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Insulin prevents pulmonary vascular leakage by inhibiting transglutaminase 2 in diabetic mice

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ABSTRACT

Aims: Insulin is a central peptide hormone required for carbohydrate metabolism; however, its role in diabetes-associated pulmonary disease is unknown. Here, we investigated the preventative effect of insulin against hyperglycemia-induced pulmonary vascular leakage and its molecular mechanism of action in the lungs of diabetic mice.

Main methods: Vascular endothelial growth factor (VEGF) activated transglutaminase 2 (TGase2) by sequentially elevating intracellular Ca^{2+} and reactive oxygen species (ROS) levels in primary human pulmonary microvascular endothelial cells (HPMVECs).

Key findings: Insulin inhibited VEGF-induced TGase2 activation, but did not affect intracellular Ca^{2+} elevation and ROS generation. Insulin prevented VEGF-induced vascular leakage by inhibiting TGase2-mediated c-Src phosphorylation, disassembly of VE-cadherin and β-catenin, and stress fiber formation. Insulin replacement therapy prevented hyperglycemia-induced TGase2 activation, but not ROS generation, in the lungs of diabetic mice. Insulin also prevented vascular leakage and cancer metastasis in the diabetic lung. Notably, vascular leakage was not detectable in the lungs of TGase2-null (*Tgm2*$^{-/-}$) diabetic mice.

Significance: These findings demonstrate that insulin prevents hyperglycemia-induced pulmonary vascular leakage in diabetic mice by inhibiting VEGF-induced TGase2 activation rather than ROS generation.

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder induced by chronic hyperglycemia due to insufficient insulin secretion or action. Hyperglycemia in diabetic patients is associated with progressive blood vessel damage and dysfunction, resulting in microvascular and macrovascular complications [1]. Additionally, it has been proposed that the lung may be affected by DM [2]. Pulmonary dysfunction including reduced lung volume and diffusing capacity has been reported in type 1 and type 2 DM [3,4]. DM may also increase the risk of chronic obstructive pulmonary disease (COPD) and asthma [5,6], and the pathophysiologies of DM and COPD are related [6]. However, the effect of hyperglycemia on pulmonary disease and the underlying mechanism(s) remain unclear.

Vascular permeability is tightly regulated by the actin cytoskeleton and intercellular adherens junctions, which limit macromolecule passage across the microvasculature [7]. Vascular permeability is stimulated by growth factors such as vascular endothelial growth factor (VEGF) and inflammatory cytokines [8]; it is involved in the progression of various conditions including diabetic complications [1] and cardiovascular disease [9]. Lung capillaries are densely packed, and thus vascular permeability can also contribute to pulmonary disease [10]. Increased vascular permeability may contribute to pulmonary diseases such as acute respiratory distress syndrome (ARDS), cancer, and inflammation [10]. The vascular permeability factor VEGF contributes to small-airway remodeling that is a pathological feature of COPD [11]. It is therefore important to inhibit pulmonary vascular leakage to prevent pulmonary disease progression.

Insulin is essential for controlling blood glucose concentration [12]. Insulin has differential effects in each organ, although this hormone

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affects key metabolic biological processes like glucose and lipid metabolism [13]. Insulin therapy is widely used to treat diabetes [14] and reportedly exerts beneficial effects against ischemic/reperfusion renal injury [15]. In brain, the potential effects of insulin have been described for the treatment of central nervous system (CNS)-related metabolic disorders including dementia and cognitive dysfunction [16]. Interestingly, insulin may not be involved in preventing diabetic complications, although it has a major function in controlling blood glucose levels [1]. Moreover, insulin therapy exacerbates retinopathy in type 2 diabetic patients [17,18]. Insulin is important in the development of the lung [13]. However, the function of insulin in pulmonary disease is not clear.

In this study, we demonstrate the preventative effect of insulin against hyperglycemia-induced pulmonary vascular leakage in diabetic mice. We hypothesized that insulin inhibits transglutaminase 2 (TGase2) and prevents pulmonary vascular leakage in the diabetic mice. TGase2 is involved in VEGF-induced vascular leakage through VE-cadherin disruption in the retina of diabetic mice, but its role in pulmonary vascular integrity is not known [19]. We found that insulin inhibited VEGF-induced molecular events including vascular leakage by inhibiting TGase2 activation, but it did not affect reactive oxygen species (ROS) generation, in primary human pulmonary microvascular endothelial cells (HPMVECS). We also found that insulin replacement therapy prevented hyperglycemia-induced TGase2 activation and vascular leakage, but not ROS generation, in the lungs of diabetic mice. Our findings suggest that insulin can prevent hyperglycemia-induced vascular leakage in the diabetic lung.

2. Materials and methods

2.1. Cell culture

HPMVECs were obtained from the Applied Cell Biology Research Institute (Cell Systems, Kirkland, WA) and grown on 2% gelatin-coated plates in M199 medium supplemented with 20% FBS, 3 ng/mL basic fibroblast growth factor, 5 U/mL heparin, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO_2 incubator. Cells were authenticated by STR analysis and seven to eleven passage subconfluent cells were used in the experiments. For *in vitro* experiments, cells were incubated for 6 h in low-serum medium supplemented with 1% FBS and antibiotics. B16F10 cells, obtained from American Type Culture Collection (Manassas, VA, USA), were maintained at 37 °C in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO_2 incubator.

2.2. Measurement of intracellular Ca^{2+} levels and ROS generation

Intracellular Ca^{2+} levels and ROS generation were determined using Fluo-4-AM and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Thermo Fisher Scientific, Waltham, MA), respectively, as previously described [19]. Fluorescence intensity peaks at the single-cell level were determined for 10 randomly selected cells/experiment.

2.3. Measurement of in situ TGase activity

In situ TGase transamidating activity was determined by a confocal microscopic assay as previously described [20]. Briefly, cells were incubated at 37 °C with 10 ng/mL VEGF for 2 h and treated with 1 mmol/L 5-(biotinamido)pentylamine (BAPA), a TGase pseudosubstrate, for the last 1 h. After fixing with 3.7% formaldehyde in PBS for 30 min, cells were permeabilized with 0.2% Triton X-100 in PBS for 30 min and stained with fluorescein isothiocyanate (FITC)-conjugated streptavidin (1:200; MilliporeSigma, Billerica, MA) for 1 h. The fluorescence intensities of single stained cells were determined by confocal microscopy (FV-300; Olympus, Tokyo, Japan) for 10 randomly selected cells/experiment.

2.4. Transfection with human TGM2-specific siRNA

HPMVECs were transfected with 100 nmol/L human *TGM2*-specific siRNA or control siRNA (Dharmacon, Inc., Lafayette, CO) using siLentFect lipid reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions and as described previously [19].

2.5. Visualization of VE-cadherin, β-catenin, and actin filaments in HPMVECs

VE-cadherin and β-catenin were visualized as previously described [19]. HPMVECs were incubated with various inhibitors for 30 min and treated with 10 ng/mL VEGF for 90 min. Fixed cells were incubated overnight at 4 °C with monoclonal antibodies against VE-cadherin or β-catenin (1:200; Santa Cruz Biotechnology, Dallas, TX) and probed with FITC-conjugated goat anti-mouse antibodies (MilliporeSigma). Labeled cells were visualized by confocal microscopy (Olympus). Adherens junctions were quantitatively analyzed at the single-cell level for 10 randomly selected cells/experiment.

