Prevention of VEGF-mediated microvascular permeability by C-peptide in diabetic mice

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1. Introduction

Human C-peptide has a beneficial effect on the prevention of diabetic neuropathy, nephropathy, and vascular complications; however, its role in protection against increased vascular permeability in diabetes remains unclear. Our purpose was to explore the potential protective role of C-peptide against microvascular permeability mediated by vascular endothelial growth factor (VEGF)-induced reactive oxygen species (ROS) generation in diabetes.

Methods and results

Generation of intracellular ROS, real-time changes in intracellular Ca2⁺, ROS-dependent stress fibre formation, and the disassembly of the adherens junctions were studied by a confocal microscopy in human umbilical vein endothelial cells (HUVECs). VEGF-induced vascular leakage was investigated in the skin of diabetic mice using a Miles vascular permeability assay. Microvascular leakage in the retina of streptozotocin diabetic mice was investigated using a confocal microscopy after left ventricle injection of fluorescein isothiocyanate (FITC)-dextran. C-peptide inhibited the VEGF-induced ROS generation, stress fibre formation, disassembly of vascular endothelial cadherin, and endothelial permeability in HUVECs. Intradermal injection of C-peptide prevented VEGF-induced vascular leakage. Consistent with this, intravitreal injection of C-peptide prevented the extravasation of FITC-dextran in the retinas of diabetic mice, which was also prevented by anti-VEGF antibody and ROS scavengers in diabetic mice.

Conclusions/interpretation

C-peptide prevents VEGF-induced microvascular permeability by inhibiting ROS-mediated intracellular events in diabetic mice, suggesting that C-peptide replacement is a promising therapeutic strategy to prevent diabetic retinopathy.

Keywords

C-peptide • Diabetes mellitus • Retinopathy • VEGF • Microvascular permeability

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(ROS)-mediated apoptosis of endothelial cells. However, the mechanism(s) by which C-peptide provides a beneficial effect for protection against diabetic vascular complications is not clearly understood.

Retinopathy is one of the major microvascular complications induced by diabetes and is the leading cause of blindness in adults; retinopathy develops in nearly all type 1 DM patients and in >60% of patients with type 2 DM. Vascular endothelial growth factor (VEGF) is a potent angiogenic and vascular permeability factor, and increased expression of VEGF in the retina during diabetes induces neovascularization and macular oedema. Elevation of VEGF can increase ROS production and macular oedema. Elevation of VEGF can increase ROS production and macular oedema. Elevation of VEGF can increase ROS production and macular oedema.

2. Methods

2.1 Cell culture

HUVECs were isolated from the human umbilical cord vein according to the Declaration of Helsinki as previously described and cells from passages 3 to 6 were used in the following experiments. Cells were grown at 37 °C in a humidified 5% CO2 incubator in M199 culture media supplemented with 20% FBS, 3 ng/mL LfFGF (Millipore, Billerica, MA, USA), 5 U/mL heparin (Sigma, St Louis, MO, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. For experiments, cells were incubated for 6 h in low-serum medium (M199 supplemented as above, but with only 1% FBS) and treated with 10 ng/mL VEGF (Millipore) in the presence or absence of various concentrations of human C-peptide (American Peptide Company, Sunnyvale, CA, USA).

2.2 Measurement of intracellular ROS generation and Ca2+ levels

Intracellular ROS generation and Ca2+ levels were measured by a confocal microscopy as previously described. Briefly, HUVECs were pre-incubated with 1 mM N-acetyl cysteine (NAC), 0.5 μM Trolox, 5 μM BAPTA-AM, or various concentrations of C-peptide for 30 min and treated with VEGF for 10 min. Cells were then incubated with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR, USA) in phenol red-free media for last 10 min. The level of intracellular ROS was determined by comparing the fluorescence intensities of treated cells with those of control cells.

