Original Paper

Heat-Induced Epithelial Barrier Dysfunction Occurs via C-Src Kinase and P120ctn Expression Regulation in the Lungs

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Key Words

Heat • Thermal inhalation injury • P120ctn • C-Src • Cell junction

Abstract

Background/Aims: Thermal injury causes pulmonary edema and can lead to death. Intercellular junctions are composed of adhesive (p120ctn, E-cadherin, α -catenin and β -catenin) and compact (occludin and ZO-1) junctions. Heat deteriorates intercellular junctions and increases cell gaps to ultimately induce pulmonary edema, but the underlying mechanism remains elusive. **Methods:** Mouse lung epithelial (MLE-12) cells pre-treated with the c-Src inhibitor PP2, p120ctn catenin (p120ctn) small interfering RNA and p120ctn catenin (p120ctn) complementary DNA were subjected to heat treatment. Western blotting and real-time polymerase chain reaction assays were used to evaluate junction protein expression changes after heat treatment, and co-immunoprecipitation was used to test the binding state of junction proteins. In addition, hematoxylin and eosin staining and immunohistochemistry were used to evaluate changes in junction protein expression and lung injury in a Wistar rat model of thermal inhalation injury. **Results:** Heat increased cell permeability; induced ZO-1, occludin, α -catenin and β -catenin degradation; and decreased E-cadherin distribution in cell membranes. Heat also activated c-Src and decreased both p120ctn expression levels and occludin and ZO-1 association. c-Src inhibitor (PP2) treatment and p120ctn overexpression reversed these effects and attenuated lung injury *in vivo*. **Conclusion:** Heat induces junction protein degradation and dissociation to increase membrane permeability and cause lung edema via c-Src kinase activation and p120ctn expression downregulation.

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Introduction

Heat is a major risk factor for lung damage in thermal inhalation injury, which is common in burn patients and can induce acute lung injury [1]. Thermal injury causes pulmonary

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edema, which can lead to acute respiratory distress syndrome, sepsis, multiorgan failure and even death [2]. Alveolar epithelial cells are the main barrier protecting the lungs from endogenous and exogenous damage. Heat causes cell junction deterioration and increases gaps between these cells, ultimately inducing pulmonary edema, which is the leading cause of mortality in thermal inhalation injury [3]. However, the mechanism through which heat destroys cell connections and leads to pulmonary dysfunction remains elusive. Treatments that maintain alveolar membrane integrity and pulmonary vascular permeability after heat exposure are crucial for patients with thermal inhalation injury [4]. Therefore, a detailed understanding of cell junction protein structure and function is highly significant for preventing and treating heat-induced lung injury.

Pulmonary edema and lung injury occur through a series of reactions and inflammatory responses induced by heat [5, 6]. Alveolar barrier function loss is a hallmark of severe lung injury because the junctions between endothelial and epithelial cells regulate paracellular fluid flux. Heat also reportedly induces intestinal epithelial barrier dysfunction [7]. The destabilization of cell-cell contacts involved in maintaining endothelial barrier function can lead to increased endothelial permeability. This increase in endothelial permeability results in the anarchical movement of fluid, solutes and cells out of the vasculature and into the surrounding tissues, thereby contributing to various diseases, such as stroke or pulmonary edema [8, 9]. The results of studies performed during the past three decades have shown that hyperthermia changes junction protein expression and increases paracellular permeability in *in vitro* culture models. The most direct indicator of permeability is cell connectivity, which is mediated by adherens junctions and tight junctions and plays an indispensable and key role in maintaining the integrity of the epithelial barrier. The main components of adherens junctions include p120ctn, E-cadherin, α -catenin and β -catenin, whereas compact junctions are composed primarily of occludin and ZO-1 [10]. In epithelial cells, external stimuli, such as inflammation and hypoxia, can activate intracellular tyrosine kinases (tyrosine pressure protein kinase or c-Src) and trigger a series of inflammatory responses [11]. c-Src is a member of the Src family, which is involved in intracellular signal transduction [12]. Studies have shown that PL20 is a c-Src kinase substrate that contributes to the degradation and synthesis of tight junction proteins. c-Src kinase activation can initiate p120ctn degradation to disrupt alveolar epithelial barrier integrity in ventilator-induced lung injury [12]. However, the functions of c-Src and p120ctn in thermal inhalation injury require further research.

