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Nanomechanical Characterization of E-Cigarette-Induced Lung Endothelial Dysfunction: Roles of Cortactin and Mitochondrial ROS

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Abstract

E-cigarettes (E-cigs) are increasing in popularity and are considered as a potentially safer alternative to traditional cigarettes. However, prior studies have demonstrated that inhalation of nicotine-containing e-cigs can cause substantial pathophysiologic changes, and “vaping” of some substances has led to severe lung damage. Our group recently described the role of cortactin (*CTTN*), a cytoskeletal actin-binding regulatory protein, in mediating cigarette smoke (CS) and E-cig-induced lung endothelial apoptosis and mitochondrial dysfunction. In the current study, we advance this work by characterizing the effects of E-cig on lung endothelial nanomechanical properties and barrier function. Lung EC exposure to E-cig extract (50 µg/ml) resulted in disruption of endothelial barrier properties as assessed by Electric Cell Substrate Impedance (ECIS). Since excess mitochondrial ROS (mitoROS) is an important marker of mitochondrial dysfunction, we next assessed the effect of Mito-TEMPO (10 µM, 3 h), a cell-permeable antioxidant, on E-cig-induced endothelial permeability. Pretreatment with Mito-TEMPO provided EC barrier protection after E-cig challenge, suggesting a key role of mitoROS in E-cig-induced EC permeability. E-cig exposure induces cytoskeleton rearrangement leading to gap formation in lung EC and significantly alters EC elastic properties as assessed by atomic force microscopy (AFM). Reduction in *CTTN* expression by siRNA further augmented the injurious effects of E-cig on EC permeability and elastic properties. This is the first study to explore the role of *CTTN* in evaluating the effect of E-cigarette exposure on lung endothelium using AFM and provides novel mitochondrial and biophysical characterization of the effects of E-cig exposure on human lung EC. This work advances our understanding of the pathophysiologic effects of E-cig exposure.

Keywords: mitochondrial dysfunction; permeability; elastic properties; atomic force microscopy

1. Introduction

Electronic cigarettes (E-cig) are gaining popularity, especially among youth, and have been considered as a potentially safer alternative to traditional cigarettes [1,2]. E-cigarettes (E-cig) first entered the US market in 2006, while pod mode forms such as “JUUL” were introduced in 2015. In 2019 the Centers for Disease Control and Prevention (CDC) and Food and Drug Administration (FDA) declared an “E-cigarette epidemic” due to a national outbreak of E-cig-induced acute lung injury (EVALI) cases [3]. E-cig inhalation causes lung tissue damage thereby increasing the risk of chronic lung disease [4–6] including COPD. The underlying mechanisms by which E-cigs contribute

to lung disease are incompletely understood; however, recent reports suggest that E-cigs cause inflammation, apoptosis, and tissue damage [7,8].

Despite the growing interest in their potential negative effects, it is unknown how E-cig exposure may alter the biomechanical properties of lung endothelial cells (EC), which play a critical role in maintaining vascular barrier integrity [9]. EC dysfunction is a hallmark of various pulmonary diseases, including those associated with cigarette and E-cig exposure [1,9,10]. Cytoskeletal changes are central to EC responses to injurious stimuli such as cigarette smoke (CS) [11]. CS exposure directly modulates EC barrier permeability, necrosis, and apoptosis through multiple signaling pathways involving RhoA, FAK, and p38, which all participate in cytoskeletal rearrangements [12–14]. Our group has identified cortactin (CTTN), a central regulator of the actin cytoskeleton, as an important modulator of lung EC function and lung injury responses [15,16]. Understanding the potential detrimental effects of E-cigs on EC cytoskeletal organization and associated biomechanical properties may provide novel insights into EVALI pathophysiology.

