 3A).

Next, our examination of whether the inhibition of PKCδ activity and function counteracts the cytotoxic effects of 4-HNE on HUVECs, revealed that rottlerin, a selective PKCδ inhibitor, significantly reduced 4-HNE-induced cell damage in a dose-dependent manner (Fig. 3B and C). Collectively, these results support the concept that 4-HNE induces endothelial cell injury through PKCδ activation.

Interaction between JNK and PKCδ in 4-HNE-induced endothelial cell injury

Since, as shown here, both JNK and PKCδ are involved in 4-HNE-induced endothelial cell injury, we examined whether these kinase pathways are, subsequently or independently, involved in the cytotoxic effects of 4-HNE. Pre-treatment with SP600125 suppressed the phosphorylation of PKCδ, suggesting that early-phase JNK activation occurs upstream of PKCδ (Fig. 3D). Taken together with the results of Fig.3B and C, early-phase JNK activation is, at least partly, requisite for the cytotoxicity of 4-HNE through PKCδ activation.

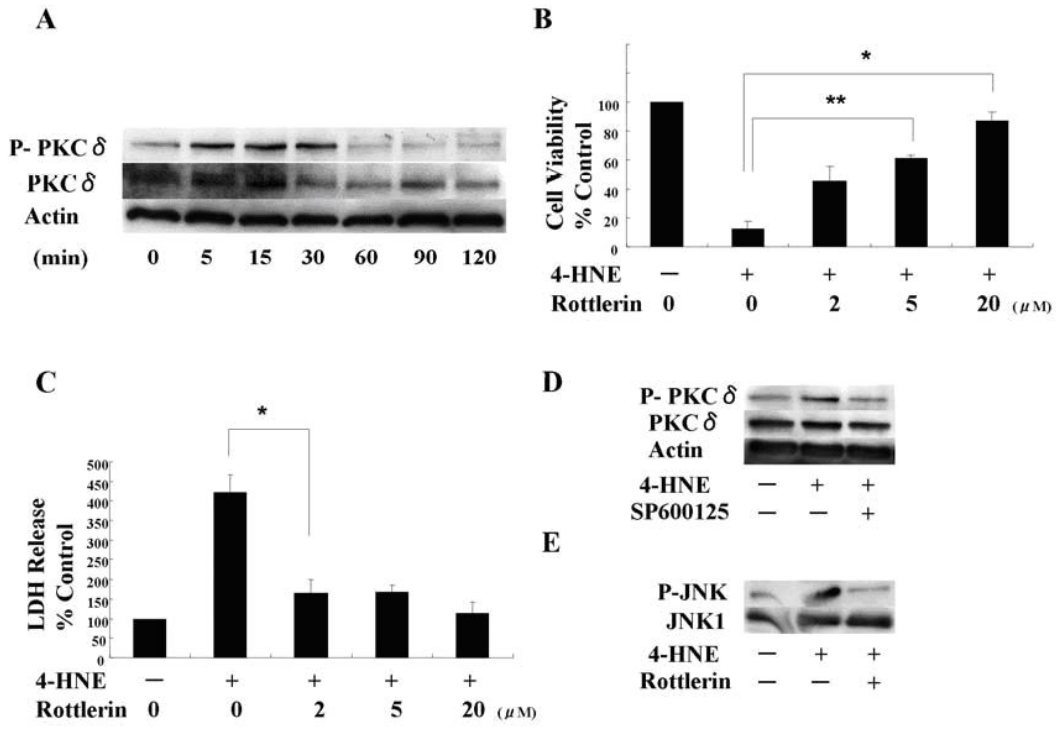

Figure 3: PKCδ involvement in 4-HNE-induced endothelial cell injury. (A) The phosphorylation of PKCδ after exposure to 50 μM 4-HNE for the durations indicated. (Upper panels) Phospho-PKCδ (Lower panels) total PKCδ and actin as loading controls. The results are representative of three separate experiments with similar results. (B) WST-8 assay and (C) LDH assay of HUVECs pretreated with or without a PKCδ inhibitor (Rottlerin) and then exposed to 50 μM 4-HNE. Graphic results are expressed as a percentage of control value, mean ± SD (n=3). Each figure is representative of three separate experiments with similar results. *$P < 0.01$, **$P < 0.05$. (D) Effects of 20 μM SP600125 pretreatment on the phosphorylation of PKCδ elicited by exposure to 50 μM 4-HNE. The results are representative of three separate experiments with similar results. (E) Effects of 20 μM rottlerin on the late phase of JNK phosphorylation 240 min after exposure to 50 μM 4-HNE. The results are representative of three separate experiments with similar results.

Inversely, rottlerin suppressed late-phase JNK phosphorylation 240 min after exposure to 4-HNE (Fig. 3E); it did not, however, decrease early-phase phosphorylation of JNK (data not shown). Taken together, these results indicate that early-phase activation of JNK by 4-HNE phosphorylates PKCδ, and subsequently leads to late-phase activation of JNK resulting in endothelial cell injury (Fig. 4).