In the present study, we hypothesized that heat degrades membrane integrity and increases pulmonary vascular permeability in thermal inhalation injury. Specifically, heat induces c-Src activation to cause p120ctn degradation, which disturbs cell protein junctions and reduces the association of occludin and ZO-1, thus causing cause alveolar epithelial barrier dysfunction and pulmonary hyperpermeability.

Materials and Methods

Cell culture and treatment

MLE-12 cells (Cell Resource Center, Beijing, China) were cultured at 37°C in a 5% CO₂ humidified-atmosphere in DMEM (Dulbecco's modified Eagle's medium) (Gibco, CA, USA) containing 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were incubated at 52°C for 5 min for the *in vitro* heat exposure model. PP2 and SU66656 (Thermo Scientific, Fremont, CA, USA) were dissolved in dimethyl sulfoxide (DMSO) and freshly diluted for each experiment. The DMSO concentration in the culture medium was less than 0.1% in all the experiments. The cells were treated with the PP2 inhibitor (10 μ m) and SU66656 (50 nm) 4 h before the heat treatment. MLE-12 cells were incubated at 47°C for 5 min to produce a heat exposure damage model. To investigate the changes in lung epithelial cell integrity after heat treatment, we used FITC-dextran and TER to measure the lung epithelial cell permeability.

Animals and thermal inhalation injury

The animal studies were approved by the Animal Ethics Committee of the Third Military Medical University and were conducted according to the approved guidelines. Thermal inhalation injury was induced

as previously described [13]; adult male Sprague-Dawley rats were used for the experiments. All procedures were approved by the Animal Ethics Committee of Jinan University and were carried out in accordance with the approved guidelines. The thermal inhalation injury procedure was modified from a previously described method used in dogs [13]. Adult male Sprague-Dawley rats were placed under general anesthesia by constant inhalation of isoflurane (5%) via a face mask. Tracheotomies were performed under anesthesia, and a 1-cm catheter was inserted into the trachea; preheated dry air was blown through the catheter into the airway by a heat gun (TAK-3316E, Shenzhen Takgiko Technology, Shenzhen, China) at a flow rate of 190 L/min for a total of 10 min with 1 min intervals in between each minute of heat inhalation. The air was preheated to 110°C inside the heat gun and was determined to be approximately 50°C when blown into the end of the catheter. Then, the rats were closed, and the wounds were sutured; acetylpromazine (0.75 mg/kg body weight) was injected intraperitoneally to relieve pain.

Western blotting

RIPA lysis buffer was used to extract protein from cells and lung tissue (80–120 mg). After protein concentration determination, equal amounts of protein were subjected to 6–8% polyacrylamide-sodium dodecyl sulfate gel electrophoresis and transferred as previously described. Membrane strips were blocked with 5% non-fat milk for 1 h at room temperature and incubated with primary antibodies overnight at 4°C. The following antibodies were used for detection: E-cadherin, occludin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β -catenin and α -catenin (Life Technologies, Germany). β -actin or β -tubulin (Santa Cruz Biotechnology) was detected on the same membrane as the control to ensure equal protein loading. After incubation with secondary horseradish peroxidase-labeled antibodies (Abcam, UK; diluted 1:5000 in blocking buffer), the membranes were washed three times, incubated with an enhanced chemiluminescence substrate developing solution and detected with a FluorChem E System (Alpha Innotech, San Leandro, CA, USA). Densitometry was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA). All experiments were performed in triplicate.

Immunoprecipitation

Protein was extracted on ice for 30 min using RIPA lysis buffer; the samples were ultrasonicated and centrifuged at $16,000 \times g$ for 5 min at 4°C. After protein concentration determination, the lysates were mixed with mouse immunoglobulin (Ig) G and agarose A/G on a shaker for 5 h at 4°C. Then, the samples were centrifuged at $13,000 \times g$ for 5 min. After the supernatants were collected, a primary antibody was added to pull down the protein complexes, and the mixtures were incubated overnight at 44°C on a shaker. The agarose was washed five times with centrifugation at 1300 rpm and then dissolved in sodium dodecyl sulfate gel sample buffer. The samples were analyzed using the western blotting protocol.

p120ctn small interfering RNA (siRNA) and p120ctn complementary DNA (cDNA) transfection

Mouse lung epithelial (MLE-12) cells (70% confluent) were cultured in optim media without serum for 2 h. Then, the scrambled siRNA and p120ctn siRNA (Santa Cruz Biotechnology) were transfected using Lipofectamine® RNAiMAX transfection reagent (Invitrogen, MA, USA) according to the manufacturer's protocol [7]. The p120ctn 1A cDNA and the empty vector (GeneChem Inc., Shanghai, China) were transfected using Lipofectamine 2000 cDNA reagent (Invitrogen, MA, USA) according to the manufacturer's protocol. After 12 h, the cell culture media was exchanged for fresh complete media. Forty-eight hours after transfection, the cells were used for the experiments.