In prior work, we have shown that atomic force microscopy (AFM) is an invaluable tool to characterize nanoscale mechanical changes in living cells including endothelium [9,10]. AFM offers the sensitivity to detect early or subtle cytoskeletal disruptions that may precede overt morphological damage. AFM is a high-resolution scanning probe technique designed to investigate interatomic forces and surface topography at the nanoscale [10]. Using force-indentation curves and contact mechanics models such as Hertz or Sneddon, AFM enables the precise measurement of local elastic responses from cell membranes and underlying cytoskeletal structures [9,10,17]. For most cell types, Young's modulus typically ranges between 1 and 100 kPa [18,19], with stiffness largely governed by the integrity of the actin cytoskeleton and internal mechanical forces. These nanomechanical insights are critical, as alterations in cell stiffness are closely linked to changes in cellular function and are often associated with pathological processes. For example, malignant cancer cells may appear morphologically similar to benign ones, they can often be differentiated by their lower elastic modulus—reflecting cytoskeletal remodeling that facilitates invasion and metastasis [20]. This softening is often driven by upregulation of actin-regulating proteins such as Arp2/3, cortactin, and cofilin, promoting the formation of invasive structures like podosomes and invadopodia [20–23]. These findings highlight AFM's sensitivity in detecting biomechanical signatures of disease, particularly those driven by cytoskeletal reorganization. However, accurate interpretation of AFM-derived mechanical data depends on the probe geometry, indentation models, applied forces, and cell type, along with calibration for reproducibility.

In this study, we apply AFM to evaluate the nanomechanical properties of lung endothelial cells exposed to E-cigarette extract. We characterize cell stiffness through spatial force mapping and analyze elastic modulus, providing insight into how E-cigarette exposure disrupts endothelial mechanical integrity. This work provides novel insight into the mechanical underpinnings of E-cig-induced endothelial dysfunction and further advances our understanding of the pathophysiological effects of E-cigs on the lung vasculature.

2. Materials and Methods

2.1. Cell Culture

Human pulmonary artery endothelial cells (HPAECs) were purchased from Lonza (Walkersville, MD) and cultured in Endothelial Cell Growth Medium-2 (EGM-2) (Lonza) supplemented with 10% fetal bovine serum (FBS) (Sigma, St Louis, MO). Cells were maintained at 37 °C in a 5% CO₂ incubator and used at passages 6–8 for all experiments. Cells were starved for 2 hours in 2% FBS media prior to treatments.

2.2. E-Cigarette Preparation

“JUUL” E-cigarette was obtained commercially. The 5.0% nicotine, unflavored pod was used for our experiments and contains nicotine, propylene glycol, glycerin, and benzoic acid. Cells were treated with 50 µg/ml of E-cig liquid for 24 hours.

2.3. siRNA Transfection

HPAECs were transfected with scrambled RNA or *CTTN* siRNA (100 nM) using the DharmaFECT 1 transfection reagent. 48 hours after transfection, EC were challenged with E-cig. Transfection efficiency was determined by western blotting.

2.4. Immunofluorescence Microscopy

HPAECs were grown on 8 well glass chamber slide to 80-90% confluence in EGM-2 medium. After indicated treatments, cells were fixed with 3.7% paraformaldehyde for 10 min followed by three washes with PBS. The cells were then permeabilized with 0.25% Triton X 100 for 5 min and rinsed with PBS for 5 mins followed by incubation in blocking buffer (1% BSA-PBS) for 1 hour. Cells were then incubated with cortactin antibody for 1 hour, washed with PBS and then incubated with secondary antibody-Alexa Fluor 488 and Alexa 594-Phalloidin (F-actin staining) for 1 hour. After washing at least four times, the coverslips were mounted with profound gold DAPI (Invitrogen). Images were taken using a Zeiss confocal microscope at 40x magnification.

2.5. Reagents

Horseshoe Peroxidase (HRP)-linked anti-mouse and anti-rabbit secondary antibodies, anti-β actin antibodies and MitoTEMPO were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). SiRNA (control and *CTTN*) and DharmaFECT1 transfection reagent were purchased from Dharmacon (Horizon Inspired Cell Solutions). Anti-*CTTN* antibody was purchased from Sigma Aldrich.

2.6. Trans-Endothelial Monolayer Electrical Resistance (TEER) Measurements

Electrical cell impedance sensing (ECIS) (Applied Biophysics, Troy, NY) was used to evaluate the integrity of the EC monolayer, as described in our previous work [24]. Briefly, HPAEC were grown to confluence on gold microelectrodes, and the cells were subjected to a weak electric current for the continuous measurement of TER. After 1 h of measurement (to establish a stable baseline resistance), the indicated stimuli were added to the wells and measurement continued for 20 h. The data were analyzed using custom-designed Epool software by normalizing each resistance value to the starting resistance for that electrode as we have previously described [24].