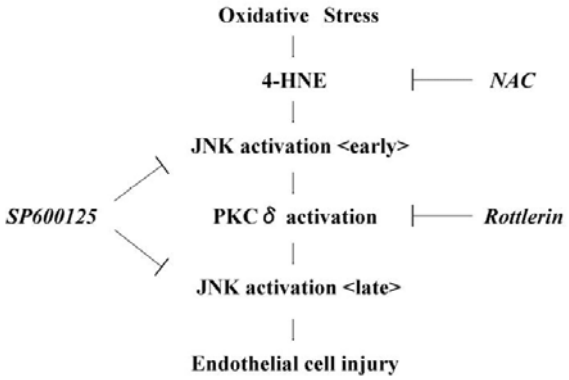

Figure 4: Mechanisms of 4-HNE-induced endothelial cell injury. 4-HNE induces a biphasic activation of JNK in HUVECs. Early-phase JNK activation results in PKCδ phosphorylation, then in late-phase JNK activation leading to endothelial cell injury.

DISCUSSION

Accumulating evidence suggests that 4-HNE exerts cytotoxic effects on various kinds of cells (Liu et al., 2000); however, the mechanisms of 4-HNE-induced endothelial injury have not been clearly defined. Here, we investigated the signaling cascade of the cytotoxic effects of 4-HNE on HUVECs. 4-HNE induced both PKCδ and biphasic JNK activation, and specific inhibitors of JNK and PKCδ reduced the cytotoxic effects of 4-HNE. Moreover, even post-treatment with the JNK inhibitor, which only suppressed late-phase JNK activation, effectively reduced the cytotoxic effects of 4-HNE. Furthermore, inhibition of JNK reduced PKC phosphorylation, whereas inhibition of PKC reduced late-phase JNK phosphorylation without any influence on early-phase JNK phosphorylation. Collectively, these results indicate that 4-HNE induces endothelial cell injury, at least partly, by biphasic JNK activation intermediately through the PKC pathway.

In this study, 4-HNE, which exerted cytotoxic effects on HUVECs, is known to regulate intracellular signaling cascades through the induction of oxidative stress by the production of intracellular ROS or by the depletion of glutathione (GSH) (Leonarduzzi et al., 2004). 4-HNE can also modulate cell signaling by Michael protein adduct formation (Leonarduzzi et al., 2004). Our results demonstrated that NAC, but no antioxidants, suppressed the cytotoxicity of 4-HNE. Since NAC exerts such effects not only as an antioxidant, but also as a thiol protectant that inhibits adduct formation, it is likely that the cytotoxic effects of 4-HNE on HUVECs are dependent more on adduct formation than on oxidative stress.

4-HNE induces JNK activation in HUVECs, which is consistent with previous reports (Kutuk and Basaga, 2007), where JNK is activated through ROS generated by 4-HNE. In this study, however, it is more likely that JNK was activated through Michael adduct formation than through oxidative stress. This mechanism is similar to that in a recent study demonstrating that 4-HNE interacts directly with JNK isoforms (Parola et al., 1998). Nonetheless, further studies are needed to determine whether JNK activation by 4-HNE in HUVECs occurs through direct interaction or through upstream pathways.

In HUVECs, 4-HNE elicits biphasic JNK activation, as several other stimuli also do (Ventura et al., 2006). In particular, late and sustained JNK activation is involved in cell death pathways (Ventura et al., 2006), which is consistent with our observation; however, only few reports have shown the roles of biphasic JNK activation. Early-phase JNK activation by TNF-α signals cell survival, while late-phase JNK activation mediates proapoptotic signaling (Ventura et al., 2006). Conversely, it has very recently been demonstrated that both

early- and late-phase JNK activation are essential for cell death induced by co-treatment with TNF-α and CCN1, secreted matricelluar protein (Chen et al., 2007), which is consistent with our results. Additional studies are fundamental to elucidating the reasons for the biphasic activation requisite for signal transduction.

In this study, PKCδ activation was elicited by 4-HNE in HUVECs. PKCδ is activated by tyrosine phosphorylation or by enzymatic cleavage to a constitutively active catalytic fragment in response to diverse stimuli (Choi et al., 2006). We found the full-length form of PKCδ decreased at 60 min after exposure to 4-HNE, suggesting that the enzymatic cleavage of PKCδ into the active form could occur. 4-HNE induces a marked increase in PKCδ activity through ROS generation or through the direct interaction of aldehyde with the thiol-rich regions of the kinase regulatory domain (Leonarduzzi et al., 2004). In contrast, our results clearly demonstrated that neither mechanism is likely to be involved in 4-HNE-induced PKCδ activation but that it is dependent on early-phase JNK activation. To the best of our knowledge, this is the first study demonstrating that JNK activates PKCδ, whereas a substantial number of documents show that PKCδ regulates JNK activation.

Recent studies showing that PKCδ modulates apoptosis through the regulation of JNK (Mitsutake et al., 2001), are consistent with our observations. Our results demonstrated that late-phase JNK activation is, at least partly, dependent on the PKCδ pathway. It is generally accepted that late-phase JNK activation is due to the inactivation of MAPK phosphatases (MKPs) (Ventura et al., 2006). It has been demonstrated that PKCδ suppresses MKP activity (Choi et al., 2006). Collectively, these reports suggest that PKCδ could induce late-phase JNK activation through the inactivation of MKP.

To summarize, we have shown that 4-HNE induces endothelial cell injury through PKCδ and biphasic JNK activation. Since 4-HNE is involved in the pathogenesis of several lung and cardiovascular diseases, our findings indicate that blocking PKCδ and JNK pathways would provide a new therapeutic approach.

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