To monitor intracellular Ca2+ levels, cells were co-incubated with 0.5 nM C-peptide or inhibitors and 1 μM Fluo-4 AM (Molecular Probes) for 30 min. Stained live cells on coverslips were mounted on perfusion chambers and scanned every 10 s with 10 ng/mL VEGF by a confocal microscopy (FV-300, Olympus, Tokyo, Japan). Serial images from the scan were processed to analyze changes in intracellular Ca2+ levels at the single-cell level. Results were expressed as the relative fluorescence intensity (RFI).

2.3 Visualization of actin filaments

HUVECs were treated with C-peptide or inhibitors for 30 min, and incubated with 10 ng/mL VEGF at 37 °C for 1 h. Cells were fixed with 3.7% formaldehyde in phosphate buffer saline (PBS) for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 30 min, and stained with rhodamine-phalloidin (Molecular Probes) in PBS for 1 h. Actin filaments were visualized by a confocal microscopy.

2.4 Visualization of VE-cadherin

HUVECs were incubated for 90 min with 10 ng/mL VEGF, 0.5 nM C-peptide, or with 10 ng/mL VEGF in the presence of 0.5 nM C-peptide or inhibitors. Cells were fixed with 3.7% formaldehyde in PBS for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 30 min, and stained overnight at 4 °C with a monoclonal VE-cadherin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were then probed with goat anti-mouse antibody conjugated with FITC (Sigma) and VE-cadherin was visualized using a confocal microscopy. Adherens junctions are represented by histograms of VE-cadherin as indicated by dotted lines.

2.5 In vitro endothelial cell monolayer permeability assay

Cells were grown on gelatin-coated inserts (0.4 μm polycarbonate membrane) of Transwell Permeable Supports (Costar, Corning, NY, USA) up to confluence as described. Cells on the inserts were pre-treated with 0.5 nM C-peptide, 0.5 nM C-peptide, 1 mM NAC, 0.5 μM Trolox, or 5 μM BAPTA-AM for 30 min, incubated with VEGF for 90 min, and incubated with 1 mg/mL 40 kDa FITC-dextran (Sigma) for the last 60 min. The amount of FITC-dextran diffused through the endothelial monolayer into the lower chamber was measured by a microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA).

2.6 Animals

Six-week-old male C57BL/6 mice were obtained from Nara Biotech (Seoul, Korea). Mice were maintained in temperature-controlled clean racks with a 12-h light/dark cycle. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Ethics Committee of Kangwon National University.

2.7 Generation of diabetic mouse model

Diabetic mice were generated by a single ip injection of streptozotocin (150 mg/kg body weight; Sigma) as previously described. Streptozotocin was freshly prepared in 100 mM citrate buffer (pH 4.5). After injection, mice were supplied with 10% sucrose overnight to prevent sudden hypoglycaemic shock. Sufficient hyperglycaemia was observed 2 days after injection, as determined by measuring blood glucose using the Accu-Check Active blood glucose monitor (Roche Diagnostics, Germany) and glucose assay using Ursican (TD Diagnostics, Young-In, Korea). After 1 week, mice with non-fasting blood glucose levels >16 mM, polyuria, and glucosuria were defined as diabetic and used for the experiments.

2.8 Miles vascular permeability assay

A Miles vascular permeability assay was performed using diabetic mice as described previously. Mice were anaesthetized using 250 μL/mouse of 2.5% avertin (Sigma) and shaved. After 2–3 days, mice were intravenously injected with 150 μL of 1% Evans blue solution. After 15 min, 15 μL of VEGF
(10 ng) solution containing 0, 138, or 276 nM C-peptide was injected intra-dermally into the shaved back skin of the mice. PBS was injected for the control. After 30 min, the mice were killed by cervical dislocation and an area of skin that included the blue spot resulting from leakage of the dye was dissected. The Evans blue dye was eluted from the dissected skin by incubation with formamide at 56°C for 2 days. The amount of dye was quantified by spectrophotometry at 620 nm.