2.7. AFM Imaging

Asylum MFP-3D-Bio atomic microscope (Oxford Instruments Asylum research, Santa Barbara, CA) and PNP-TR-Au-1 triangular silicon nitride cantilevers (with tip height 3.5 µm and radius of curvature 10 nm) were purchased from Asylum Oxford Research Group. Imaging for cells in liquid was performed in contact mode. Cantilevers with spring constant of $k=0.08\text{N/m}$ or 0.17N/m were used for imaging and elasticity measurements. Mechanical properties of the cell cytoskeleton were acquired in tapping force mode. The tip velocity was set at $1.99\text{ }\mu\text{m/s}$. The cell indentation part of the force curves were fitted to the Hertz model [25] and yield the Young's elastic modulus as a measure of cell stiffness, E .

2.8. Statistical Significance and Data Analysis

All data are expressed as mean $\pm$ SEM from at least three independent experiments. Statistical analysis was performed using the GraphPad Prism 8 software. Student's t-test or two-way ANOVA

(Tukey's or Dunnett's post hoc tests) were used to compare two or more groups respectively. Values of $*p < 0.05$ were considered statistically significant.

3. Results

3.1. E-Cigarette Exposure Induces Cytoskeletal Rearrangement and Gap Formation in Lung ECs

We first evaluated the effects of E-cig on the structure of critical cytoskeletal proteins cortactin (CTTN) and actin in cultured human lung EC [15]. As revealed by immunofluorescence imaging, E-cig stimulation of HPAEC causes redistribution of actin and CTTN that decreases their expression at the cell periphery (Figure 1). This pattern is similar to that we previously reported in response to CS exposure and is associated with decreased cell-cell interaction, gap formation between cells, and increased EC permeability [11,26,27].

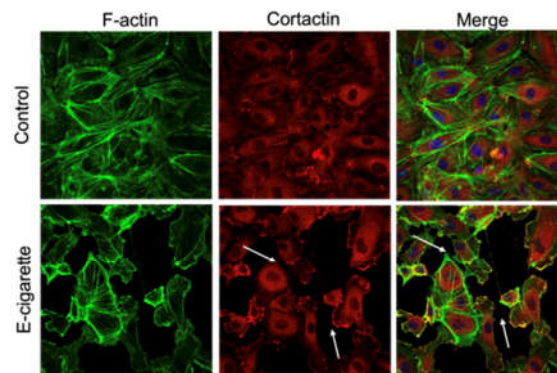


Figure 1. E-cigarette induces loss of cell-cell interaction in endothelial cells.

HPAECs were treated with vehicle or E-cig (25 $\mu\text{g/mL}$) for 3h, fixed and subjected to immunofluorescence analysis. Confocal images were taken at 40X after staining with Alexa488-CTTN (red), and Alex 594-Phalloidin (F-actin staining, green). Arrows indicate areas of gap formation and loss of cell-cell interactions.

3.2. Trans-Endothelial Resistance Is Decreased by E-Cigarette Exposure in a Dose-Dependent Manner in Lung ECs

Trans-endothelial electrical resistance (TER) measurements were obtained to investigate the effects of E-cig on EC barrier function, a primary physiologic role for the endothelium [17,28]. Dose-dependent E-cig exposures resulted in prolonged disruption of EC barrier function with exposure to 25 $\mu\text{g/mL}$ or higher of E-cig (Figure 2A).