For actin filament visualization, cells were treated with 10 ng/mL VEGF, fixed, and permeabilized. Cells were incubated with Alexa Fluor 488 phalloidin (1:200; Thermo Fisher Scientific) for 1 h at room temperature, and actin filaments were observed with a confocal microscope [19].

2.6. Western blot analysis

Proteins were extracted from pulmonary cells and tissues, subjected to SDS-PAGE, and transferred to polyvinylidene fluoride membranes. The membranes were blocked and probed with a phosphor-Src (Tyr^{416}) antibody (1:2000; Cell Signaling Technology, Danvers, MA) and then incubated with horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized using a chemiluminescent substrate (Bio-Rad, Hercules, CA).

2.7. In vitro permeability assay

In vitro permeability was assessed as previously described [21]. HPMVECs were grown to confluence on gelatin-coated inserts (Costar, Corning, NY). Cells were treated with 10 ng/mL VEGF for 90 min and incubated with 1 mg/mL 40-kDa FITC-dextran (MilliporeSigma) for the last 60 min. The amount of FITC-dextran in the lower chamber was measured with a microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA).

2.8. Generation of diabetic mice

Six-week-old male C57BL/6 mice were obtained from DBL (EumSeong, Korea). *Tgm2*$^{-/-}$ mice (C57BL6), prepared as previously described [19], were kindly given by Dr. Soo-Youl Kim (National Cancer Center, Korea). Mice were housed in conventional cages with a 12:12 h light-darkness cycle. Diabetic mice were generated by intraperitoneal injections of streptozotocin (200 mg/kg body weight, MilliporeSigma) freshly prepared in 100 mmol/L citrate buffer (pH 4.5), as previously described [22]. Mice with nonfasting blood glucose concentrations ≥19 mmol/L, polyuria, and glycosuria were considered diabetic. All animal experiments conformed to National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals) and were approved by the Institutional Animal Care and Use Ethics Committee of Kangwon National University.

2.9. Insulin supplementation with osmotic pumps

One week after streptozotocin injections, diabetic mice were anesthetized with isoflurane. Anesthesia was monitored *via* the pain reflex reaction. Mice were implanted with Alzet Mini-osmotic pumps (model

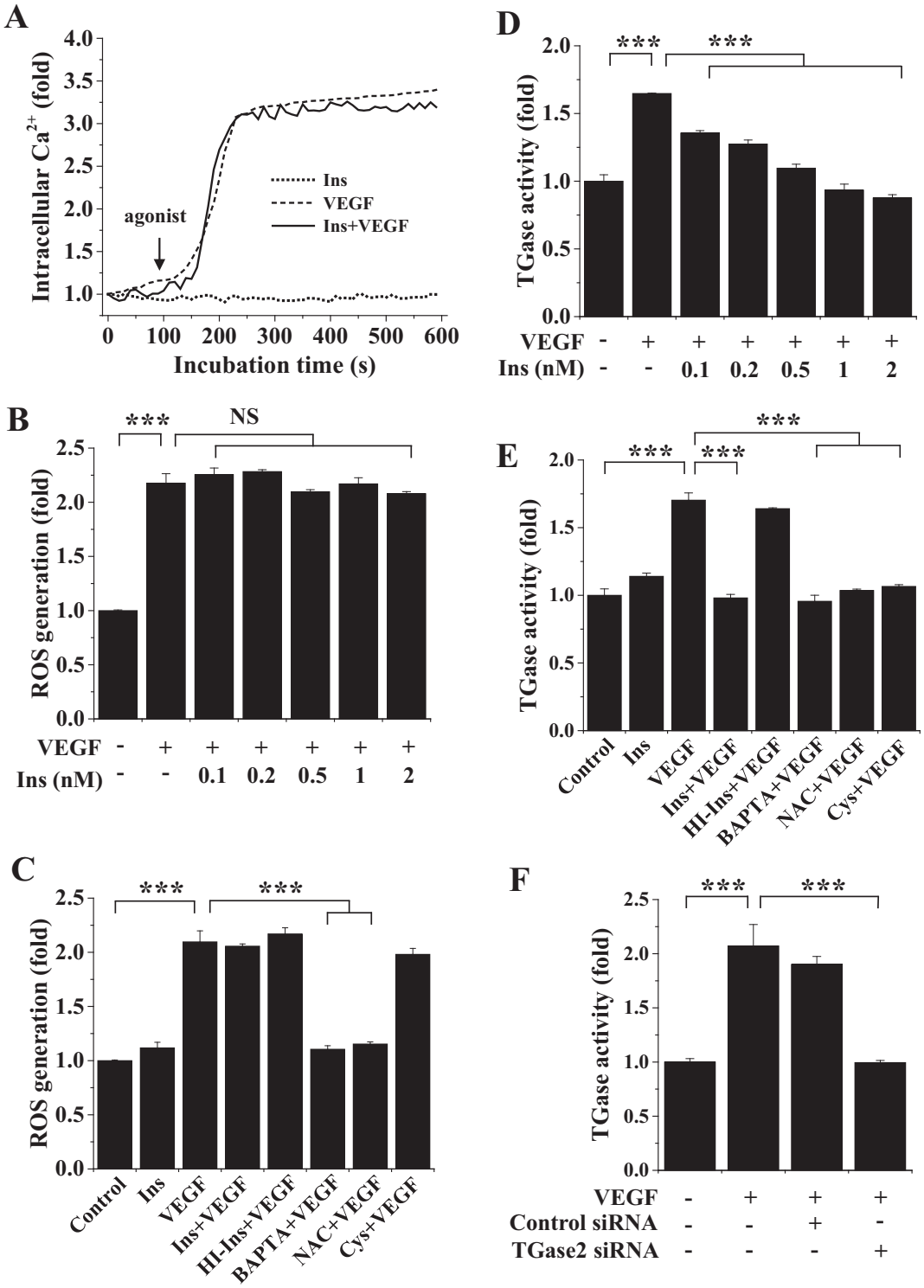

Fig. 1. Insulin inhibits VEGF-induced TGase2 activation but has no effect on intracellular Ca^{2+} elevation or ROS generation in HPMVECs.
A: Time course of VEGF-induced intracellular Ca^{2+} increase. HPMVECs were incubated with 1 nmol/L insulin (Ins) and 10 ng/mL VEGF, and intracellular Ca^{2+} levels were monitored by confocal microscopy. B and C: Intracellular ROS generation. HPMVECs were incubated with the indicated concentrations of insulin for 30 min (B) or incubated with 1 nmol/L insulin (Ins), 1 nmol/L heat-inactivated insulin (HI-Ins), 5 μmol/L BAPTA-AM, 1 mmol/L NAC, or 50 μmol/L cystamine (Cys) (C). Cells were then treated with 10 ng/ml VEGF for 10 min, and ROS levels were determined with confocal microscopy. D and E: *In situ* TGase activity. HPMVECs were incubated with 10 ng/mL VEGF for 2 h in the presence of the indicated concentrations of insulin (D) or 1 nmol/L insulin, 1 nmol/L heat-inactivated insulin, 5 μmol/L BAPTA-AM, 1 mmol/L NAC, or 50 μmol/L cystamine (E). *In situ* TGase activity was determined by confocal microscopy. F: *TGM-2* siRNA prevention of VEGF-induced TGase activation. HPMVECs were transfected with 100 nmol/L control or human *TGM2* siRNA and treated with 10 ng/mL VEGF for 2 h. Results are expressed as the mean ± SD from three independent experiments. ***$P < 0.001$. NS, non-significant.