To overexpress p120ctn *in vivo*, we injected liposome-based vectors into the retinal vein plexus for the systemic and targeted delivery of the p120ctn cDNA complex. After p120ctn siRNA and p120ctn cDNA transfection, we treated lung epithelial cells with heat at 47°C for 5 min and then returned them to normal cell culture conditions. After 2 days, we measured E-cadherin expression levels in the cell membrane and cytoplasm. Western blotting was used to detect the transfection efficiency 2 days later; at this point, we established the inhalation injury model. In some experiments, the animals were intravenously administered the c-Src inhibitor PP2 (2 μ g/kg) dissolved in dimethyl sulfoxide 1 h before inhalation injury.

Quantitative real-time reverse transcription-polymerase chain reaction (PCR)

Total RNA was extracted from 30 μ g of cell protein and 100 mg of lung tissue using Trizol reagent. After the total RNA concentration was measured, 1 μ g of total RNA was used for reverse transcription.

Subsequently, quantitative real-time reverse transcription-PCR was carried out using a cDNA synthesis kit (Thermo Scientific, Fremont, CA, USA) and the SGExcelUltraSYBR Mixture (Sangon Biotech, Shanghai, China).

Measurement of transepithelial electrical resistance (TER)

MLE-12 cells were seeded at 2.0×10^6 cells per well on polycarbonate membranes in Transwell inserts (6.5-mm diameter, 0.4- μ m pore size; Corning, Cambridge, MA, USA). Data were collected when the TER values were consistently above 300 Ω/cm^2 . The TER values for the filter-grown MLE-12 intestinal monolayers were measured with a Millicell ERS (electrical resistance system; Millipore, Bedford, MA, USA). All TER values are expressed as Ω/cm^2 .

Epithelial paracellular permeability assay

MLE-12 paracellular permeability across cell monolayers was measured using the flux of fluorescein isothiocyanate (FITC)-labeled dextran with a molecular mass of 4 kDa (FD4, Sigma-Aldrich). FD4 (1 mg/mL) was added to the medium in the apical chambers of Transwells (Corning). After 2 h, the medium was collected from the bottom chamber, and the fluorescence was measured with a fluorometer (excitation, 492 nm; emission, 520 nm; BioTek).

Histopathologic analysis

Lung samples (sham, Heat, DMSO, PP2 and P120cDNA) were isolated and fixed in 4% paraformaldehyde (4 h) and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin as previously described [14]. The slides were observed using a fluorescence microscope (Instruments Inc., USA) and quantified by randomly counting 30 areas from three independent experiments. The degree of lung pathological injury was scored based on the following variables: inflammatory cell infiltration, lung edema, hemorrhaging, hyaline membrane structure and atelectasis. The degree of each abnormality was graded numerically from 0 (normal) to 4 (diffuse injury) according to the following criteria (19): no injury = 0; injury to 25% of the field = 1; injury to 50% of the field = 2; injury to 75% of the field = 3; diffuse injury = 4. Blind analyses were performed to determine the lesion degree of all samples [15].

Immunohistochemical staining

Immunohistochemistry analyses were performed using paraffin-embedded lung sections (2–4 mm). The sections were deparaffinized in xylene and dehydrated in a gradient of ethanol dilutions. A proteolytic enzyme solution was used for antigen retrieval. Then, the sections were incubated with rabbit IgG, anti-occludin (Santa Cruz Biotechnology) and anti-ZO-1 (Invitrogen, Paisley, UK) antibodies with 5% non-fat milk overnight. On the second day, the slides were washed three times and incubated with biotinylated anti-rabbit IgG goat IgG (Sigma-Aldrich, Shanghai, China) and then with an ABC-alkaline phosphatase kit (Vector, Burlingame, CA, USA). Images were taken with a Leica Microsystems apparatus (DFC300FX, Leica, Switzerland) and quantified with Image Pro-Plus. Five random fields were examined per animal to detect ZO-1 and occludin expression levels [16]. ZO-1 and occluding expression levels were also quantified using ImageJ software. Briefly, after the figures were transformed into 8-bit images, immunohistochemistry images were acquired using identical parameters, and the background intensity was subtracted. Then, the ZO-1 staining intensity was measured. The reported values are the averages of different images from five rats [17].

