Suppressing PTEN Activity by Tobacco Smoke Plus Interleukin-1β Modulates Dissociation of VE-Cadherin/β-Catenin Complexes in Endothelium

Silvia S. Barbieri, Luca Ruggiero, Elena Tremoli, Babette B. Weksler

Objectives—Tobacco smoke (TS) interacts with inflammatory cytokines to produce endothelial dysfunction. We hypothesized that interleukin-1β (IL-1β) plus TS (TS/IL-1β) induces disassembly of endothelial junctional complexes of VE-cadherin/β-catenin by suppression of PTEN activity and investigated molecular mechanisms that modulate PTEN-deactivation in this situation.

Methods and Results—TS/IL-1β exposure, which disrupted adherens junctions and induced nuclear β-catenin accumulation, increased tyrosine phosphorylation (p-Tyr) of VE-cadherin and β-catenin, and reduced PTEN activity. Overexpression or silencing of PTEN modulated p-Tyr of both VE-cadherin and β-catenin, changed assembly of adherens junction complexes, and altered nuclear β-catenin accumulation. In addition, inhibiting ROS production stimulated by TS/IL-1β, decreased activation of Src, EGFR and p38MAPK, phosphorylation of PTEN, VE-cadherin and β-catenin, and abrogated the effect of TS/IL-1β to disorganize adherens junctions, resulting in reduced endothelial permeability and decreased nuclear β-catenin accumulation. Finally, exposure of ApoE$^{-/-}$ mice to cigarette smoke–induced phosphorylation of Src, EGFR, p-38MAPK, PTEN, and β-catenin, and disrupted VE-cadherin/β-catenin complexes in cardiovascular tissue.

Conclusions—TS interaction with IL-1β modulates PTEN activity though the ROS/Src/EGFR-p38MAPK pathway. PTEN deactivation is essential to increase VE-cadherin and β-catenin p-Tyr and to disassemble VE-cadherin/β-catenin membrane complexes, events that lead to accumulation of β-catenin within the nucleus. (***Arterioscler Thromb Vasc Biol*. 2008;28:000-000.)**

Key Words: smoke ■ PTEN ■ tyrosine phosphorylation ■ VE-cadherin ■ β-catenin

Smoking reduces the human life span and is involved at many stages in the progression of inflammatory pathologies including cancer and cardiovascular diseases. Carcinogenesis and arteriosclerosis have much in common: both endothelial dysfunction and neovascularization in atherosclerosis resemble the neoangiogenesis in carcinogenesis. The initial stage of atherogenesis is associated with formation of neovessels characterized by paucity of tight junctions and by discontinuous basement membrane.[1]

Although mechanisms by which smoking promotes cancer and cardiovascular diseases are not entirely understood, one consistent finding in cigarette smoke–related diseases is endothelial dysfunction.[2]

In vivo and in vitro studies suggest that cigarette smoke modulates both endothelial function and structure, increasing permeability to macromolecules, accumulation of lipoproteins, and endothelial damage,[3–5] crucial events in atherogenesis and angiogenesis. The endothelial cell–specific membrane protein VE-cadherin, the major adhesive protein of endothelial adherens junctions, is required for vascular integrity. VE-cadherin is linked to the actin cytoskeleton via the armadillo family members β and γ-catenin.[6] VE-cadherin and β-catenin function are controlled by cytoskeletal dynamics and by protein phosphorylation events. Tyrosine phosphorylation (p-Tyr) of both VE-cadherin and β-catenin is important in disassembly of endothelial adherens junctions.[7–10] Increasing VE-cadherin p-Tyr results in disruption of VE-cadherin/β-catenin binding, with consequent nuclear translocation of β-catenin where it modulates gene transcription.

Several lines of evidence suggest that PTEN (phosphatase and tensin homolog deleted on chromosome 10) interacts indirectly with β-catenin by binding scaffolding proteins containing a PDZ domain, and thus participates in regulating cell-cell junctions and vascular permeability.[11–14] PTEN inhibits PI3-kinase pathway by dephosphorylation of phosphoinositide second messengers.[15] PTEN activity is negatively regulated by its oxidation and phosphorylation.[13,16,17]

The role of tobacco smoke (TS) in modulation of PTEN activity has not been explored. We previously reported that

Original received August 8, 2007; final version accepted January 3, 2008.

From the Department of Pharmacological Sciences (S.S.B., E.T.), University of Milan, Italy; the Division of Hematology-Medical Oncology (S.S.B., B.B.W.), Weill Medical College of Cornell University, New York; and the Monzino Cardiologic Center I.R.C.C.S (L.R., E.T.), Milan, Italy.

Correspondence to Babette B. Weksler, MD, Division of Hematology-Medical Oncology, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10065. E-mail babette@med.cornell.edu

Arterioscler Thromb Vasc Biol. **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.107.159434**

TS augments cytokine effects on endothelial permeability and VE-cadherin/β-catenin complexes.[3] Here, we show that TS potentiation of cytokines operates through suppression of PTEN activity, leading to p-Tyr and dissociation of VE-cadherin/β-catenin complexes in endothelium.