2.9 Measurement of vascular leakage in retina
After anaesthetizing using 2.5% avertin (Sigma), diabetic mice were intravitreally injected with 2 µL C-peptide (1.037 µM), a monoclonal VEGF antibody (100 µg/mL; R&D Systems, Minneapolis, MN, USA), NAC (500 mM), Trolox (2 µM), and BAPTA-AM (5 mM) into one eye, and an equal volume of PBS was injected into the contralateral eye (n = 6 per group). After 24 h post-injection, retinal vascular leakage was quantified using fluorescein angiography. For the experiment, mice were anaesthetized using 2.5% avertin and injected with 1.25 mg of 500 kDa FITC-dextran (Sigma) into the left ventricle and the dye was allowed to circulate for 5 min. Mice were killed by cervical dislocation and the eyes were enucleated and immediately fixed with 4% paraformaldehyde for 45 min. Retinas were dissected, cut in the Maltese cross-configuration, and flat-mounted onto slide glass. Retinas were incubated with 4% paraformaldehyde in PBS and stored overnight in methanol at −20°C. Retinas were dissected, blocked with 2% bovine serum albumin in Tris-buffered saline containing 0.1% Triton X-100 and incubated with rabbit-polyclonal CD11b antibody (Santa Cruz Biotechnology) for 2 days. Retinas were incubated with FITC-conjugated rabbit anti-rabbit IgG overnight and observed by a confocal microscopy (FV300).

2.10 Measurement of leucocyte infiltration in retina
Diabetic mice were intravitreally injected with 2 µL C-peptide (1.037 µM) into one eye, and an equal volume of PBS was injected into the contralateral eye (n = 8 per group). After 24 h post-injection, eyes (n = 8 per group) were immediately enucleated and fixed with 4% paraformaldehyde in PBS and stored overnight in methanol at −20°C. Retinas were dissected, blocked with 2% bovine serum albumin in Tris-buffered saline containing 0.1% Triton X-100 and incubated with rabbit-polyclonal CD11b antibody (Santa Cruz Biotechnology) for 2 days. Retinas were incubated with FITC-conjugated rabbit anti-rabbit IgG overnight and observed by a confocal microscopy (FV300).

2.11 Statistical analysis
Data processing was performed using Origin 6.1 (OriginLab, Northampton, MA, USA). Statistical significance was determined using the t-test and ANOVA. A P-value of <0.05 was considered statistically significant.

3. Results
3.1 C-peptide inhibits VEGF-induced intracellular ROS generation, but has no effect on VEGF-induced intracellular Ca2+ elevation
We investigated the protective effect of C-peptide against VEGF-induced ROS generation and thereby prevention of vascular leakage, using HUVECs. VEGF generated intracellular ROS (P < 0.01); and this ROS generation was abolished by treatment with the ROS scavengers Trolox and NAC (Figure 1A). C-peptide inhibited the VEGF-induced generation of intracellular ROS in a dose-dependent manner, with complete prevention observed at 0.5 nM (Figure 1B). The Ca2+ chelator BAPTA-AM also blocked VEGF-induced intracellular ROS generation, indicating that VEGF produces intracellular ROS by elevating intracellular Ca2+. VEGF induced a rapid increase in intracellular Ca2+ and the elevated intracellular Ca2+ level was maintained until 600 s (Figure 1C). However, C-peptide did not increase intracellular Ca2+ and had no effect on VEGF-induced changes in intracellular Ca2+ (Figure 1C). BAPTA-AM blocked changes in intracellular Ca2+ levels in response to VEGF (Figure 1D). Trolox and NAC had no effect on VEGF-induced elevation of intracellular Ca2+, indicating that intracellular ROS are not involved in the VEGF-induced elevation in intracellular Ca2+. Taken together, VEGF generates intracellular ROS via elevating intracellular Ca2+ levels, whereas C-peptide inhibits the VEGF-induced elevation of intracellular ROS without affecting intracellular Ca2+ levels.