Determination of the wet-to-dry weight (W/D) ratio

Wet weights were obtained immediately after fresh lung tissues were isolated. Then, 100 mg of lung tissue was dried in an oven at 60°C for 2 days; at this point, the stable weight was recorded as the dry weight. Then, the W/D ratio was calculated.

Statistical analysis

Statistical analyses were performed by using t-tests and one-way ANOVA. The data are presented as the means $\pm$ standard error of three independent experiments for each group and were analyzed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). All tests were two-tailed. A P value of <0.05 was considered significant.

Results

Decreased junction protein expression levels and increased paracellular permeability in lung epithelial cells after heat treatment

As shown in Fig. 1A, no changes in these parameters were observed at time points 0 h and 12 h after heat treatment. However, the paracellular permeability was increased on days 1 and 3. To further evaluate the damage to junction proteins caused by heat exposure, we assessed the expression levels of ZO-1, occludin, β -catenin and α -catenin in lung epithelial cells after heat treatment. The real-time PCR results indicated that the levels of these factors were decreased on days 1 and 3 after heat treatment (Fig. 1B). Consistently, the protein expression levels were also decreased on days 1 and 3 (Fig. 1C). Furthermore, we isolated cell membrane and cytoplasmic proteins to examine the effect of heat on E-cadherin distribution. On days 1 and 3, the E-cadherin levels in the cell membrane were decreased significantly, whereas the levels in the cytoplasm were increased compared with those in the control group (Fig. 1D).

Decrease in junction protein binding in heat-treated lung epithelial cells

Junction protein binding states are critical for maintaining cell permeability. To further explore the mechanism through which heat affects junction proteins, we analyzed the binding states of junction proteins with co-immunoprecipitation. As shown in Fig. 2A, the association of PL20 and E-cadherin was decreased on days 1 and 3 after heat treatment. Similarly, occludin and ZO-1 protein binding was reduced. These results indicate that the heat treatment negatively affected the junction binding states of the lung epithelial cells (Fig. 2B).

Fig. 1. Heat treatment increased permeability and decreased junction protein expression levels in lung epithelial cells. (A) Paracellular permeability was analyzed with fluorescein isothiocyanate-dextran and transepithelial electrical resistance. ns, no significant difference, ** $P < 0.01$ and *** $P < 0.001$ vs. control ($n = 3$). (B) Real-time polymerase chain reaction analysis and quantification of the expression of the cell junction proteins ZO-1, occludin, β -catenin and α -catenin. Relative messenger RNA (mRNA) levels were normalized to the 18s ribosomal RNA levels. ns, no significant difference, ** $P < 0.01$ and *** $P < 0.0001$ vs. control ($n \geq 3$). (C) Representative western blot and quantification data for the cell junction proteins ZO-1, occludin, p120ctn, β -catenin and α -catenin. $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$ vs. control. (D) Representative western blot and quantification data for E-cadherin in the cell membrane and cytoplasm. ns, no significant difference, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$ vs. control.

Involvement of p120ctn and c-Src in heat-induced junction protein degradation

Heat treatment degraded p120ctn and increased phosphorylated (p)-c-Src expression levels, which means that heat treatment activated Src activity (Fig. 3A). To further elucidate the role of p120ctn and c-Src in heat-induced junction damage, we used p120ctn siRNA, p120ctn cDNA, PP2 (a c-Src inhibitor) and SU6656 to regulate the expression of p120ctn and c-Src. Under normal conditions, P120ctn cDNA and P120ctn siRNA respectively increased and decreased P120ctn expression levels (Fig. 3B). In addition, the P120ctn cDNA group expressed higher levels of P120 than the control group after the heat treatment (Fig. 3C). As we expected, both PP2 and SU6656 decreased (p)-c-Src expression, which was activated by the heat treatment (Fig. 3D and Fig. 3E). Notably, in the normal cells, we found that c-Src kinase activity was higher in the p120ctn siRNA treatment group than in the control group (Fig. 3F). Furthermore, P120ctn cDNA inhibited (p)-c-Src activation in the heat damage model, which indicated that p120ctn regulates c-Src activity (Fig. 3G).