Our data also show that TS/IL-1β mediate production of reactive oxygen species (ROS) that stimulate phosphorylation of Src, with consequent increased activation of EGFR and p38MAPK. Therefore, activation of the ROS-dependent Src/EGFR-p38MAPK pathway inhibits PTEN activation through its phosphorylation. Blocking the PTEN/PI3K pathway triggers p-Tyr of VE-cadherin and β-catenin, leading to dissociation of adherens junction complexes with translocation of β-catenin into the nucleus. We show for the first time the existence of a link between smoke exposure, PTEN, phosphorylation events, and adherens junction integrity in the regulation of endothelial cell barriers and nuclear β-catenin accumulation.

Materials and Methods

For detailed descriptions of the Materials and Methods, please see supplemental Material and Methods (available online at http://atvb.ahajournals.org).

Reagents and Antibodies

PBS, medium D-MEM, HEPES, L-glutamine, penicillin/streptomycin were from GIBCO Laboratories. IL-1β, FBS, NAC (N-Acetyl-Cysteine), phosphatase and protease inhibitors, fluorescein isothiocyanate (FITC)-conjugated dextran, DCFH-DA, and DAF-2 were from Sigma. The PI3K inhibitor LY294002, EGFR inhibitor AG1478, p38MAPK inhibitor SB202190, and Src inhibitor PP1 were purchased from Calbiochem. For Western blot analysis, the following antibodies were used: primary antibody against EGFR, phospho-EGFR (Tyr-1068), Akt, phospho-Akt (Ser-473), PTEN, phospho-PTEN (Ser-380), Src, phospho-Src (Tyr-416), p38MAPK, phospho-p38MAPK (Thr180/Tyr182), β-catenin (Cell Signaling); VE-cadherin, β-catenin, SV40-T, intercellular adhesion molecule-1 (ICAM-1), phosphotyrosine (Santa Cruz); β-actin, tubulin (Sigma); phosphotyrosine (PY20) anti-mouse and anti-rabbit peroxidase-conjugated secondary antibodies (Calbiochem), anti-mouse secondary antibodies labeled with Cy2 (Jackson Laboratory). Protein A-Sepharose beads (Zymed). BCA protein assay kit (Bio-Rad). All chemicals were of analytical grade and purchased from Sigma if not otherwise stated.

Results

TS/IL-1β exposure disrupted adherens junctions, increased ROS production, and p-Tyr of VE-cadherin and β-catenin.

Our previous in vitro and in vivo studies showed in mouse cardiac endothelial cells (MCECs) that tobacco smoke (TS) cooperates with IL-1β to cause barrier dysfunction with disruption of VE-cadherin/β-catenin complexes, and enhancement of nuclear β-catenin accumulation (please see supplemental Figure IA and IB).[3] We showed that β-catenin in unstimulated cells was mainly localized at the intercellular junctions. However, within 10 minutes' exposure to TS or IL-1β, β-catenin redistributes away from the cell-cell contacts with gaps developing between the endothelial cells. This effect was most striking after cotreatment with TS and IL-1β (Figure 1A).

Because production of reactive oxygen species (ROS) and tyrosine phosphorylation (p-Tyr) of junction proteins critically affect stable junction formation,[7–10] we investigated whether TS interacts with IL-1β to modulate ROS and protein p-Tyr in MCECs. As shown in Figure 1B, treatment with TS alone did not alter ROS production, whereas exposure to IL-1β caused a 1.3-fold induction of peroxide production. In contrast, ROS production increased 4.7-fold when MCECs were exposed to the same concentrations of TS and IL-1β (TS/IL-1β) combined (Figure 1B and supplemental Figure IC). TS/IL-1β increased ROS production in a time-dependent manner within 5 to 15 minutes, an effect that declines partially after 30 minutes (supplemental Figure ID and IE).

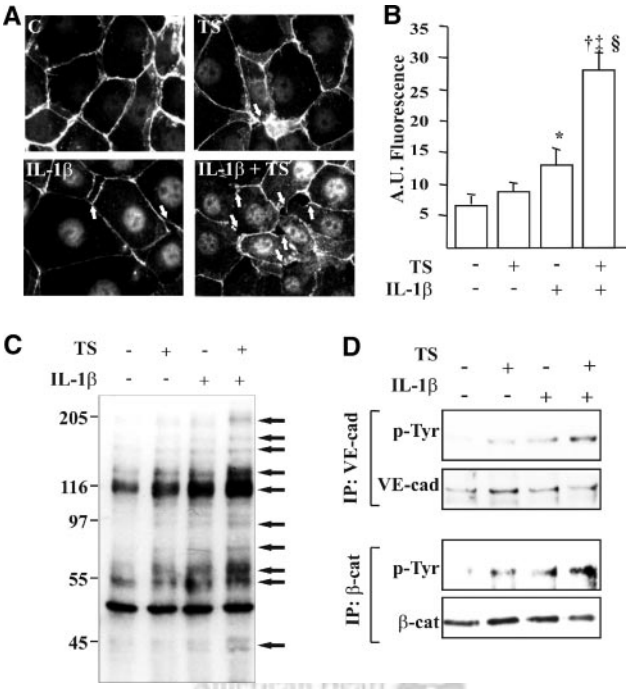

Figure 1. Effect of TS/IL-1β exposure on adherens junction stability, ROS production, and protein p-Tyr. MCECs incubated with TS (6.4 puffs/L) or IL-1β (2 μg/L). A, β-catenin staining redistributed from cell junctions in 10 minutes. B, ROS production (mean±SD of triplicates, n=3; *$P<0.05$ and †$P<0.01$ vs Control, ‡$P<0.01$ vs TS, §$P<0.01$ vs IL-1β). C, Lysate protein p-Tyr. D, Lysates immunoprecipitated with anti–VE-cadherin or with anti–β-catenin and immunoblotted as shown.