3.2 C-peptide inhibits VEGF-induced stress fibre formation in endothelial cells
To investigate the protective role of C-peptide in VEGF-induced stress fibre formation, actin filaments were visualized in HUVECs by staining with rhodamine-phalloidin. VEGF activated the formation of stress fibres extending over the cytoplasm, which was sufficiently suppressed by treatment with C-peptide (Figure 2). VEGF-activated stress fibre formation was also inhibited upon treatment with the ROS scavengers NAC and Trolox and the Ca2+ chelator BAPTA-AM, indicating that intracellular ROS and Ca2+ are essential for the VEGF-induced formation of stress fibres. Considering the inhibition of VEGF-stimulated ROS generation by C-peptide, these results demonstrate that C-peptide inhibits VEGF-induced stress fibre formation by preventing intracellular ROS generation in endothelial cells.

3.3 C-peptide inhibits VEGF-induced disassembly of adherens junctions
VEGF stimulated the disassembly of VE-cadherin, and this disassembly was inhibited by C-peptide (Figure 3A). The VEGF-induced disassembly of VE-cadherin was also prevented by NAC, Trolox, and BAPTA-AM, indicating that intracellular ROS and Ca2+ mediate the VEGF-induced disruption of VE-cadherin. The changes in permeability are further represented by histograms displaying RFI, as indicated by white lines that cross two cell–cell contacts (Figure 3B). The VEGF-induced decrease in the fluorescence intensity of the adherens junctions was recovered by treatment with C-peptide, the ROS scavengers, and BAPTA-AM. Consistent with the preventive effect of C-peptide against VEGF-induced disassembly of VE-cadherin, intravitreal injection of C-peptide inhibited the diabetes-induced disassembly of adherens junctions in the microvessels of the diabetic mouse retina (data not shown). Thus, C-peptide protects against VEGF-induced disassembly of VE-cadherin at cell junctions by inhibiting intracellular ROS generation in endothelial cells.

3.4 C-peptide inhibits VEGF-induced endothelial permeability
We investigated the preventive effect of C-peptide on VEGF-induced increase in endothelial permeability using FITC-dextran flux in HUVECs. VEGF increased in vitro endothelial permeability, which was inhibited by C-peptide (Figure 3C). The VEGF-induced endothelial permeability was prevented by the ROS scavengers NAC and Trolox, demonstrating that intracellular ROS mediates the VEGF-induced endothelial permeability. BAPTA-AM also inhibited the VEGF-induced endothelial permeability. Thus, VEGF induced disassembly of adherens junctions and endothelial permeability by intracellular ROS and Ca2+, whereas C-peptide protects against VEGF-induced endothelial leakage by inhibiting intracellular ROS generation.
3.5 C-peptide protects against microvascular leakage in skin of streptozotocin diabetic mice

Our in vitro findings in cultured endothelial cells demonstrate that C-peptide protects against VEGF-induced disassembly of adherens junctions and endothelial permeability through inhibiting intracellular ROS generation. To confirm the in vitro finding, we further investigated the preventive effect of C-peptide against microvascular leakage by a Miles vascular permeability assay in vivo in the skin of streptozotocin diabetic mice. Intradermal injection of VEGF significantly induced vascular permeability in diabetic mice, which was suppressed by C-peptide in a dose-dependent manner (Figure 4A). However, C-peptide alone did not cause a significant change in vascular permeability. The VEGF-induced vascular leakage of Evans blue dye from plasma into the interstitial space was quantified in skin-core biopsies by dye extraction and spectrophotometric absorbance measurements (n = 12, P < 0.01; Figure 4B). These results demonstrate C-peptide prevention of microvascular leakage in peripheral vessels of streptozotocin diabetic mice.