Heat can lead to the degradation of junction proteins (ZO-1, occludin, β -catenin and α -catenin). However, compared with the heat treatment group, the PP2 and p120ctn cDNA treatment groups showed dramatically lower cell junction degradation. To avoid pharmacological agent off-target effects, we also used SU6656 as a c-Src inhibitor. We found that SU6656 can dramatically inhibit cell junction degradation. These results demonstrate that p120ctn/c-Src regulated the junction protein changes after heat treatment, and p120ctn was upstream of c-Src in the regulation of c-Src activity (Fig. 3I).

Effect of p120ctn on E-cadherin distribution and junction protein binding in vitro

Under normal conditions, p120ctn siRNA transfection significantly decreased E-cadherin expression levels in the cell membrane. However, the expression levels in the cytoplasm were increased compared with those in the scrambled siRNA group (Fig. 4A). After heat treatment, E-cadherin expression levels in the cell membrane and cytoplasm decreased and increased, respectively. Notably, p120ctn cDNA treatment reversed the distribution of E-cadherin (Fig. 4B). All of these results indicate that the loss of P120ctn can decrease E-cadherin expression in the cell membrane, and heat can decrease P120ctn to cause E-cadherin departure from the membrane. These findings are compatible with the results that heat can decrease P120ctn expression (Fig. 3A) and cause E-cadherin transfer from the membrane to the cytoplasm (Fig. 1D).

We also performed co-immunoprecipitation analyses to examine junction protein binding after p120ctn cDNA and siRNA transfection. As shown in Fig. 4C and Fig. 4D, the degradation of occludin was significantly increased in the P120CTN siRNA transfection group, and the results of the co-immunoprecipitation analyses suggested that p120ctn siRNA transfection further reduced the association of ZO-1 and occludin. Conversely, the overexpression of p120ctn reduced the degradation of occludin and increased the association of ZO-1 and occludin.

Fig. 2. Heat decreased the junction protein binding state of lung epithelial cells. (A) Co-immunoprecipitation (Co-IP) analyses were used to determine the binding of p120ctn and E-cadherin. Representative western blot and quantification data for p120ctn and E-cadherin. $P^* < 0.05$ and $***P < 0.001$ vs. control ($n \geq 3$). (B) Co-IP was used to analyze the binding of occludin and ZO-1. Representative western blot and quantification data for occludin and ZO-1. $*P < 0.05$, $***P < 0.001$ vs. control ($n \geq 3$).

Fig. 3. Heat decreased p120ctn expression levels and activated c-Src phosphorylation. (A)

Representative western blot and quantification data for p120ctn, phosphorylated c-Src (p-c-Src), c-Src and β -actin at various time points after heat treatment. ns, no significant difference, *** $P < 0.0001$ vs. control.

(B) Representative western blots of p120ctn after p120ctn small interfering RNA (siRNA) or p120ctn complementary DNA (cDNA) treatment in normal cells. (C) Representative western blots of p120ctn after p120ctn complementary DNA (cDNA) transfection in heat-treated cells. (D) Representative western blots of p-c-Src and c-Src after PP2 treatment in heat-treated cells. (E) Representative western blots of p-c-Src and c-Src after SU6656 treatment in heat-treated cells. (F) Representative western blots of p-c-Src and c-Src after p120ctn small interfering RNA (siRNA) treatment in normal cells. (G) Representative western blots of p-c-

Src and c-Src p120ctn after p120ctn complementary DNA (cDNA) transfection in heat-treated cells. (H) Representative western blot and quantification data for the cell junction proteins ZO-1, occludin, β -catenin and α -catenin after p120ctn siRNA, p120ctn cDNA and PP2 treatment. (I) Representative western blot and quantification data for the cell junction proteins ZO-1, occludin, β -catenin and α -catenin after SU6656 treatment. ns, no significant difference, *** $P < 0.0001$ vs. control.