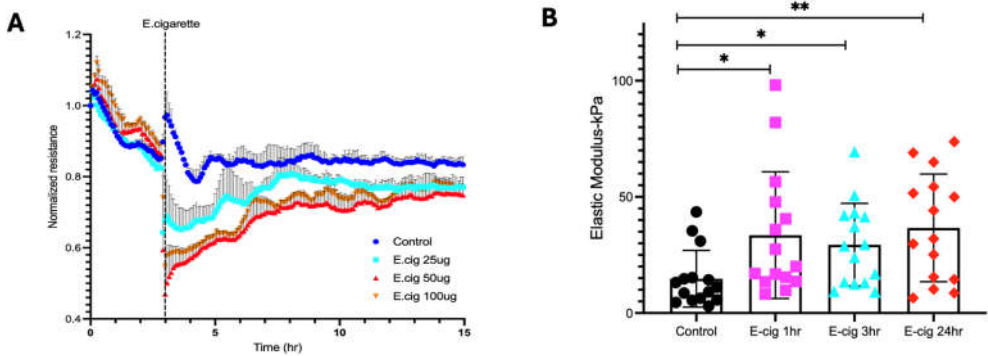


Figure 2A: E-cigarette causes loss of endothelial barrier function. HPAECs were grown on gold electrodes to ~90% confluence and then challenged with E-cigarette at three different concentrations (25 μ g/ml, 50 μ g/ml, and 100 μ g/ml). HPAEC monolayer permeability dynamics were assessed over 15 hours by continuous TER measurements. Shown is an ECIS tracing from a representative experiment. **Figure 2B: Elastic modulus of cytoplasm of HPAECs in response to E-cig in a time-dependent manner.** E-cig induces an increase in Young's modulus in HPAECs compared to control at 1 hr, 3 hr and 24 hr. Shown is a representative bar graph for 15-20 cells per condition.

3.3. Elastic Modulus Magnitude Is Increased in Lung ECs by E-Cig Exposure

We next characterized the mechanical properties of EC via AFM. Sub-confluent EC were first imaged using AFM contact mode, and then force curves were subsequently generated. AFM analyses of untreated cells were used as controls and compared to EC exposed to E-cig in a time-dependent manner (1 hr, 3 hr, and 24 hr). AFM measurements show that E-cig exposure significantly increased cell stiffness of ECs as compared to controls without E-cig exposure (Figure 2B). For the maximum loads applied during our experiments (~2nN), indentation made on ECs by the AFM tip was typically ~200-600 nm.

3.4. Cortactin Expression Modulates the Lung Barrier Effects of E-Cigarettes

Lung EC permeability is regulated by CTTN expression and function [15,27,29–31]. To investigate its role in lung EC barrier integrity during E-cig exposure, ECs were transfected with control or CTTN siRNA for 48 hours and then seeded on TEER plates. Western blotting confirmed significant reduction in protein expression of CTTN via siRNA (data not shown). TEER measurements demonstrated that E-cig-induced barrier disruption is significantly increased in ECs treated with CTTN siRNA (Figure 3). These data support an important role for CTTN expression in attenuating EC barrier disruption caused by E-cig.

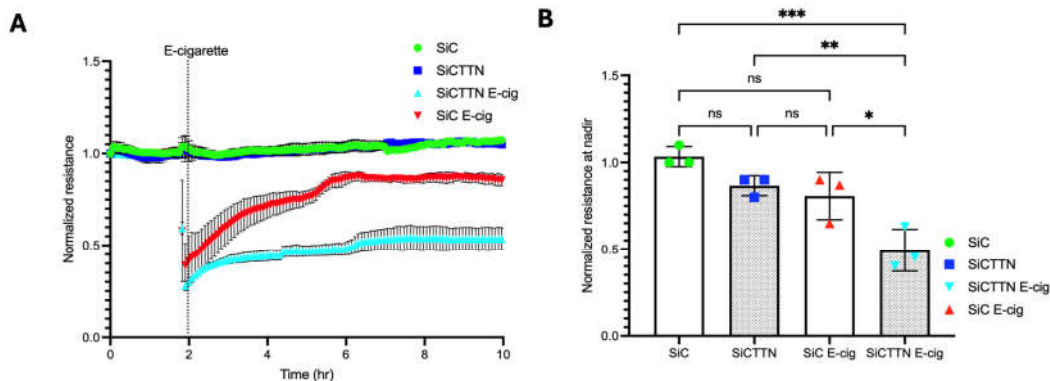


Figure 3. E-cig-induced permeability is regulated by CTTN expression in HPAEC. A; HPAECs grown on ECIS plates were transfected with control or CTTN specific siRNA and then challenged with E-cig (25 μ g/ml). TER was measured and the resistance in each well was normalized. B; quantification of normalized resistance at nadir. Shown is a tracing from a representative experiment.