Moreover, either TS or IL-1β alone stimulated protein p-Tyr only slightly, whereas cotreatment with TS/IL-1β strongly increased protein p-Tyr detected by Western blotting. TS/IL-1β induced intense p-Tyr at 40 kDa, 60 to 90 kDa, and 120 to 200 kDa (Figure 1C). Intriguingly, the molecular weights of VE-cadherin and β-catenin are 92 kDa and 130 kDa, respectively. In addition, we studied the effects of TS/IL-1β on p-Tyr specifically of VE-cadherin and β-catenin. TS or IL-1β alone induced only modestly increased p-Tyr of VE-cadherin and β-catenin, whereas TS/IL-1β combined strongly induced p-Tyr of both proteins (Figure 1D and supplemental Figure IF).

Thus, TS cooperates with IL-1β to increase ROS production, to modify VE-cadherin/β-catenin complexes at the endothelial cell plasma membrane, and augment their p-Tyr.

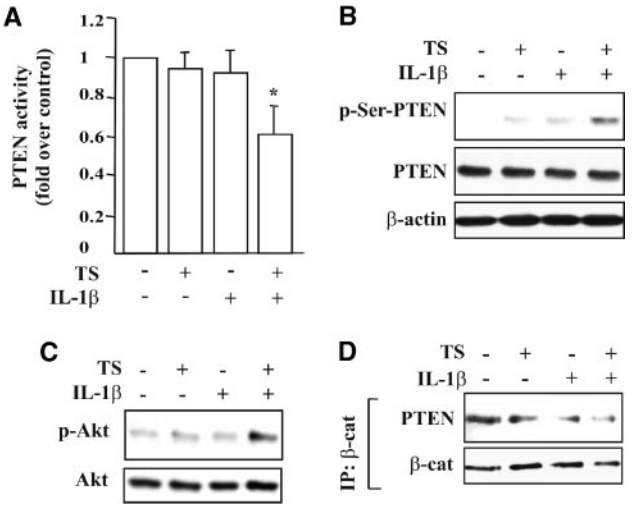

Figure 2. TS/IL-1β treatment modulates PTEN activity. A, TS/IL-1β decreased PTEN activity (mean$\pm$SD of duplicate, n=4, *$P<0.01$). MCECs treated 5 minutes were lysed and processed to detect PTEN and p-Ser-PTEN (B) or Akt, p-Akt (C). D, MCECs treated 15 minutes were lysates, immunoprecipitated with anti-β-catenin, immunoblotted with anti-PTEN, or anti-β-catenin.

TS/IL-1β Suppressed PTEN Activity, Though Its Phosphorylation of Ser Residues Not Its Oxidation

Activation of PTEN may prevent p-Tyr of β-catenin[14] and PI3K signaling,[15] events required to modulate permeability of endothelial monolayers. We showed that TS/IL-1β exposure reduced PTEN activity by 40%, but no change in PTEN resulted when MCECs were treated with TS or IL-1β alone (Figure 2A). PTEN is known to be negatively regulated in a redox-dependent manner,[16,17] by p-Tyr,[18] or by phosphorylation of residues Ser380 and Thr382/383 (p-Ser/Thr).[13] We observed that H_2O_2 treatment of MCECs shifted PTEN toward oxidized form, whereas no oxidized PTEN was detected in MCECs treated with TS/IL-1β (supplemental Figure IIA). Moreover, no changes in p-Tyr of PTEN were observed after exposure to TS/IL-1β (supplemental Figure IIB). In contrast, 5 minutes of TS/IL-1β costimulation resulted in increased p-Ser of PTEN (p-Ser-PTEN), compared with treatment with TS or IL-1β alone (Figure 2B). We tested whether TS/IL-1β exposure increased phosphorylation of Akt and affected PTEN recruitment into a complexes with β-catenin.[11] Increased p-Ser of PTEN induced by TS/IL-1β is coupled to enhanced p-Akt (Figure 2C) and decreased PTEN binding with β-catenin (Figure 2D). Treatment with TS or IL-1β alone did not alter either the level of p-Akt or the amount of PTEN bound to β-catenin (Figure 2C and 2D). Collectively, these data suggest that TS/IL-1β exposure inhibits PTEN activity primarily through p-Ser of PTEN.

N-Acetylcysteine (NAC) Prevented p-Ser of PTEN, p-Tyr of VE-Cadherin and β-Catenin, Stabilized Adherens Junction Complexes, Reduced Trafficking of β-Catenin, and Decreased Permeability of Endothelial Monolayers

Treatment of MCECs with the antioxidant NAC almost completely abolished the generation of peroxide induced by TS/IL-1β (data not shown), and inhibited p-Ser-PTEN and p-Akt (supplemental Figure IIIA). Furthermore, exposure to NAC strongly attenuated p-Tyr of both β-catenin and VE-cadherin, and blocked the dissociation of VE-cadherin/β-catenin complexes (supplemental Figure IIIB). Additionally, ROS inhibition resulted in decreased nuclear β-catenin accumulation (supplemental Figure IIIC), and restored normal endothelial monolayer permeability (supplemental Figure IIID). Based on these observations, we suggest that ROS induced by TS/IL-1β are required to modulate the PTEN/AKT pathway, p-Tyr of VE-cadherin and β-catenin, adherens junction complex disruption, and increased endothelial permeability.