Heat treatment decreased junction protein expression levels and increased paracellular permeability *in vivo*

Because heat can disturb junction protein integrity and increase cell permeability *in vitro*, we investigated the effect of heat on lung injury *in vivo*. The W/D weight ratio of lung tissue was used to quantify pulmonary edema. After thermal inhalation injury, the W/D weight ratios were increased on days 3 and 5, which means that pulmonary edema occurred in the thermal inhalation injury model (Fig. 5A). As shown in Fig. 5B, compared with the sham group, the injury group had significantly lower mRNA expression levels of occludin, ZO-1, α -catenin and β -catenin on days 2 and 5. Similarly, tight junction protein expression levels were the lowest on days 2 and 5 (Fig. 5C).

Fig. 4. Effect of p120ctn on E-cadherin distribution and junction binding in the heat treatment model. (A) Representative western blot and quantification data for E-cadherin in the cell membrane and cytoplasm after p120ctn siRNA treatment. ns, no significant difference, $P < 0.05$, $**P < 0.01$ and $***P < 0.0001$ vs. control ($n \geq 3$). (B) Representative western blot and quantification data for E-cadherin in the cell membrane and cytoplasm after p120ctn cDNA treatment. ns, no significant difference, $*P < 0.05$, $**P < 0.01$ and $***P < 0.0001$ vs. control ($n \geq 3$). (C) Binding between occludin and ZO-1 after p120ctn siRNA transfection was analyzed with Co-IP. $*P < 0.05$ and $***P < 0.001$ vs. control ($n \geq 3$). (D) Binding between occludin and ZO-1 after p120ctn cDNA transfection was analyzed with Co-IP. $*P < 0.05$ and $***P < 0.001$ vs. control ($n \geq 3$).

Effects of p120ctn and c-Src on thermal inhalation lung injury

Compared with the heat group, the p120ctn cDNA and PP2 groups had significantly lower W/D ratios (Fig. 6A). Furthermore, the expression levels of ZO-1, occludin, β -catenin and α -catenin were higher in the P120CTN cDNA and PP2 treatment groups (Fig. 6C), which indicated that p120ctn cDNA and PP2 treatment may have protective effects on the junction protein degradation induced by thermal inhalation injury.

Epithelial barrier dysfunction in the lungs can induce inflammatory cell infiltration and lung edema and increase lung permeability. Lung pathological injury scores can indicate lung permeability [15]. The degree of lung pathological injury was scored based on the following variables: inflammatory cell infiltration, lung edema, hemorrhaging, hyaline membrane structure and atelectasis. As shown in Fig. 6D, lung pathological injury scores were three times higher than those of the control group. The histopathologic examination results showed increased numbers of inflammatory cells and red cells in the lungs from the thermal inhalation injury group. The epithelial integrity and lung permeability were significantly increased after heat treatment. Notably, all of these effects were markedly limited in the p120ctn cDNA and PP2 treatment groups (Fig. 6D). As shown in Fig. 6E and Fig. 6F, occludin and ZO-1 expression was detected mainly in the cytoplasmic membranes or peripheral cytoplasm of endothelial cells and the alveolar epithelium, and staining was more

Fig. 5. Heat treatment disturbed junction protein expression and increased paracellular permeability in vivo. (A) The wet-to-dry (W/D) weight ratio was determined as detailed in the Materials and Methods section. The data shown are the means $\pm$ SEM of at least three independent experiments ($n = 6$ in each group). ns, no significant difference, ** $P < 0.01$ and *** $P < 0.0001$ vs. the sham group. (B) Real-time polymerase chain reaction and quantification data for the cell junction proteins ZO-1, occludin, β -catenin and α -catenin. Relative mRNA levels were normalized to the 18S ribosomal RNA levels. ns, no significant difference, ** $P < 0.01$ and *** $P < 0.0001$ vs. the sham group ($n \geq 3$). (C) Representative western blots and quantification data for the cell junction proteins ZO-1, occludin, p120ctn, β -catenin, p-c-Src and α -catenin. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$ vs. the sham group.

intense at the junctions of adjacent cells. Compared with the heat treatment group, the PP2 and p120ctn cDNA treatment groups had significantly higher levels of occludin and ZO-1 expression.

Discussion

In the present study, we found that heat-induced c-Src activation led to p120ctn degradation, which reduced the disassociation and binding of junction proteins *in vitro* and *in vivo*. In a thermal inhalation injury model, heat treatment induced junction protein degradation and dissociation, resulting in increased alveolar membrane permeability and lung edema via c-Src kinase activation and p120ctn expression downregulation. This study is the first to elucidate the mechanism through which heat affects paracellular lung permeability and induces lung edema, and these findings may open new avenues for the therapy of thermal inhalation injury.