3.5. Role of MitoROS in E-Cig-Induced Lung EC Permeability

Our recent report demonstrated a novel role for CTTN in mitoROS generation and lung endothelial apoptosis [11]. MitoTEMPO is a scavenger of mitoROS and an effective tool for assessing its functional significance [32]. Here we investigated the role of mitoROS in E-cig-induced lung EC permeability by pretreating ECs with MitoTEMPO (20 μ M for 3 hrs) prior to E-cig challenge (25 μ g/ml). TER measurements demonstrated partial attenuation of E-cig-induced barrier disruption in EC pretreated with MitoTEMPO, confirming an important functional role for mitoROS in E-cig-induced barrier disruption (Figure 4).

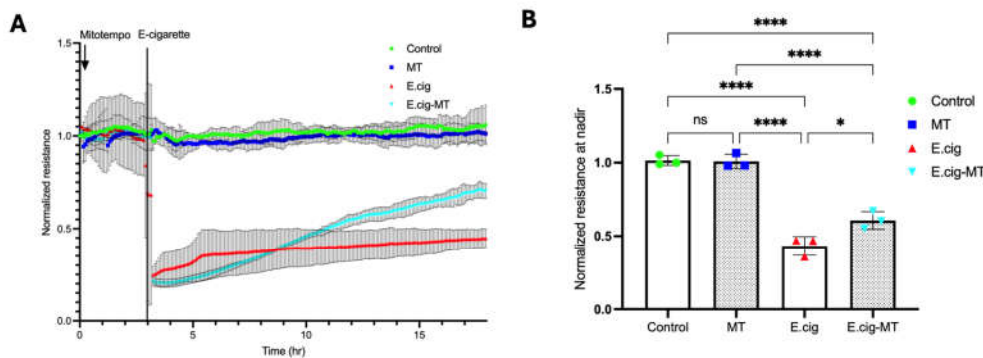


Figure 4. MitoTEMPO restores lung endothelial barrier function after E-cig challenge.
A; HPAECs grown on gold electrodes to ~90% confluence were pretreated with MitoTEMPO (20 μ M for 3 hours) followed by 25 μ g/mL of E-cig. Real time changes in TER were measured continuously over 15 hours using ECIS. B; statistical analysis was performed at the TER nadir; normalized resistance data were obtained from three independent experiments.

3.6. Mitochondrial ROS Participates in E-Cig-Induced Elastic Modulus Changes in Lung ECs

We next characterized the role of mitochondrial ROS in EC nanomechanical responses to E-cig using AFM. Sub-confluent EC were first imaged using contact mode, and then force-distance maps were subsequently generated. AFM analyses of untreated cells were used as controls and compared to EC pretreated with MitoTEMPO (20 μ M, 3 h) and then challenged with E-cig for 1hr. AFM measurements were used to generate elastic maps to determine Young's modulus. For maximum loads applied during our experiments (~2nN), deformation of the ECs by the AFM tip was typically ~200-600 nM. EC elastic modulus was significantly increased by E-cig exposure, but this increase was significantly attenuated in EC pretreated with MitoTEMPO, supporting an important functional role for mitochondrial ROS in E-cig-induced EC nanomechanical responses (Figure 5A).

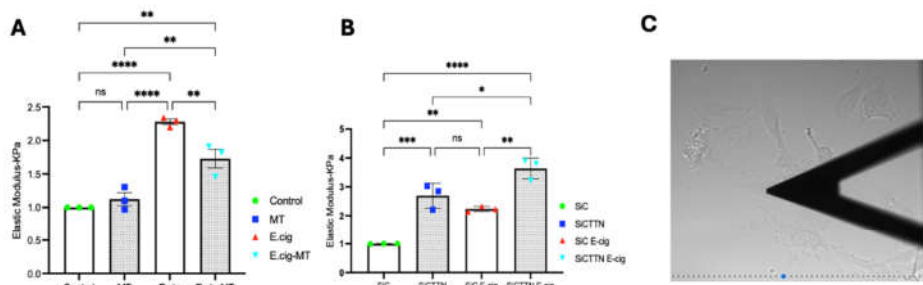


Figure 5A: MitoTEMPO attenuates EC elastic modulus responses to E-cig. E-cig induces a significant increase in Young's modulus in HPAECs compared to control, but pretreatment with MitoTEMPO (20 μ M, 3 hrs) lowers the elastic modulus in E-cig-induced ECs. Shown is a representative bar graph from three independent experiments (15-20 cells per condition/experiment). **5B: Elastic modulus responses to E-cig are enhanced by cortactin depletion in lung endothelial cells.** Inhibition of CTTN expression by siRNA (48 hours) enhances this elastic modulus response to E-cig in HPAECs compared to control. The bar graph represents data from three independent experiments (15-20 cells per condition/experiment). **5C:** White field image showing cantilever tip measuring elastic modulus of HPAECs.