PTEN Activity Was Required to Modify p-Tyr, VE-Cadherin/β-Catenin Complexes, and Nuclear β-Catenin Accumulation

To examine whether PTEN is required to modulate PI3K/Akt/nuclear β-catenin accumulation pathway we used 2 different approaches: we transfected MCECs with PTEN-specific siRNA, or exposed MCECs to TS/IL-1β after transfection with pcDNA3-PTEN (pc-PTEN). Transfection of PTEN siRNA but not control siRNA significantly suppressed PTEN levels, and increased Akt phosphorylation (supplemental Figure IVA). Transfection with PTEN siRNA had the same effect as TS/IL-1β treatment, ie, induced p-Tyr of β-catenin and VE-cadherin, disrupted VE-cadherin/β-catenin complexes (supplemental Figure IVB), and increased nuclear β-catenin accumulation (supplemental Figure IVC). In contrast, overexpression of PTEN completely prevented phosphorylation of Akt induced by TS/IL-1β compared with induction of both the events in control cells transfected with pcDNA3 vector alone (supplemental Figure IVD). Moreover, induction by TS/IL-1β of β-catenin p-Tyr was completely abrogated in cells transfected with pc-PTEN (Figure 3A). Surprisingly, PTEN overexpression decreased p-Tyr of VE-cadherin, inhibited the disruption of VE-cadherin/β-catenin complexes (Figure 3A), and blocked nuclear β-catenin accumulation (Figure 3B). We confirmed that PTEN activity modulated p-Tyr and dissociation of VE-cadherin/β-catenin membrane complexes, by pretreating MCECs with the PI-3K inhibitor LY294002 (LY). LY treatment of MCECs completely blocked Akt phosphorylation (supplemental Figure IVE), and inhibited β-catenin p-Tyr induced by TS/IL-1β (Figure 3C). Furthermore, inhibition of PI3K reversed p-Tyr of VE-cadherin and prevented disruption of VE-cadherin/β-catenin complexes induced by cotreatment (Figure 3C). Both the increased permeability of endothelial monolayers and β-catenin trafficking into the nucleus caused by TS/IL-1β were significantly diminished when PI-3K was inhibited (supplemental Figure IVF and IVG).

Direct inhibition of PI3K, an immediate downstream target blocked by activated PTEN, has the same negative effect on TS/IL-1β mediated p-Tyr of VE-cadherin and β-catenin, and modification of VE-cadherin/β-catenin interactions as does overexpressing PTEN. Taken together, these observations demonstrate that reduction of PTEN activity mediated by its

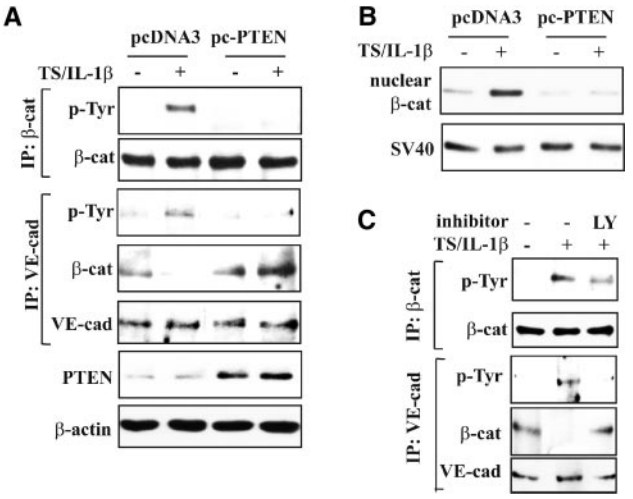

Figure 3. Effect of PTEN on protein p-Tyr, disruption of VE-cadherin/β-catenin complexes, and nuclear β-catenin accumulation. MCECs were transfected with pc-PTEN or pcDNA3 (A, B) or preincubated or not for 1 hour with LY294002 (LY, 2.5 nmol/L) and then stimulated or not with TS/IL-1β (C). A and C, Immunoprecipitation with anti–β-catenin or anti–VE-cadherin, immunoblotting with anti–p-Tyr, anti–β-catenin or anti–VE-cadherin. At bottom: PTEN level in total lysates. B, β-catenin in nuclear fraction.