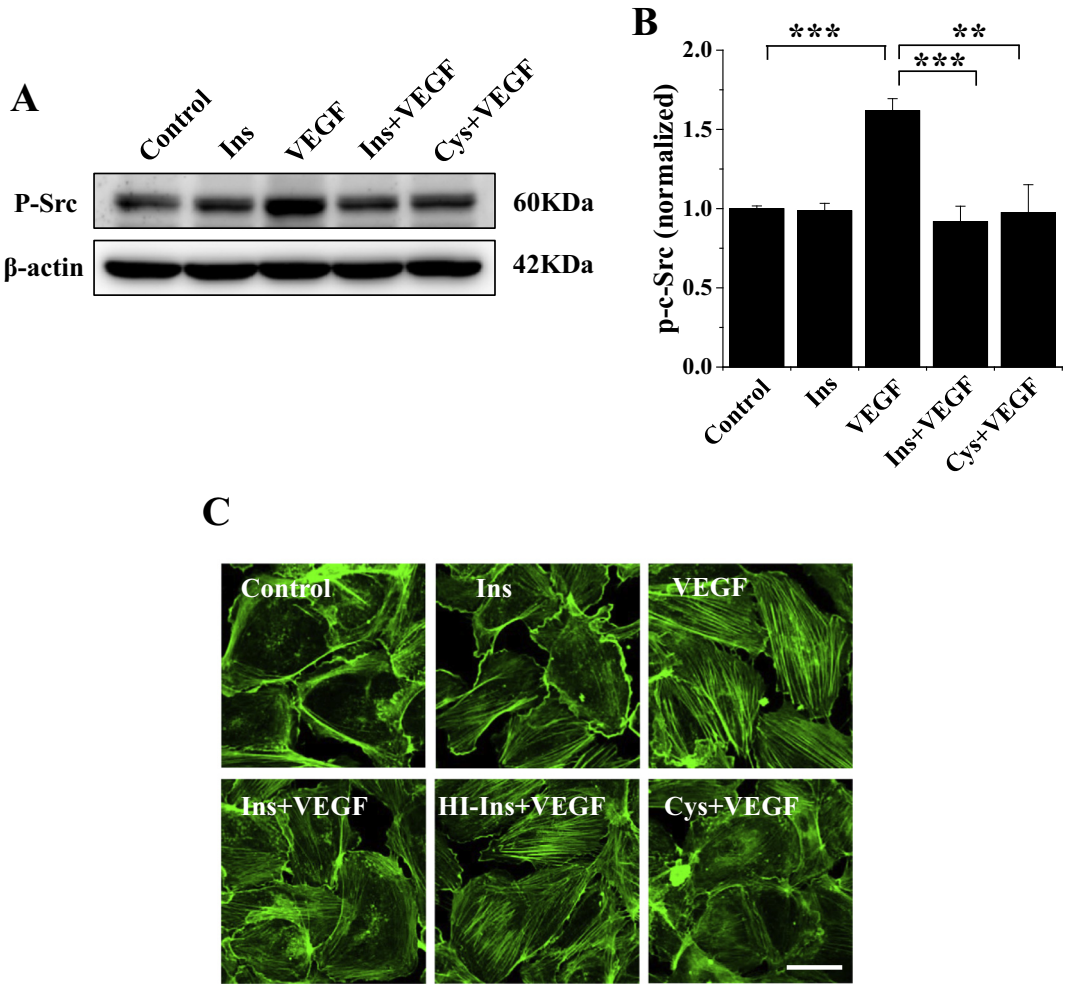

Fig. 2. Insulin inhibits VEGF-induced c-Src phosphorylation and stress fiber formation in HPMVECs.
HPMVECs were incubated with 1 nmol/L insulin (Ins), 1 nmol/L heat-inactivated insulin (HI-Ins), or 50 μmol/L cystamine (Cys) for 30 min and treated with 10 ng/mL VEGF for 10 min (A and B) or 90 min (C). A and B: c-Src phosphorylation at Tyr^{416} was analyzed by Western blot and quantified by densitometry ($n = 3$). Results are expressed as the mean ± SD from three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$. **C**: Stress fibers were stained with Alexa 488-conjugated phalloidin and observed by confocal microscopy. Scale bar, 20 μm.

2004; Durect Corp., Cupertino, CA) to deliver human insulin (MilliporeSigma) at a rate of 58.4 pmol/min/kg [23]. Control and unsupplemented diabetic mice underwent sham operations. Four weeks after streptozotocin injection, mice were subjected to measurements of ROS levels, *in vivo* TGase activity, and vascular leakage in the lungs.

2.10. Measurement of ROS generation in mouse lungs

ROS levels were determined as previously described [24]. Briefly, the lungs were dissected and quickly frozen in OCT compound (Sakura Finetek, Torrance, CA). Unfixed cryosections (60-μm thick) were incubated with 10 μmol/L dihydroethidium (Thermo Fisher Scientific) in PBS for 30 min at 37 °C. The ROS levels were also measured by incubating with 10 μmol/L H_2DCFDA in PBS for 10 min at 37 °C as previously described. Stained sections were observed by confocal microscopy, and ROS levels were quantitatively analyzed *via* fluorescence intensities ($n = 8$/group).

2.11. Measurement of in vivo TGase activity in mouse lungs

In vivo TGase activity was determined by confocal microscopy [24]. Briefly, mice were deeply anesthetized, and 48 μL of 100 mmol/L BAPA was injected into the right ventricle of each mouse and allowed to circulate for 10 min. Mice were killed by cervical dislocation, and their lungs were fixed overnight with 4% paraformaldehyde at 4 °C and permeabilized with 0.2% Triton X-100 in PBS for 30 min at room temperature. The lungs were treated with FITC-conjugated streptavidin (1:200 [vol/vol]) for 1 h and then embedded in a cryosectioning medium. Cryosections of stained lungs (20-μm thick, $n = 8$/group) were observed by confocal microscopy (K1-Fluo; Nanoscope Systems, Taejon, Korea), and *in vivo* TGase activities were quantified using fluorescence intensities.

2.12. Measurement of vascular leakage in mouse lungs

Microvascular leakage in mouse lungs was investigated by confocal microscopy [24]. Briefly, 1.25 mg of 500-kDa FITC-dextran (MilliporeSigma) was injected into the right ventricle of each mouse and allowed to circulate for 5 min. The lungs were dissected, fixed, and embedded in OCT compound (Sakura Finetek). Cryosections (20-μm thick) of lungs ($n = 8$/group) were stained with Alexa 647-isolectin B4 (1:1000; Thermo Fisher Scientific). Microvascular leakage was quantitated by determining the fluorescence intensities of FITC-dextran (Nanoscope Systems).

Vascular leakage was also assessed with the lung Evans blue extravasation assay [25]. Briefly, 100 μl of 1% Evans blue dye was injected into the tail vein ($n = 9$, normal and diabetic mice; $n = 7$, insulin-supplemented diabetic mice) and allowed to circulate for 40 min.