Several studies have shown that lung edema, acute lung injury and acute respiratory distress syndrome are caused by barrier malfunctions [18]. The disruption of intercellular junctions caused by heat is a major factor in epithelial permeability [19]. Accumulating evidence in animal and cell culture models indicates that the junctional malfunction of the lung barrier contributes significantly to deleterious outcomes. Moreover, heat has long been reported to induce intestinal barrier dysfunction through various pathways [20]. Consistent with these results, we found that in thermal inhalation injury, heat induces structural and cytosolic changes in alveolar epithelial cells and ultimately causes alveolar epithelial barrier dysfunction and pulmonary hyperpermeability.

In a conflagration, the temperature of the air surrounding the fire source is approximately 800°C [21, 22]. However, the temperature of the air in the bronchi is approximately 48.3°C

Fig. 6. Heat treatment disturbed junction protein expression and increased paracellular permeability in vivo. (A) The W/D weight ratios after PP2 and p120ctn cDNA treatment were determined as detailed in the Materials and Methods section. The data shown are the means $\pm$ SEM of at least three independent experiments ($n = 6$ in each group). ns, no significant difference, ** $P < 0.01$ and *** $P < 0.0001$ vs. the sham group. (B) Western blot data for p-c-Src and p120ctn after PP2 and p120ctn cDNA treatment in vivo. (C) Representative western blot and quantification data for the cell junction proteins ZO-1, occludin, β -catenin and α -catenin. $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$. (D) Hematoxylin and eosin staining showing lung permeability after inhalation injury. (E) Representative immunohistochemistry quantification data showing the localization and expression of ZO-1 in lung tissues after inhalation injury. (F) Representative immunohistochemistry quantification data showing the localization and expression of occludin in lung tissue after inhalation injury.

because more than 90% of the inhaled heat is released after passing through the upper airway. In the intestines, heat stress (39–41°C) can disturb junction proteins and barrier function [23]. Accordingly, we created a heat damage model with cell exposure to 47°C for a short time to mimic the temperature at which inhalation injury occurs in burn patients.

In lung epithelial cells and the lung barrier, heat stress can induce lung intercellular junction degradation and increase lung permeability [2, 18, 24]. Some studies of intestinal cells have found that after heat treatment, junction protein expression levels increase initially and then decrease dramatically approximately 12 h after treatment. However, we observed no change in junction protein expression immediately after heat treatment; instead, we found a dramatic decrease on day 1 in the *in vitro* model and on day 2 in the *in vivo* model [25]. This difference may be attributed to differences in the cell lines used and the organs studied. Clinically, lung edema usually occurs on day 5 after thermal inhalation injury, which is consistent with our results.

Cell adherens junctions and tight junctions are key components in maintaining lung epithelial membrane integrity and permeability [26]. In particular, tight junctions are located on the outside of pulmonary capillary endothelial and epithelial cells and are composed of protein complexes containing claudins, ZO-1, junctional adhesion molecules (JAMs) and occludins; these junctions seal epithelial barriers to maintain pulmonary vascular permeability [27, 28]. Claudin and JAM-1 were shown to be associated with ZO-1 through both non-PDZ and PDZ domains [29]. Although more proteins are present, the major types are ZO-1 and the occludins, which can bind to the actin component of the cytoskeleton. Thus, occludin and ZO-1 are always used as the major makers for tight junctions [30]. Mark et al. found that the downregulation or redistribution of occludin and ZO-1 in intestinal epithelial cells impaired the function of the intestinal epithelial barrier, causing malabsorption and diarrhea [26, 31]. Adherens junctions depend on the associations of p120ctn, E-cadherin, β -catenin and α -catenin, which mediate the transduction of stress signals to vascular endothelial cells [32]. β -catenin acts by anchoring the actin cytoskeleton to the junctions. α -catenin participates in the formation and stabilization of adherens junctions by binding to β -catenin-cadherin complexes in the cell [33]. Additional catenins, such as γ -catenin and δ -catenin, have been identified. Most studies investigating catenin actions focus on α -catenin and β -catenin [34]. However, it is unclear whether the regulation of occludin and ZO-1 is related to lung tissue permeability in thermal inhalation injury. Therefore, we measured ZO-1, occludin, β -catenin and α -catenin expression after heat treatment. Our data showed that occludin and ZO-1 expression levels were decreased in thermal inhalation injury, reaching their lowest levels 2 days after injury; these decreases induced lung tissue permeability. These changes correlated with changes in lung tissue permeability, which suggests that structural abnormalities in junction proteins were responsible for the lung damage.