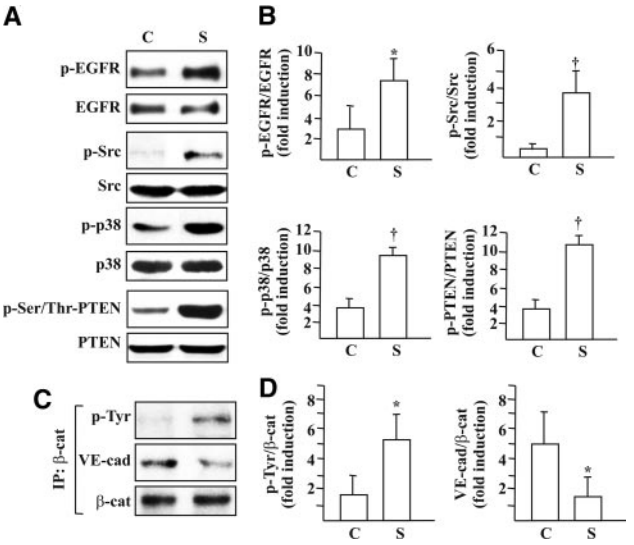

Figure 4. Effect of cigarette smoke on p-EGFR, p-Src, p-p38, p-PTEN, p-Tyr of β-catenin and disruption of VE-cadherin/β-catenin complexes in ApoE$^{-/-}$ mice. A, Expression of p-EGFR, p-Src, p-p38, and p-Ser-PTEN in cardiovascular tissue (Western blotting). B and D, Changes in protein content (mean±SD; n=5 group; *$P<0.05$, †$P<0.01$ vs control). C, Tissue lysates immunoprecipitated with anti–β-catenin then immunoblotted as shown.

silencing or by treatment with TS/IL-1β is required to modify endothelial adherens junctional complexes.

By Modulating PTEN Activity, TS/IL-1β Regulates the Src/EGFR/p38 Pathway, p-Tyr of Junctional Proteins, and Dissociation of VE-Cadherin and β-Catenin Complexes

We analyzed phosphorylation of Src, EGFR, and p38MAPK (p38) by TS and IL-1β as representative kinases activated by tobacco smoke.[3,18–21] TS or IL-1β alone had either no significant effect or resulted in slightly increased protein phosphorylation levels. In contrast, cotreatment with TS/IL-1β resulted in significantly enhanced phosphorylation of Src, EGFR, and p38 (data not shown).

To identify possible interactions among these kinases, we pretreated MCECs with SB202190 (SB), PP1, and AG1478 (AG), selective inhibitors of p38, Src, and EGFR, respectively. Although no changes in phosphorylation levels of Src and EGFR induced by TS/IL-1β were detected when MCECs were pretreated with SB (data not shown), pretreatment with PP1 dramatically reduced phosphorylation of Src, EGFR, and p38 (data not shown). In contrast, AG inhibited phosphorylation of EGFR but had no effect on the phosphorylation of Src and p38 (data not shown).

Because Src regulates phosphorylation of VE-cadherin and β-catenin,[21] we tested whether activation of the Src, EGFR, and p38 modulated PTEN/PI3K activity and consequently the status of VE-cadherin/β-catenin complexes in endothelial cells. Pretreatment of MCECs with PP1, AG, and SB inhibited phosphorylation of PTEN and of AKT (supplemental Figure VA and B), as well as p-Tyr of β-catenin (supplemental Figure VC) and VE-cadherin, and restored VE-cadherin/β-catenin complexes to the unstimulated state (supplemental Figure VC). The inhibitors individually blocked enhanced endothelial permeability (supplemental Figure VD, VE, and VF), as well as nuclear trafficking of β-catenin (supplemental Figure VG) induced by TS/IL-1β. No change was observed in total Akt protein (data not shown). We then determined whether activation of Src, EGFR, and p38 by TS/IL-1β is under redox regulation. NAC treatment of MCECs blocked TS/IL-1β induced phosphorylation of Src, EGFR, and p38 (supplemental Figure VH) without altering total protein levels of EGFR, p38 (data not shown), and Src. Based on these observations, we suggest that in MCECs, TS/IL-1β act to modulate the Src/EGFR-p38 pathway with consequent PTEN inactivation. This in turn rapidly leads to increased p-Tyr of VE-cadherin and β-catenin, with dissociation of adherens junction and accumulation of β-catenin into the nucleus, permitting subsequent gene activation.

Exposure of Mice to Cigarette Smoke Increased Src/EGFR/p38/PTEN Phosphorylation, pTyr of β-Catenin and Decreased VE-Cadherin/β-Catenin Complexes

Our previous in vivo studies showed that in mice exposed to cigarette smoke 1 hour daily for 15 days Akt phosphorylation and nuclear β-catenin accumulation increased in cardiovascular tissue (CT), as did serum IL-1β levels.[3] Now, in the same tissue lysates, we observed a statistically significant increase in phosphorylation of Src (+277%, $P<0.01$), EGFR (+253%, $P<0.05$), p-38 (+250%, $P<0.01$), and PTEN (+297%, $P<0.01$) (Figure 4A and 4B). Moreover, induction of p-Tyr of β-catenin (+278%, $P<0.05$) and decreases in VE-cadherin/β-catenin complexes (−70%, $P<0.05$; Figure ●●C and ●●D) were observed in cardiovascular tissue of

smoke-exposed ApoE$^{-/-}$ mice compared with control mice. Taken together, our data suggest that exposing live animals to cigarette smoke induces activation of the Src/EGFR/p-38/PTEN pathway, tyrosine phosphorylation of β-catenin, and dissociation of adherent junction VE-cadherin/β-catenin complexes in cardiovascular tissue.