3.7. CTTN Expression Regulates E-Cig-Induced Elastic Modulus Responses in Lung Endothelial Cells

We further explored the role of CTTN in E-cig-induced elastic modulus responses using AFM. EC were transfected with control or CTTN-specific siRNA for 48 h and then were challenged with E-cig for 1 h. Young's modulus was then determined as above. Elastic modulus was again increased upon E-cig challenge. This increase was significantly enhanced in siCTTN cells, further demonstrating an important role for CTTN in regulating E-cig-induced EC nanomechanical responses (Figure 5B).

4. Discussion

In 2019 the CDC/FDA declared an E-cigarette epidemic due to a national outbreak of E-cigarette-induced acute lung injury cases [E-cig or Vaping product use-associated acute lung injury (EVALI)] [33]. The underlying mechanisms by which E-cig contribute to lung disease remain incompletely understood, but it is likely that pathophysiologic effects on lung endothelial function are mechanistically involved. However, the effects of E-cig on lung ECs are only now beginning to be explored. Cortactin (CTTN) is an actin-binding protein that regulates cytoskeletal dynamics such as cell migration, adhesion, and apoptosis [11,13,15,34]. It plays a critical role in vascular integrity and has been studied in various diseases such as cancer and both acute and chronic lung disorders [21]. The potential mechanistic association between CTTN and E-cig-induced endothelial permeability is of particular interest given the essential barrier role of the lung endothelium. In our previous work, we demonstrated that CTTN plays a critical role in regulating lung endothelial apoptosis and mitochondrial ROS. Specifically, silencing CTTN or blocking its SH3 domain significantly increased endothelial cell apoptosis, providing mechanistic insights into COPD pathogenesis [11,15]. Building on these findings, the current study integrates atomic force microscopy (AFM), transendothelial electrical resistance (TEER) permeability assays, and immunofluorescence imaging to examine how E-cigarette exposure alters cytoskeletal organization, cell-cell interactions, and the biomechanical properties of endothelial cells (Figure 6). The major findings of this study are i) E-cig induces loss of cell-cell interaction causing gap formation in ECs, ii) E-cig causes loss of endothelial barrier function and increases elastic modulus (Y), indicative of increased cellular stiffness, iii) EC "Y" is attenuated by pretreatment with MitoTEMPO (MT), iv) EC "Y" is enhanced in CTTN-silenced EC during E-cig challenge, v) Pretreatment with MT protects the endothelial barrier during E-cig challenge, and vi) CTTN reduction by siRNA increases barrier disruption after E-cig challenge.

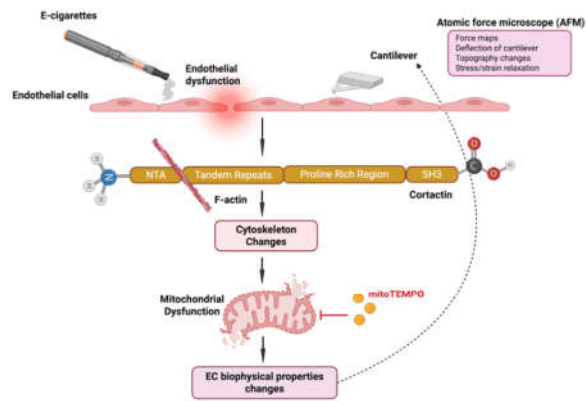


Figure 6. Schematic representation of the role of cortactin (CTTN) in e-cigarette-induced lung endothelial dysfunction. CTTN, an actin-binding protein, contributes to e-cigarette-mediated injury by promoting actin cytoskeletal rearrangement and mitochondrial dysfunction, leading to biomechanical changes. E-cigarette-induced barrier dysfunction is evaluated through measurement of elastic properties using atomic force microscopy. The mitochondrial antioxidant mitoTEMPO mitigates these effects by reducing ROS production and preventing barrier dysfunction. This study highlights a previously uncharacterized mechanism of E-cigarette-induced Lung endothelial barrier integrity and the role of CTTN in this mechanism.