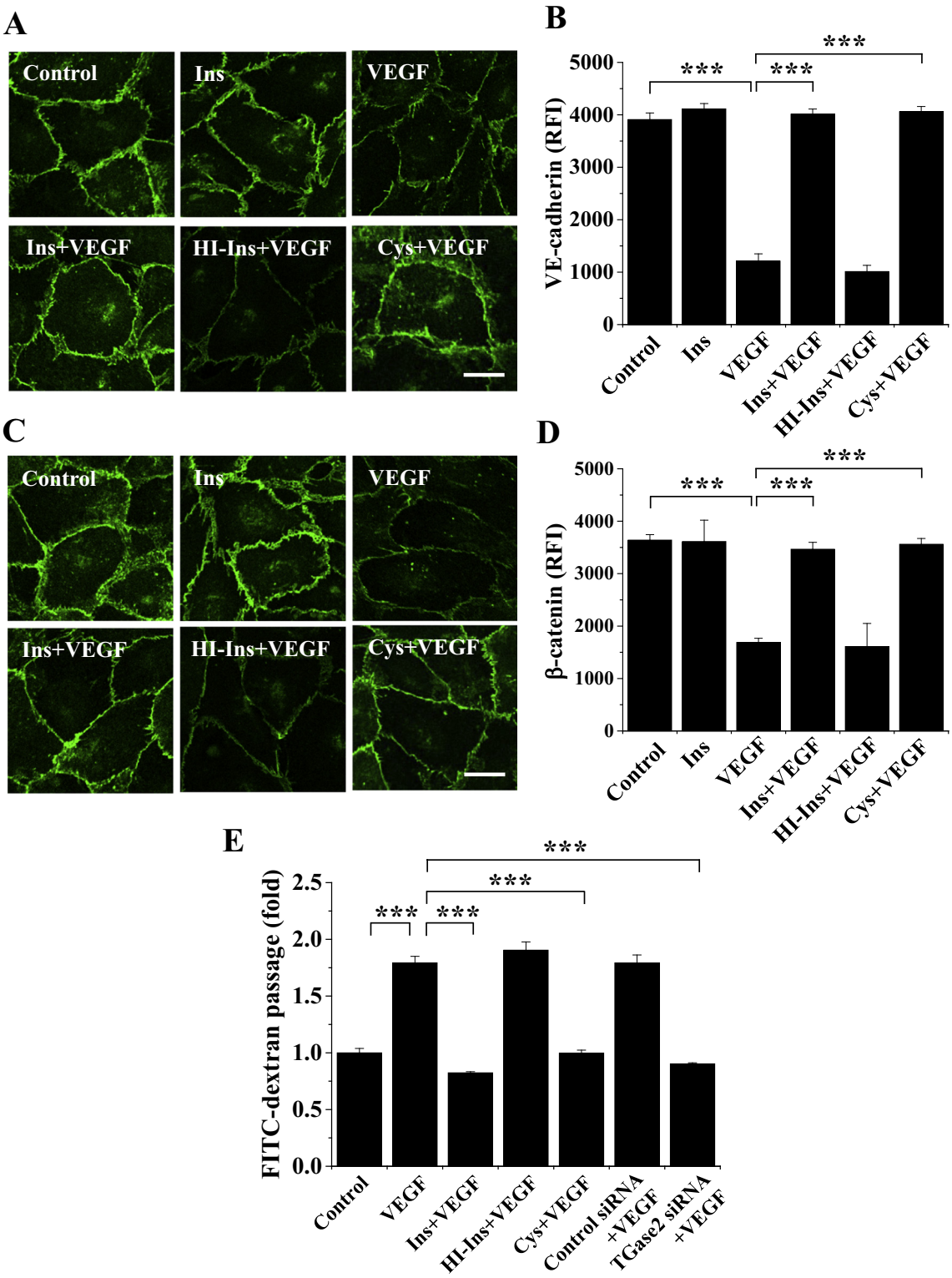

Fig. 3. Insulin inhibits VEGF-induced adherens junction disruption and endothelial cell permeability in HPMVECs.
HPMVECs were incubated with 1 nmol/L insulin (Ins), 1 nmol/L heat-inactivated insulin (HI-Ins), or 50 μmol/L cystamine (Cys) for 30 min or transfected with 100 nmol/L control or human *TGM-2* siRNA and treated with 10 ng/mL VEGF for 90 min. A–D: VE-cadherin (A and B) and β-catenin (C and D) were stained and visualized by confocal microscopy. Scale bar, 20 μm. VE-cadherin (B) and β-catenin (D) were quantitatively analyzed using peak fluorescence intensities at the single-cell level. E: *In vitro* endothelial cell monolayer permeability assay. Results are expressed as the mean ± SD from three independent experiments. ***$P < 0.001$.

To remove the dye from circulation, mice were perfused by injecting 40 mL of PBS containing 2 mM EDTA through the left ventricle of heart, allowing the blood to flow out by puncturing the right ventricle. Lungs were isolated and incubated at 78 °C with formamide for 2 days, and the amount of dye was quantitated by spectrophotometry at 620 nm.