Discussion

Endothelial dysfunction represents a common link among many diseases induced by cigarette smoking,[1] ranging from chronic pulmonary inflammation to arteriosclerosis and carcinogenesis.[2,22] The mechanisms by which smoking activates and alters vascular endothelium have not been clearly elucidated. We showed that aqueous extracts of cigarette smoke (TS) interacted with the inflammatory cytokine, IL-1β, to increase production of nitric oxide (NO) and expression of intercellular cell adhesion molecule (ICAM-1), and induce monocyte adhesion to endothelium (supplemental Figure VI). Moreover, we previously demonstrated that TS/IL-1β produce rapid increase in vascular permeability and promote accumulation of nuclear b-catenin in the nucleus, acting as a cofactor for upregulation of inflammatory and proproliferative genes.[3] We have now explored signaling pathways involved in these smoke-induced events. Altered endothelial barrier integrity and translocation of β-catenin into the nucleus favor entry of lipids and moreover of leukocytes into the vessel wall promoting formation of more "active" and "vulnerable" plaque rather than calcified, more "inactive" plaque. We demonstrate here that exposing endothelial cells to TS/IL-1β, decreases PTEN activity, permitting activation of the PI-3K/Akt pathway.

Little is known about how tobacco smoking affects signaling pathways that regulate PTEN activity, nor how smoke mediates PTEN function in the β-catenin activation pathway.

Preformed reactive oxygen species (ROS) present in cigarette smoke are unlikely to explain endothelial dysfunction observed on exposure to an aqueous solution of tobacco smoke, as ROS are short-lived and rapidly decompose into innocuous end products in a physiological milieu.[23] Evidence rather suggests that stable compounds in cigarette smoke increase NADPH oxidase activity within cells to produce intracellular ROS,[24] that may interact with NO resulting in induction of peroxynitrite ($ONOO^-$), a strong oxidant capable of modifying most biological molecules, including amino acids such as tyrosine, that contribute to the progression of cardiovascular disease.[25] Furthermore, ROS may oxidize and inactivate PTEN by forming disulfide bonds between Cys 124, the active site and Cys 71.[16,17] We demonstrate that ROS produced when MCECs are exposed to TS/IL-1β regulate PTEN activity. Remarkably, ROS do not induce the oxidized form of PTEN or its tyrosine phosphorylation but, rather, inactivate PTEN through phosphorylation of serine residues of the PTEN tail, suggesting an indirect modulation. Phosphorylation of PTEN by casein kinase-2 (CK2) reduces catalytic activity of PTEN with activation of PI3K/Akt pathway,[26] and stabilizes PTEN in a monomeric "closed" conformation with low affinity for β-catenin/scaffolding protein complexes.[13] ROS/NADPH-mediated decreases in PTEN activity may be modulated through oxidative activation of intermediate protein kinases such as Src, rather than by direct PTEN inactivation. Although it is known that ROS produced by NADPH oxidase are crucial for Src activation in endothelial cells,[27] and that activated Src decreases the stability and the activity of PTEN, how this occurs is not clear. Our findings suggest that induction of serine/threonine phosphorylation and inactivation of PTEN modulated by TS/IL-1β is regulated through CK2 because Src, EGFR, and p38MAPK increase phosphorylation and catalytic activity of CK2.[28–30] The activation of this pathway in endothelial cells is important in multiple physiological processes including cardiovascular morphogenesis, postnatal neovascularization, and tumor angiogenesis.[31]

Recent literature demonstrates that Tyrosine phosphorylation of VE-cadherin that is stimulated by PAF, VEGF, thrombin, or histamine correlates with a rapid disruption of adherens junctions, a critical step in both angiogenesis and inflammation.[8] Conversely, we have shown that overexpression of PTEN and consequent inhibition of PI3K activity is sufficient to diminish TS/IL-1β–induced p-Tyr of VE-cadherin and β-catenin, prevent disruption of adherens junctions, and block translocation of β-catenin into the nucleus after MCECs are exposed to TS/IL-1β. The involvement of PTEN/PI3K activation in p-Tyr of both VE-cadherin and β-catenin and consequent modulation of cell-cell adhesion has been controversial. We can suggest that in our model PTEN does not directly modulate p-Tyr of VE-cadherin and β-catenin, but that p-Tyr of VE-cadherin/β-catenin complexes is regulated through PI3K. Indeed, LY294002, selective inhibitor of PI3K, decreased p-Tyr and dissociation of adherens junction complexes. In agreement with our data, cell-cell interactions and p-Tyr of β-catenin mediated by transforming growth factor (TGF)-β in pancreatic carcinoma cell lines have been shown to be directly regulated by PTEN.[14] In contrast, wild-type PTEN reverses the increased cell-cell adhesion mediated by overexpression of Src in MDCK cells, but in this model no significant effect on p-Tyr of β-catenin was observed.[32]

Of interest, we have shown that TS/IL-1β in combination rapidly activate the AKT/GSK-3β pathway with consequent Ser/Thr phosphorylation of β-catenin, leading to its decreased degradation in the cytoplasm and its increased accumulation in the nucleus.[3] Therefore, PTEN inactivation mediated by TS/IL-1β may also further regulate trafficking of β-catenin from membrane to the nucleus by regulating the latter's p-Tyr as well as reducing its degradation as a consequence of Ser/Thr phosphorylation mediated by GSK-3β deactivation (supplemental Figure VII).