Mechanical properties regulate cell development and function, and the relationship between force generation and cell architecture is an emerging area for providing fundamental insights [35,36]. The current study focuses on exploring the role of CTTN structure/function (Figures 3 and 5B) in modulating nanomechanical and rheological responses during E-cig-induced lung EC dysfunction [24,34]. External mechanical forces impact EC permeability through cytoskeleton rearrangement, junctional complex disassembly, and altered actomyosin contractility [17]. Here we identify that E-cig exposure leads to loss of cell-cell interaction and increases in EC permeability (Figures 1 and 2). To further investigate the role of CTTN and mitoROS in E-cig-induced lung EC dysfunction, AFM was utilized to characterize biomechanical properties in lung EC after manipulation of CTTN expression/function (via siRNA) and ROS inhibitor (MitoTEMPO). The silicon nitride cantilever used for these AFM measurements assesses the rheological property of EC in a minimally-invasive manner [37]. We employed AFM contact mode to generate force maps to calculate Young's modulus and determine topographical changes in lung EC upon E-cig or CSC challenge. We hypothesized that reducing CTTN by siRNA, or inhibition of mitoROS generation with MitoTEMPO, alters E-cig responses in lung EC in a manner that contributes to our understanding of their pathogenetic effects.

Our data indicate E-cig exposure increases the elastic modulus in lung ECs (as estimated with the Hertzian model) compared to vehicle controls. Force-distance mapping assesses the deflection of the AFM cantilever as it approaches the sample, demonstrating that cantilever deflection is lower in E-cig compared to vehicle (Figure 5A–C). Reduction of CTTN by siRNA results in an increase in the elastic modulus, and this effect is accentuated further upon E-cig exposure (Figure 5B). In contrast, scavenging mitoROS with MitoTEMPO ameliorates this E-cig effect on elastic modulus levels, suggesting a functional role in E-cig induced lung endothelial effects (Figure 5A). Elasticity is defined as a substance's ability to resist deformation. To complement our AFM data, we measured trans-endothelial resistance (TEER) using Electric Cell-substrate Impedance Sensing (ECIS) to demonstrate that E-cig exposure reduces EC barrier function in a dose-dependent fashion when compared to vehicle (Figure 2). Reduction in CTTN expression by siRNA enhanced this pathophysiologic disruption of EC permeability by E-cig (Figure 3), while MitoTEMPO inhibition of mitoROS resulted in some EC barrier protection [32,38,39] (Figure 4).

In summary, these data indicate that E-cig exposure induces a series of pathophysiologic events in lung EC, including increased elastic modulus, loss of intercellular interaction, decreased resistance, and increased EC permeability (Figure 6). Mechanistically, these responses are modulated in part by CTTN expression and mitoROS generation. These observations add to our previous work describing CSC- and E-cig-induced apoptotic changes [11] and further support an important functional role for CTTN in mediating these responses. In addition, prior reports have demonstrated spatially-specific alterations of the EC cytoskeleton in response to various agonists, with differential effects at the cell periphery, cytoplasm and nucleus [9]. Further studies are therefore needed to measure the E-cig-induced deformations at different subcellular regions such as nucleus, cytoplasm, and periphery to better understand the nanomechanical properties within the cell. Next steps also may include CTTN overexpression studies to determine if this approach is protective against E-cig-induced EC dysfunction. In vivo correlation is likewise needed and may include the use of genetically modified CTTN mice and AFM measurements of lung tissue after E-cigarette exposure. Future work may focus on assessing other EC biomechanical properties with AFM such as height and other morphometric changes after E-cig exposure. These assessments can determine EC shrinking and loss of cell-cell interaction. Direct comparisons between the effects of CSC and E-cig also can be made. Finally, the potential signaling connection between CTTN structure/function and mitoROS needs further characterization.

Author Contributions: Conceptualization: SMD, JGN, MB. Methodology: MB, XG, JL. Data acquisition and investigation: MB. Data analysis: MB. Writing—original draft preparation: MB. Writing—review and editing: MB, SMD, JGN, JL. Reagents: SMD, JGN. Supervision: SMD; JL. All authors have read and agreed to the published version of the manuscript.

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