3.6 C-peptide prevents against microvascular leakage in retinas of streptozotocin diabetic mice

To evaluate the possible beneficial effect of C-peptide on diabetic retinopathy, streptozotocin diabetic mice were intravitreally injected with C-peptide and retinal vascular leakage was investigated using fluorescence angiography. C-peptide concentrations in the vitreous chamber were maintained within the physiological range (0.9–2.0 nM) for 12 h after intravitreal injection (data not shown). Considerably high levels of extravasation of FITC-dextran were observed in the retinas of diabetic mice (n = 6); this leakage was blocked in the retinas of the C-peptide-injected contralateral eyes (n = 6; Figure 5A). C-peptide prevention against vascular leakage in the retinas of diabetic mice was quantitatively analysed by determining the fluorescence intensity of FITC-dextran in whole retina tissues (n = 6, P < 0.01, Figure 5B). Intravitreal injection of a monoclonal anti-VEGF antibody significantly inhibited microvascular leakage in the retinas of diabetic mice (Figure 6A). Intravitreal injection of the ROS scavengers NAC and Trolox and the Ca²⁺ chelator BAPTA-AM also prevented retinal vascular leakage in diabetic mice, demonstrating...
that intracellular ROS and Ca\(^{2+}\) are involved in vascular leakage in the retina of diabetic mice. Additionally, increased leucocyte infiltration was observed in the retinas of diabetic mice (n = 8); this infiltration was blocked by intravitreal injection of C-peptide (n = 8, P < 0.01, Figure 5Q). Thus, C-peptide protects against VEGF-induced retinal vascular leakage by inhibiting intracellular ROS generation in retinal endothelial cells. Taking all our data together, we conclude that C-peptide prevents microvascular leakage in diabetic mice by inhibiting VEGF-induced intracellular ROS generation, stress fibre formation, and disassembly of the endothelial adherens junction, and micro-vascular permeability.

4. Discussion

C-peptide has emerged as a physiologically active peptide for the amelioration of diabetes-induced complications.\(^{1,2,4,5}\) Although hyperglycaemia alone can cause alterations in body homoeostasis during the diabetic state, the deficiency or absence of circulating insulin as well as C-peptide, as observed in type 1 DM or during the later stages of type 2 DM, could play a crucial role in the development and progression of hyperglycaemia-induced complications. Here, we have shown the potential role of C-peptide implicated in the prevention of microvascular permeability in streptozotocin diabetic mice, and revealed the underlying mechanism using HUVECs. We have demonstrated the essential contribution of C-peptide in protection against diabetic retinopathy through the inhibition of VEGF-induced ROS generation and microvascular hyperpermeability in endothelial cells (Figure 6).

Numerous studies in various models of diabetes indicate that hyperglycaemia in vivo induces VEGF expression and ROS production in the endothelial cells of the diabetic retina.\(^{19,30,34}\) However, the molecular mechanisms of hyperglycaemia-induced vascular leakage are not fully
Figure 3 C-peptide inhibits the VEGF-induced disruption of adherens junctions and endothelial cell monolayer permeability. (A and B) HUVECs were incubated for 90 min with 10 ng/mL VEGF or 0.5 nM C-peptide alone, or with 10 ng/mL VEGF in the presence of 0.5 nM C-peptide, 1 mM NAC, 0.5 µM Trolox, or 5 µM BAPTA-AM \( (n = 3) \). (A) VE-cadherin was stained and visualized using confocal microscopy as described in Methods. Scale bar, 20 µm. (B) Adherens junctions are represented by histograms of VE-cadherin as indicated by dotted lines. (C) In vitro endothelial cell monolayer permeability assay. HUVECs on inserts of Transwell® Permeable Supports were incubated with 0.5 nM C-peptide, 1 mM NAC, 0.5 µM Trolox, or 5 µM BAPTA-AM for 30 min, treated with 10 ng/mL VEGF for 90 min, and FITC-dextran was added to the inserts for the last 60 min. Diffused FITC-dextran to the lower chamber was measured by a microplate spectrofluorometer \( (n = 3) \). **P < 0.01.
understood. VEGF has been identified as a primary mediator of vascular alteration in diabetic retinopathy, and the expression of VEGF is increased by high glucose, hypoxia, oxidative stress, and inflammatory reactions.\textsuperscript{19–21} VEGF is also a potent promoter of physiological angiogenesis; however, VEGF was first discovered as vascular permeability factor, and it induces vascular leakage and fluid exudation into the surrounding tissue that can lead to serious diseases, such as acute lung injury and diabetic retinopathy.\textsuperscript{19,35–37} Increased vascular permeability in diabetes is a complex process involving multiple signalling pathways in endothelial cells and is stimulated by VEGF overexpression that can disrupt adherens junction proteins such as VE-cadherin, which is induced by the elevation of intracellular Ca\textsuperscript{2+} and ROS levels and stress fibre formation.\textsuperscript{24–26,38}