PTEN inactivation induced by smoking may participate in creating vascular dysfunction not only through modulation of endothelial adherens junctions and endothelial permeability, but also through upregulation of b-catenin-induced genes. Moreover, β-catenin within the nucleus may over time lead to increased transcription of the aryl hydrocarbon receptor (AhR) and increased expression of AhR protein.[33] AhR may

participate in vascular remodeling and itself act as a transcription factor,[34] in the development of atherogenesis and cardiovascular diseases.[35] Because no data are available about PTEN and AhR activation, we can only suggest that smoke-mediated changes in the ROS/PTEN/β-catenin pathway that favor trafficking of β-catenin into the nucleus may upregulate numerous different genes including AhR. In addition, other substances contained in cigarette smoke such as polycyclic aromatic hydrocarbons, agonists for AhR that are detected in arterial endothelial cells from smokers,[36] by promoting the activation of AhR may induce an array of genes involved in vascular dysfunction. Additional studies must be performed to understand whether PTEN modulation participates in AhR activation and expression.

More importantly, our in vivo experiments, confirming the in vitro data, provide evidence that smoking induces activation of Src, EGFR, and p38MAPK, phosphorylation of PTEN, VE-cadherin, and β-catenin, and disruption of VE-cadherin and β-catenin complexes.

In conclusion, we propose that suppression of PTEN activity is an important step in endothelial dysfunction caused by tobacco smoke, in particular in the presence of inflammatory cytokines, by mediating endothelial membrane junction disorganization and by promoting β-catenin-dependent gene regulation in endothelial cells. Understanding the mechanisms by which pathogenic xenobiotics such as those in tobacco smoke cooperate with inflammatory factors to enhance pathophysiologic changes in the vascular system may lead to the development of new diagnostic and therapeutic strategies.

Acknowledgments

We are grateful to Dr A. Ieraci for useful comments after reading this manuscript.

Sources of Funding

This work was supported by National Institutes of Health Grant RO-1 HL55627.

Disclosures

None.

References

1. Herrmann J, Lerman LO, Mukhopadhyay D, Napoli C, Lerman A. Angiogenesis in atherogenesis. *Arterioscler Thromb Vasc Biol*. 2006;26:1948–1957.
2. Bernhard D, Moser C, Backovic A, Wick G. Cigarette smoke–an aging accelerator? *Exp Gerontol*. 2007;42:160–165.
3. Barbieri SS, Weksler BB. Tobacco smoke cooperates with interleukin-1{beta} to alter {beta}-catenin trafficking in vascular endothelium resulting in increased permeability and induction of cyclooxygenase-2 expression in vitro and in vivo. *Faseb J*. 2007;21:1831–1843.
4. Bernhard D, Csordas A, Henderson B, Rossmann A, Kind M, Wick G. Cigarette smoke metal-catalyzed protein oxidation leads to vascular endothelial cell contraction by depolymerization of microtubules. *Faseb J*. 2005;19:1096–1107.
5. Mullick AE, McDonald JM, Melkonian G, Talbot P, Pinkerton KE, Rutledge JC. Reactive carbonyls from tobacco smoke increase arterial endothelial layer injury. *Am J Physiol Heart Circ Physiol*. 2002;283:H591–H597.
6. Breviario F, Caveda L, Corada M, Martin-Padura I, Navarro P, Golay J, Introna M, Gulino D, Lampugnani MG, Dejana E. Functional properties of human vascular endothelial cadherin (7B4/cadherin-5), an endothelium-specific cadherin. *Arterioscler Thromb Vasc Biol*. 1995;15:1229–1239.
7. Andriopoulou P, Navarro P, Zanetti A, Lampugnani MG, Dejana E. Histamine induces tyrosine phosphorylation of endothelial cell-to-cell adherens junctions. *Arterioscler Thromb Vasc Biol*. 1999;19:2286–2297.
8. Dejana E. Endothelial cell-cell junctions: happy together. *Nat Rev Mol Cell Biol*. 2004;5:261–270.
9. Haorah J, Ramirez SH, Schall K, Smith D, Pandya R, Persidsky Y. Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction. *J Neurochem*. 2007;101:566–576.
10. van Buul JD, Anthony EC, Fernandez-Borja M, Burridge K, Hordijk PL. Proline-rich tyrosine kinase 2 (Pyk2) mediates vascular endothelial-cadherin-based cell-cell adhesion by regulating beta-catenin tyrosine phosphorylation. *J Biol Chem*. 2005;280:21129–21136.
11. Kotelevets L, van Hengel J, Bruyneel E, Mareel M, van Roy F, Chastre E. Implication of the MAGI-1b/PTEN signalosome in stabilization of adherens junctions and suppression of invasiveness. *Faseb J*. 2005;19:115–117.
12. Sanchez T, Skoura A, Wu MT, Casserly B, Harrington EO, Hla T. Induction of vascular permeability by the sphingosine-1-phosphate receptor-2 (S1P2R) and its downstream effectors ROCK and PTEN. *Arterioscler Thromb Vasc Biol*. 2007;27:1312–1318.
13. Vazquez F, Grossman SR, Takahashi Y, Rokas MV, Nakamura N, Sellers WR. Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. *J Biol Chem*. 2001;276:48627–48630.
14. Vogelmann R, Nguyen-Tat MD, Giehl K, Adler G, Wedlich D, Menke A. TGFbeta-induced downregulation of E-cadherin-based cell-cell adhesion depends on PI3-kinase and PTEN. *J Cell Sci*. 2005;118:4901–4912.
15. Vazquez F, Sellers WR. The PTEN tumor suppressor protein: an antagonist of phosphoinositide 3-kinase signaling. *Biochim Biophys Acta*. 2000;1470:M21–M35.
16. Lee SR, Yang KS, Kwon J, Lee C, Jeong W, Rhee SG. Reversible inactivation of the tumor suppressor PTEN by H2O2. *J Biol Chem*. 2002;277:20336–20342.
17. Seo JH, Ahn Y, Lee SR, Yeol Yeo C, Chung Hur K. The major target of the endogenously generated reactive oxygen species in response to insulin stimulation is phosphatase and tensin homolog and not phosphoinositide-3 kinase (PI-3 kinase) in the PI-3 kinase/Akt pathway. *Mol Biol Cell*. 2005;16:348–357.
18. Lu Y, Yu Q, Liu JH, Zhang J, Wang H, Koul D, McMurray JS, Fang X, Yung WK, Siminovitch KA, Mills GB. Src family protein-tyrosine kinases alter the function of PTEN to regulate phosphatidylinositol 3-kinase/AKT cascades. *J Biol Chem*. 2003;278:40057–40066.
19. Gensch E, Gallup M, Sucher A, Li D, Gebremichael A, Lemjabbar H, Mengistab A, Dasari V, Hotchkiss J, Harkema J, Basbaum C. Tobacco smoke control of mucin production in lung cells requires oxygen radicals AP-1 and JNK. *J Biol Chem*. 2004;279:39085–39093.
20. Li JM, Cui TX, Shiuchi T, Liu HW, Min LJ, Okumura M, Jinno T, Wu L, Iwai M, Horiuchi M. Nicotine enhances angiotensin II-induced mitogenic response in vascular smooth muscle cells and fibroblasts. *Arterioscler Thromb Vasc Biol*. 2004;24:80–84.
21. Wang Y, Jin G, Miao H, Li JY, Usami S, Chien S. Integrins regulate VE-cadherin and catenins: dependence of this regulation on Src, but not on Ras. *Proc Natl Acad Sci U S A*. 2006;103:1774–1779.
22. McGill HC Jr. Smoking and the pathogenesis of atherosclerosis. *Adv Exp Med Biol*. 1990;273:9–16.
23. Pryor WA, Stone K. Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. *Ann N Y Acad Sci*. 1993;686:12–27; discussion 27–18.
24. Jaimes EA, DeMaster EG, Tian RX, Raij L. Stable compounds of cigarette smoke induce endothelial superoxide anion production via NADPH oxidase activation. *Arterioscler Thromb Vasc Biol*. 2004;24:1031–1036.
25. Turko IV, Murad F. Protein nitration in cardiovascular diseases. *Pharmacol Rev*. 2002;54:619–634.
26. Torres J, Pulido R. The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. *J Biol Chem*. 2001;276:993–998.