p120ctn plays a key role in lung injury induced by mechanical ventilation [12, 35]. It is expressed at high levels in pulmonary vascular endothelial cells, lung epithelial cells, fibroblasts, cardiomyocytes and muscle cells; in these cell types, it co-functions with E-cadherin to maintain intercellular adhesion junction stability. E-cadherin facilitates the assembly of specialized cellular junctions required to organize epithelial cells into a functional monolayer. Changes in the structure and function of the cells disturb alveolar membrane integrity and decrease cell junction protein expression, further increasing pulmonary vascular permeability [36]. The results of our study showed that heat treatment disrupted the association of p120ctn and E-cadherin. We also found that intercellular junction endocytosis and degradation were enhanced after p120ctn depletion both *in vitro* and *in vivo*. Moreover, p120ctn knockdown significantly downregulated the association of occludin and ZO-1 under normal conditions. After heat treatment, p120ctn overexpression reversed the heat-induced downregulation of occludin and ZO-1 association. *In vivo*, p120ctn overexpression decreased the lung W/D weight ratio and reversed the intercellular junction dysfunction caused by heat. Furthermore, compared with the scrambled siRNA group, the P120CTN siRNA group showed decreased E-cadherin expression levels in the cell membrane after heat treatment, and occludin and ZO-1 association was downregulated (see Fig. 4A

and 4c); these results indicate that other pathways also regulate intercellular junctions in thermal inhalation injury.

Src kinase is a critical protein for cell signal transmission, and it participates in cell adhesion, growth, migration, differentiation and necrosis [11, 37]. Moreover, Src plays a key role in regulating lung tissue inflammation. Mechanical stretch stimulation reportedly activates Src kinase and causes cell attachment protein degradation. PP2, a Src kinase-specific inhibitor, effectively blocks the mechanical stretch-induced degradation of cell junction proteins, further reducing lung injury [38]. In our study, after heat treatment, Src kinase activity was significantly increased, resulting in cell junction protein degradation and accelerated lung edema development. The c-Src inhibitor PP2 effectively limited cell junction protein degradation, which indicates that heat caused the junction protein degradation to induce lung injury by activating Src kinase. Consistent with previous studies, our results also showed that p120ctn degradation was associated with c-Src activation [27, 28]. Moreover, PP2 reversed the heat-induced degradation of p120ctn *in vitro* and *in vivo*. These results indicate that c-Src is a critical and upstream signaling protein in thermal inhalation injury models.

This study has several limitations, and further research is needed to clarify our findings. First, we used an immortal cell line for the *in vitro* studies. Because it is unethical to perform a study of this nature *in vivo* in humans, a new technique is required to improve the applicability of this research to human patients. Second, Ley and Arfors developed a protocol in which intravenous FITC-dextran was used with fluorescence microscopy to monitor changes in microvascular leakage in intact tissues. This technique could be used for further studies *in vivo* [39]. Third, temperature is likely a core factor in compromised lung barrier function, which leads to lung edema. However, the mechanism through which heat affects lung barrier permeability is complicated and includes inflammatory cytokine activity as well as endotoxemia and the innate immune dysfunction of the mitochondria or endoplasmic reticulum [5, 40], which may become a network pathway between cells. Additional research is needed to elucidate this mechanism more clearly. Fourth, a microarray experiment was devised to monitor changes in gene expression in the injured skin from 45 burn patients compared to normal skin from 15 healthy patients. According to a global gene microarray analysis of cutaneous burn wounds, there was a dataset of 1136 upregulated genes and 1003 downregulated genes during the 1–17-day period after burn trauma [41]. Jonathan et al. also present a global characterization of genes and proteins related to the lung response to heat stress in a rat model, and they found 78 differentially expressed transcripts in the lung, including transcripts related to the unfolded protein response, inflammation, and apoptosis [42]. A global gene microarray analysis was also needed for the epithelia after the heat treatment.

In summary, our findings indicate that heat treatment induces lung tissue permeability and lung edema by activating c-Src kinase, which in turn downregulates p120ctn expression. This study may provide potential targets for the development of new drugs that can protect lung function from further damage in thermal injury.

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Disclosure Statement

The authors of this article declare no conflict of interests.

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