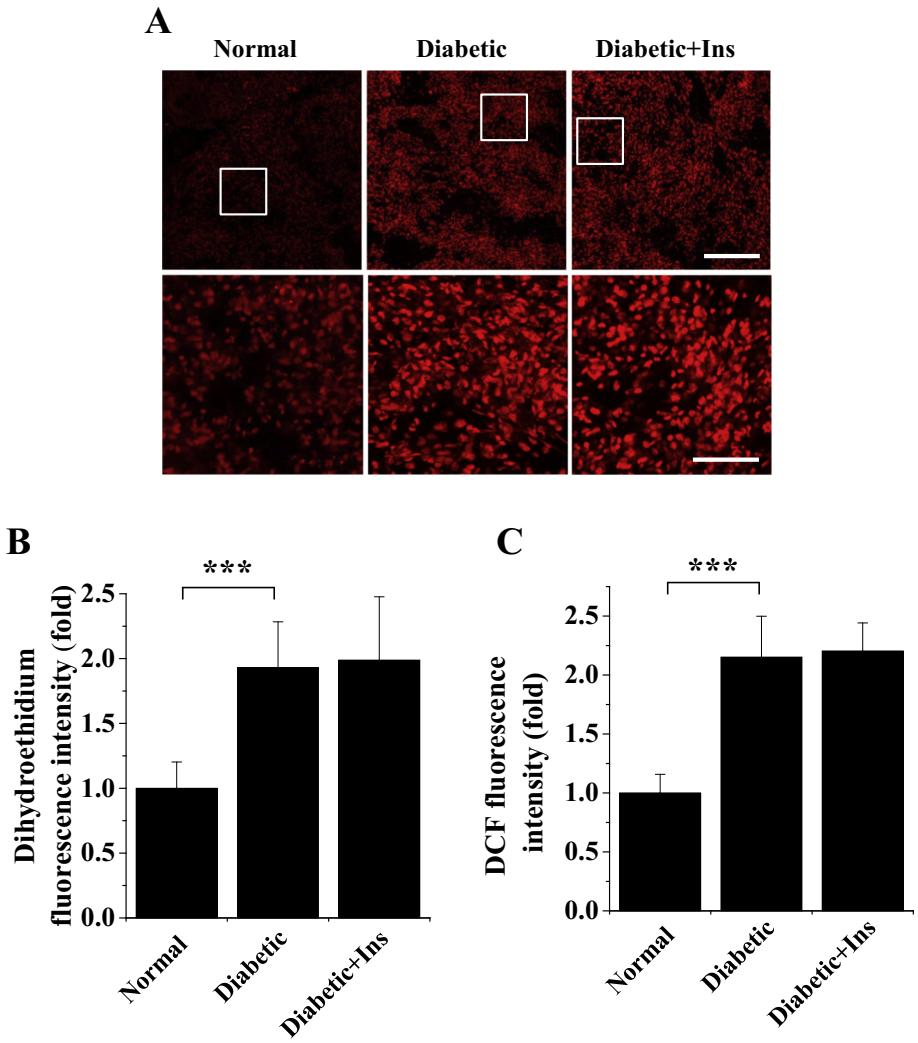

Fig. 4. Insulin supplementation does not inhibit hyperglycemia-induced ROS generation in the lungs of diabetic mice.
Diabetic mice were supplemented with insulin using osmotic pumps, and ROS generation in the lungs was visualized by confocal microscopy using dihydroethidium (A and B) or H_2DCFDA (C). A: Representative images of dihydroethidium staining. The bottom images (scale bar, 50 μm) are higher magnifications of the areas indicated in the top images (scale bar, 200 μm). B and C: ROS levels ($n = 8$ per group) were quantified by measuring the fluorescence intensities of dihydroethidium (B) and H_2DCFDA (C). ***$P < 0.001$.

2.13. Metastasis study

Lung metastasis was determined with B16F10 murine melanoma cells which induce metastasis formation in the lungs after injection into the caudal vein (24). Briefly, two weeks after streptozotocin injections, normal, diabetic, and insulin–supplemented mice ($n = 10$/group) were intravenously injected with B16F10 cells (5×10^5) in 200 μL PBS. Two weeks later, the mice were euthanized by cervical dislocation, and the lungs were removed and bleached in Fekete's solution (70% ethanol, 3.7% paraformaldehyde, 0.75 M glacial acetic acid). Metastasis was assessed by counting metastatic lung nodules under a dissection microscope and by hematoxylin and eosin staining.

2.14. Statistical analysis

Data processing was performed with Origin 6.1 software (OriginLab, Northampton, MA). Data are expressed as the mean ± standard deviation (SD) from at least three independent experiments. Statistical significance was determined using unpaired Student's *t*-tests using $P < 0.05$ as the cut-off.

3. Results

3.1. Insulin inhibits VEGF-induced TGase2 activation but has no effect on intracellular Ca^{2+} elevation and ROS generation in HPMVECs

To investigate whether insulin can prevent hyperglycemia-induced pulmonary vascular leakage in diabetic mice, we first studied its effect on VEGF-induced elevation of intracellular Ca^{2+} levels in HPMVECs. VEGF raised intracellular Ca^{2+} levels in a time-dependent manner, but this increase was not inhibited by insulin (Fig. 1A).

We next examined the effects of insulin on VEGF-induced ROS generation and *in situ* TGase activation. VEGF elevated intracellular ROS levels, but insulin had no inhibitory effect (Fig. 1B). VEGF-induced ROS generation was inhibited by BAPTA-AM or the ROS scavenger NAC ($P < 0.001$, Fig. 1C), but not by the TGase inhibitor cystamine. VEGF increased *in situ* TGase activation, which was prevented by insulin in a dose-dependent manner ($P < 0.001$, Fig. 1D). No effect was observed with heat-inactivated insulin (Fig. 1E). VEGF-induced TGase activation was inhibited by BAPTA-AM or NAC, as well as cystamine ($P < 0.001$, Fig. 1E).

We investigated the role of TGase2 in VEGF-induced TGase activation using human *TGM2*-specific siRNA [19]. Human *TGM2* siRNA fully

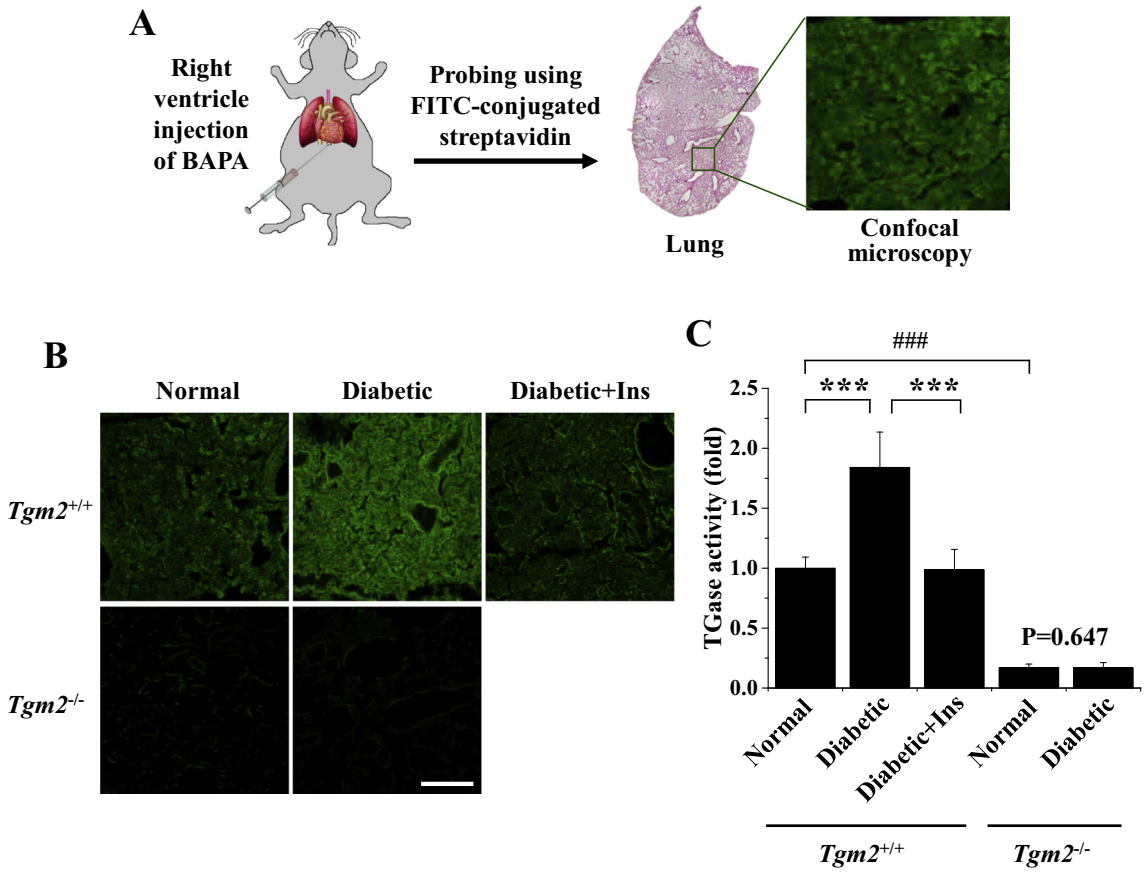

Fig. 5. Insulin supplementation inhibits hyperglycemia-induced TGase2 activation in the lungs of diabetic mice.
A: Schematic representation of *in vivo* TGase activity assay in the mouse lung. **B** and **C**: TGase2-null ($Tgm2^{-/-}$) diabetic mice were supplemented with insulin (Ins) using osmotic pumps. Normal and non-supplemented diabetic mice ($Tgm2^{+/+}$ and $Tgm2^{-/-}$) underwent sham operations. TGase activity in the lungs was visualized by confocal microscopy and quantified ($n = 8$ per group). B: Representative images of TGase activity in the lungs. Scale bar, 100 μm. C: *In vivo* TGase activity was quantified by measuring the fluorescence intensity in the lungs. ***$P < 0.001$, for diabetic *versus* normal or insulin-supplemented diabetic mice. ###$P < 0.001$, for wild-type ($Tgm2^{+/+}$) *versus* $Tgm2^{-/-}$ mice.