27. Cheng JJ, Chao YJ, Wang DL. Cyclic strain activates redox-sensitive proline-rich tyrosine kinase 2 (PYK2) in endothelial cells. *J Biol Chem*. 2002;277:48152–48157.
28. Donella-Deana A, Cesaro L, Sarno S, Ruzzene M, Brunati AM, Marin O, Vilk G, Doherty-Kirby A, Lajoie G, Litchfield DW, Pinna LA. Tyrosine phosphorylation of protein kinase CK2 by Src-related tyrosine kinases correlates with increased catalytic activity. *Biochem J*. 2003;372: 841–849.
29. Hildesheim J, Salvador JM, Hollander MC, Fornace AJ Jr. Casein kinase 2- and protein kinase A-regulated adenomatous polyposis coli and beta-catenin cellular localization is dependent on p38 MAPK. *J Biol Chem*. 2005;280:17221–17226.
30. Liu XG, Liang NC. Inhibitory effect and its kinetic analysis of tyrphostin AG1478 on recombinant human protein kinase CK2 holoenzyme. *Acta Pharmacol Sin*. 2002;23:556–561.
31. Suzuki A, Hamada K, Sasaki T, Mak TW, Nakano T. Role of PTEN/PI3K pathway in endothelial cells. *Biochem Soc Trans*. 2007;35:172–176.
32. Kotelevets L, van Hengel J, Bruyneel E, Mareel M, van Roy F, Chastre E. The lipid phosphatase activity of PTEN is critical for stabilizing intercellular junctions and reverting invasiveness. *J Cell Biol*. 2001;155: 1129–1135.
33. Chesire DR, Dunn TA, Ewing CM, Luo J, Isaacs WB. Identification of aryl hydrocarbon receptor as a putative Wnt/beta-catenin pathway target gene in prostate cancer cells. *Cancer Res*. 2004;64:2523–2533.
34. Lahvis GP, Lindell SL, Thomas RS, McCuskey RS, Murphy C, Glover E, Bentz M, Southard J, Bradfield CA. Portosystemic shunting and persistent fetal vascular structures in aryl hydrocarbon receptor-deficient mice. *Proc Natl Acad Sci U S A*. 2000;97:10442–10447.
35. Kitamura M, Kasai A. Cigarette smoke as a trigger for the dioxin receptor-mediated signaling pathway. *Cancer Lett*. 2007;252:184–194.
36. Zhang YJ, Weksler BB, Wang L, Schwartz J, Santella RM. Immunohistochemical detection of polycyclic aromatic hydrocarbon-DNA damage in human blood vessels of smokers and non-smokers. *Atherosclerosis*. 1998;140:325–331.