prevented VEGF-induced TGase activation ($P < 0.001$), whereas control siRNA showed negligible effects (Fig. 1F), demonstrating that TGase2, rather than other members of the TGase family, mostly contributed to the VEGF-induced increase in TGase activity. TGase family consists of eight isozymes, TGase1–7 and factor XIIIa. These results suggest that VEGF activates TGase2 by sequentially elevating intracellular Ca^{2+} and ROS levels in HPMVECs, and that insulin inhibits VEGF-induced TGase2 activation rather than intracellular Ca^{2+} elevation and ROS generation.

3.2. Insulin inhibits VEGF-induced c-Src phosphorylation, stress fiber formation, adherens junction disruption, and endothelial cell permeability in HPMVECs

We investigated the preventative effect of insulin against VEGF-induced vascular leakage by examining c-Src phosphorylation at Tyr^{416} and stress fiber formation. VEGF increased c-Src phosphorylation ($p < 0.001$) and this was inhibited by insulin ($p < 0.001$) or cystamine ($p < 0.01$, Fig. 2A and B). Insulin alone had no effect on c-Src phosphorylation. VEGF activated stress fiber formation, which was prevented by insulin or cystamine, but not by heat-inactivated insulin (Fig. 2C). Insulin alone induced membrane ruffling (Fig. 2C). Thus, it is likely that insulin inhibits VEGF-induced c-Src phosphorylation and stress fiber formation by inhibiting TGase2 activation.

The preventative effect of insulin against VEGF-induced vascular leakage was further explored by evaluating adherens junction disassembly and endothelial cell permeability. VEGF induced VE-cadherin disassembly, which was prevented by insulin, but not by heat-inactivated insulin (Fig. 3A). Insulin alone had no effect on VE-cadherin integrity. Cystamine prevented VEGF-induced VE-cadherin disassembly. Changes in the integrity of VE-cadherin were quantitatively analyzed by measuring the peak fluorescence intensities of line profiles (Fig. 3B). We also examined the effect of insulin on adherens junction disassembly by studying β-catenin. VEGF induced β-catenin disassembly, which was inhibited by insulin or cystamine ($p < 0.001$) (Fig. 3C and D). To confirm the preventative effect of insulin against VEGF-induced vascular leakage, we performed endothelial cell monolayer permeability assays. VEGF increased *in vitro* endothelial permeability, which was inhibited by insulin or cystamine ($p < 0.001$, Fig. 3E). Human *TGM2* siRNA fully prevented VEGF-induced endothelial permeability ($P < 0.001$), whereas control siRNA had no significant effects (Fig. 3D). These results suggest that insulin prevents VEGF-induced vascular leakage by inhibiting TGase2 activation, stress fiber formation, and adherens junction disassembly in HPMVECs.

3.3. Insulin supplementation inhibits hyperglycemia-induced TGase2 activation but does not inhibit ROS generation in the lungs of diabetic mice

To validate our *in vitro* findings, we supplemented diabetic mice with insulin using osmotic pumps and investigated ROS generation using dihydroethidium in the lungs. Intracellular ROS levels increased in the diabetic mouse lung compared to normal controls ($P < 0.001$), and this increase was not prevented by insulin (Fig. 4A and B). Similarly, there was no effect of insulin on hyperglycemia-induced ROS

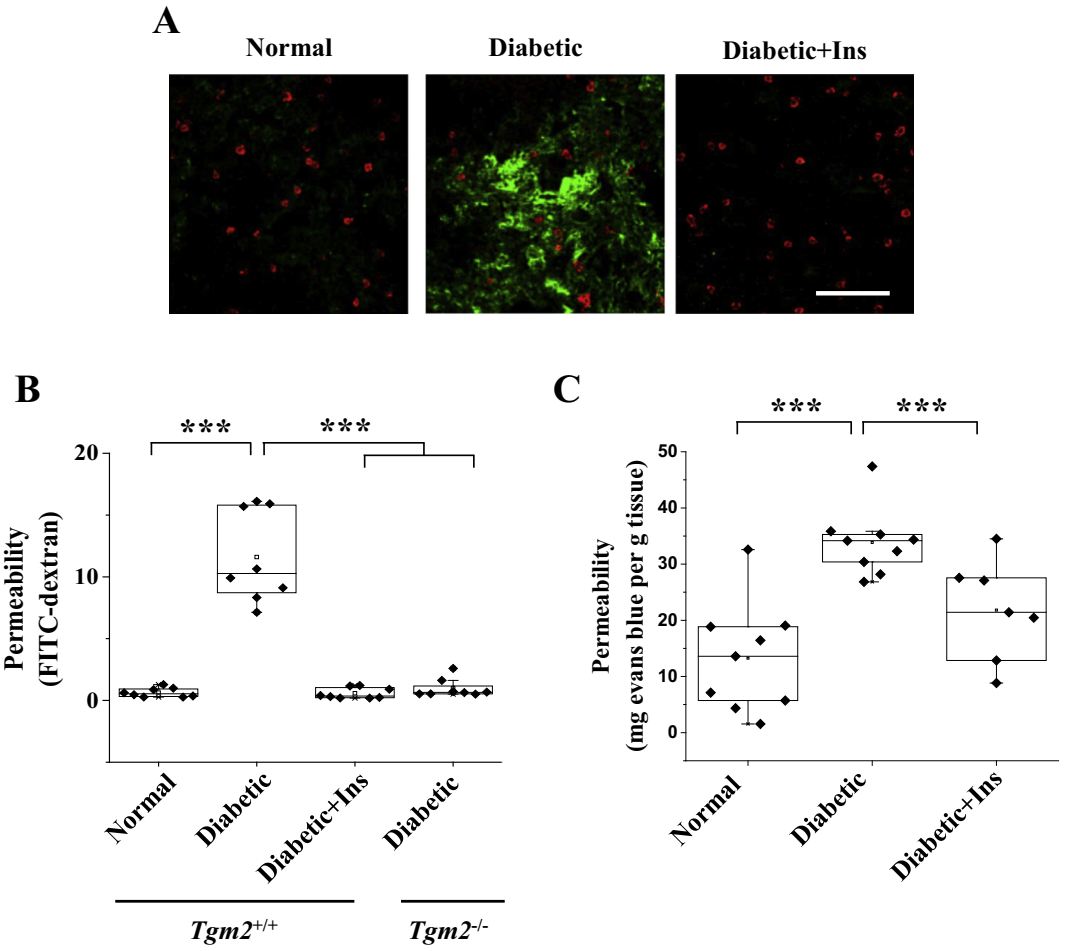

Fig. 6. Insulin supplementation inhibits hyperglycemia-induced vascular leakage in the lungs of diabetic mice.
Tgm2$^{+/+}$ diabetic mice were supplemented with insulin (Ins) using osmotic pumps. Normal and non-supplemented diabetic mice (*Tgm2*$^{+/+}$ and *Tgm2*$^{-/-}$) underwent sham operations. Vascular leakage (green) in the lungs was visualized with microvessels (red) by confocal microscopy. A: Representative fluorescent images of lung tissue. Scale bar, 100 μm. B and C: Vascular leakage was quantified by measuring the fluorescence intensity of FITC-dextran in the lungs (n = 8 per group) (B) and by the lung Evans blue extravasation assay (n = 9 for normal and diabetic mice; n = 7 for insulin-supplemented diabetic mice) (**C**). ***P < 0.001.

generation as assessed with H_2DCFDA (Fig. 4C).

We next investigated the inhibitory effect of insulin on hyperglycemia-induced TGase activation. We performed *in vivo* TGase activity assays with confocal microscopy of mouse lung (Fig. 5A). In this assay, the TGase pseudosubstrate BAPA was systemically delivered into the blood circulation by injection into the right ventricle, and biotinylated proteins in the lungs were probed using FITC-conjugated streptavidin. *In vivo* TGase activity was highly elevated in the lungs of diabetic mice compared with normal controls (P < 0.001), and this increase was blocked by insulin (P < 0.001, Fig. 5B and C). In addition, we measured *in vivo* TGase activity in *Tgm2*$^{-/-}$ mouse to confirm the specific role of TGase2 in hyperglycemia-induced TGase activation in the lungs of diabetic mice (Fig. 5B and C). TGase activity dramatically decreased in the lungs of nondiabetic *Tgm2*$^{-/-}$ mice compared with nondiabetic wild-type (C57BL/6) mice (P < 0.001), and decreased TGase activity was not changed by hyperglycemia in diabetic *Tgm2*$^{-/-}$ mice (p = 0.647). Taken together, these findings demonstrate that insulin supplementation inhibits hyperglycemia-induced TGase2 activation but not ROS generation in the lungs of diabetic mice.

3.4. Insulin supplementation inhibits hyperglycemia-induced vascular leakage and cancer metastasis in the lungs of diabetic mice

Since insulin inhibited hyperglycemia-induced TGase2 activation in the lungs of diabetic mice, we investigated its preventive effect against hyperglycemia-induced pulmonary vascular permeability. Vascular leakage in the lungs was observed as increased FITC-dextran extravasation in diabetic mice compared to normal controls (P < 0.001), and this was blocked by insulin supplementation (P < 0.001 *vs.* no insulin) (Fig. 6A and B). In contrast to the high levels of FITC-dextran extravasation in the lungs of diabetic C57BL/6 mice, there was no detectable vascular leakage in the lungs of diabetic *Tgm2*$^{-/-}$ mice (Fig. 6B), which was consistent with the *in vitro* results (Fig. 3D). The inhibitory effect of insulin against hyperglycemia-induced vascular leakage was further supported by the lung Evans blue extravasation assay (Fig. 6C). The Evans blue leakage was significantly higher in the lungs of diabetic mice compared to normal controls; however, this was prevented by insulin supplementation (P < 0.001, Fig. 6C).

We next examined whether insulin can prevent hyperglycemia-induced cancer metastasis. We used an experimental lung metastasis model by injecting metastatic B16F10 murine melanoma cells into the tail vein of normal and diabetic mice. The number of metastatic nodules was significantly increased in the lungs of diabetic mice compared with normal controls (P < 0.001), and this increase was blocked by supplementation with insulin (P < 0.001, Fig. 7A and B). Taken together, our results demonstrate that insulin prevents hyperglycemia-induced pulmonary vascular leakage and cancer metastasis by inhibiting VEGF-induced activation of TGase2, but not ROS generation, in the lungs of diabetic mice.

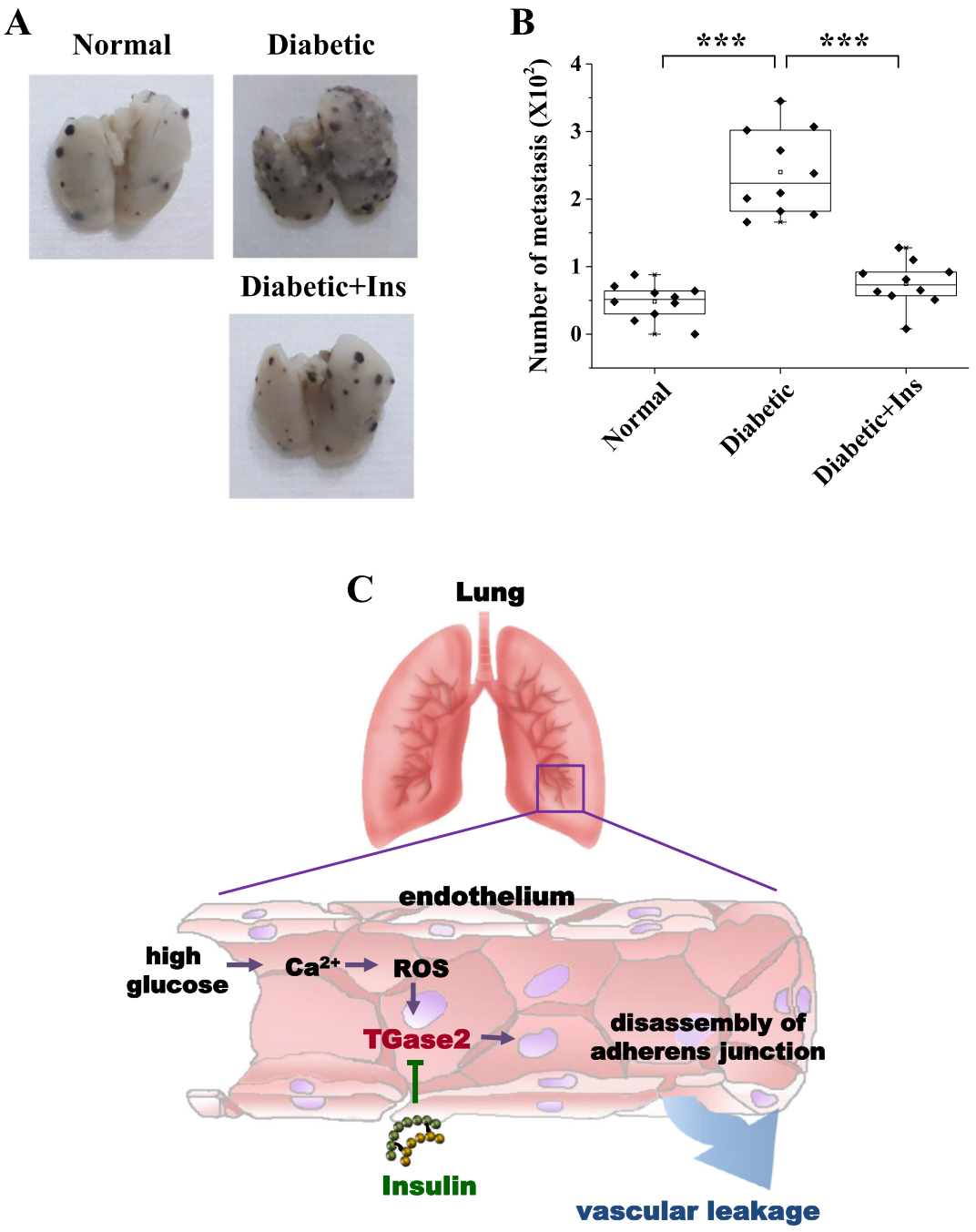

Fig. 7. Insulin prevention against hyperglycemia-induced metastasis in the lungs of diabetic mice and a schematic diagram of insulin prevention against hyperglycemia-induced pulmonary vascular leakage.
A and B: Diabetic mice were supplemented with insulin (Ins) using osmotic pumps and melanoma metastasis was investigated. A: Representative images of melanoma metastasis in lungs. B: Scatter plots of lung metastatic nodules (n = 10 per group). ***P < 0.001. C: Schematic model depicting the preventative effect of insulin on hyperglycemia-induced vascular leakage in the lungs of diabetic mice.

4. Discussion

Hyperglycemia induces diabetic microvascular and macrovascular complications in patients with diabetes [1], but its effect on pulmonary disease and the underlying mechanism remain unclear. It is reported that increased vascular leakage may contribute to pulmonary diseases including COPD, ARDS, and cancer [10]. Insulin has an important role in controlling blood glucose levels; however, its function in pulmonary disease is not clear. Here, we show that insulin prevents hyperglycemia-induced pulmonary vascular leakage and cancer metastasis by inhibiting VEGF-induced TGase2 activation rather than ROS generation, in HPMVECs and the lungs of diabetic mice (Fig. 7C). Based on these results, we propose that TGase2 is the key enzyme for hyperglycemia-induced pulmonary vascular leakage and that insulin has a potential for treating pulmonary disease associated with diabetic vascular leakage.

DM is characterized by chronic hyperglycemia that is associated with progressive blood vessel damage and dysfunction in various organs, resulting in diabetic complications such as retinopathy, nephropathy, neuropathy, foot ulcer, and stroke [1]. Despite the large capillary network in the lungs, pulmonary complications of diabetes have been relatively ignored compared with other organs [3]. It has been suggested that the lung may be a target organ affected by DM, because

pulmonary dysfunction has been reported in patients with type 1 and type 2 diabetes, including reductions in diffusing capacity, lung volume, and elastic recoil [2–4]. A possible association of DM with COPD was reported [6,26], but the effect of hyperglycemia on COPD pathogenesis is not known. Poor glycemic control may also increase the risk of developing asthma, but the pathophysiological mechanism is not known [5]. In this study, we showed that hyperglycemia induced vascular leakage through activating TGase2 in the lungs of diabetic mice. Vascular leakage can contribute to the development of pulmonary diseases, including ARDS, cancer, inflammation, and COPD [10,11]. In addition, vascular leakage plays as a critical and early event that facilitates extravasation in cancer metastasis. Thus, it is probable that hyperglycemia is associated with pulmonary disease, likely through inducing vascular leakage. Moreover, the lung might be a potential target organ of diabetic complications, resulting in so-called "diabetic pulmonopathy," although it is necessary to elucidate the effects of hyperglycemia on pulmonary disease.

Insulin is a central homeostatic hormone that regulates a number of important metabolic processes including stimulation of glucose uptake, lipid synthesis, oxidation, fat storage, and cell proliferation [13,27]. In muscle and adipose tissues, insulin regulates the metabolism of carbohydrates, lipids, and proteins [12]. In the liver, insulin inhibits glucose production and release [28]. The actions of insulin on the vasculature are complex and can be protective or deleterious. Insulin protected against renal ischemia/reperfusion injury in diabetic kidney [15]. A beneficial effect of insulin was also reported in the treatment of CNS-related metabolic disorders [16]. Defective insulin signaling is associated with disorders of memory, cognitive function, and mood, suggesting that insulin could be useful for treating CNS-associated disorders. Altered insulin signaling emerged as a potential contributor to the development of Alzheimer's disease, and insulin may improve cognitive function in patients with this condition [27,29]. In contrast, insulin may not prevent diabetic complications and could even worsen diabetic retinopathy, despite its positive effect on glycemic control [1,17,18]. Insulin therapy increased betacellulin-mediated vascular leakage and was associated with significantly increased risks of retinopathy progression and visual impairment [17,18]. In this study, we demonstrated that insulin prevented hyperglycemia-induced vascular leakage in the lungs of diabetic mice. Despite its complex effects in different organs, our findings suggest that insulin may have beneficial effect against pulmonary disease, which is associated with hyperglycemia-induced vascular leakage.

Regulating VEGF-induced intracellular events in endothelial cells is important for inhibiting hyperglycemia-induced vascular leakage in diabetes. Hyperglycemia increases VEGF levels in the retinas and lungs of diabetic mice [20,24]. Consistent with a previous study in human retinal endothelial cells [19], we found that VEGF activated TGase2 in HPMVECs by sequentially elevating intracellular Ca^{2+} and ROS levels and subsequently induced vascular leakage through adherens junction disassembly and stress fiber formation. A number of reports demonstrated that different agents prevent hyperglycemia-induced vascular leakage by differentially regulating VEGF-induced intracellular events [20,21]. The benzodiazepine anesthetic midazolam prevented hyperglycemia-induced vascular leakage in diabetic mouse retina by inhibiting elevation of intracellular Ca^{2+} levels, the initial event induced by VEGF [21]. C-peptide, a 31-amino acid polypeptide cleaved from proinsulin and released into circulation by pancreatic β-cells in equimolar concentrations with insulin [30], inhibited hyperglycemia-induced vascular leakage in the diabetic mouse retina and lung by inhibiting intracellular ROS generation, the downstream event of intracellular Ca^{2+} elevation induced by VEGF [19,31]. Cysteamine is an aminothiol with a primary amine group and a sulfhydryl group that is derived from coenzyme A degradation and metabolized into taurine [32]; it prevents hyperglycemia-induced vascular leakage by directly inhibiting TGase2 activation rather than ROS generation in the retinas of diabetic mice [20]. We found that insulin prevented hyperglycemia-induced vascular leakage by inhibiting TGase2 activation but not ROS generation in the lungs of diabetic mice. Thus, to prevent hyperglycemia-induced vascular leakage and vascular leakage-associated disease, it is likely important to inhibit VEGF-induced events such as increases in intracellular Ca^{2+}, ROS generation, and/or TGase2 activation.

In conclusion, we found that insulin prevented against hyperglycemia-induced pulmonary vascular leakage in diabetic mice. Insulin inhibited VEGF-induced TGase2 activation and a loss of vascular integrity in HPMVECs. Insulin supplementation also prevented hyperglycemia-induced TGase2 activation, pulmonary vascular leakage, and cancer metastasis in diabetic mice. Collectively, these findings suggest that insulin is a potential drug for treating vascular leakage-associated pulmonary disease.

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Declaration of competing interest

The authors declare that there is no conflict of interest.

Author contributions

Author contributions: H.-Y.J. designed and performed experiments, researched data, and wrote the manuscript. J.-A.S., S.-H.J., and Y.-J.L. performed experiments and researched data. E.-T.H., W.S.P., S.-H.H., and Y.-M.K. designed experiments and researched data. K.-S.H. conceptualized the study, designed experiments, researched data, and wrote the manuscript. All authors have approved the final version of the manuscript.

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