Our study consistently demonstrated VEGF-induced molecular events, including the elevation of intracellular Ca\textsuperscript{2+} and ROS and the formation of stress fibres. We demonstrated the VEGF-induced generation of intracellular ROS, which was sufficiently prevented by C-peptide and the calcium chelator BAPTA-AM, demonstrating that VEGF-induced ROS generation was triggered by the upstream elevation of intracellular Ca\textsuperscript{2+} (Figure 6). Our previous report suggested that C-peptide prevents high glucose-induced ROS generation in endothelial cells without affecting intracellular Ca\textsuperscript{2+} levels.\textsuperscript{16} The data from the present study also

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**Figure 4** C-peptide prevents VEGF-induced vascular leakage in the peripheral vessels of diabetic mice. Anaesthetized diabetic mice were intravenously injected with Evans blue solution and then injected intradermally with 15 \(\mu\)L C-peptide (C-Pep) or VEGF (10 ng) containing the indicated amounts of C-peptide (\(n = 12\) per group). PBS was injected as a negative control (\(n = 12\)). (A) Representative images. (B) The Evans blue dye content was eluted from the dissected skin and quantitated as described in Methods (\(n = 12\)). **P < 0.01.
consistently demonstrated that C-peptide had no inhibitory effect on the VEGF-induced elevation of intracellular $\text{Ca}^{2+}$ levels. Thus, it is likely that C-peptide inhibition of elevated ROS rather than $\text{Ca}^{2+}$ in endothelial cells plays an important role in the prevention of vasculopathy. C-peptide might inhibit VEGF-induced ROS generation by a mechanism involving AMP-activated protein kinase $\alpha$ (AMPK$\alpha$). Recently, we demonstrated that C-peptide activated AMPK$\alpha$ and prevented against hyperglycaemia-induced ROS generation and subsequent endothelial cell damage.\textsuperscript{17} VEGF increases NADPH oxidase-dependent ROS generation, which can be inhibited by C-peptide through AMPK$\alpha$ activation. Stress fibre formation downstream of several permeability-increasing mediators, such as thrombin and TNF-$\alpha$, contributes to increased permeability.\textsuperscript{26} Likewise, acting as a mediator of ROS generation, VEGF induced stress fibre formation, which was attenuated by C-peptide as well as by the ROS scavengers Trolox and NAC.

C-peptide also prevented the VEGF-induced disassembly of VE-cadherin, an essential endothelial cell-specific adhesion molecule that connects adjacent endothelial cells. The phosphorylation of VE-cadherin and $\beta$-catenin is dependent on ROS generation and stress fibre formation in decreased junctional integrity, and enhances vascular permeability.\textsuperscript{25} Our results show that VEGF can dissemble VE-cadherin at adherens junctions and that C-peptide prevents the VEGF-induced disassembly of VE-cadherin. Inhibition of the VEGF-induced disassembly of VE-cadherin by NAC and Trolox indicates that ROS inhibition by C-peptide is

**Figure 5** C-peptide prevents against vascular leakage in the retinas of diabetic mice. Streptozotocin diabetic mice were intravitreally injected with 2 $\mu$L C-peptide (Diabetic + C-pep, $n = 6$), anti-VEGF (Diabetic + anti-VEGF, $n = 6$), NAC (Diabetic + NAC, $n = 6$), Trolox (Diabetic + Trolox, $n = 6$), and BAPTA-AM (Diabetic + BAPTA) into one eye, and an equal volume of PBS into the contralateral eye (Diabetic; $n = 6$ per group). Non-diabetic mice were also intravitreally injected with 2 $\mu$L PBS into eyes (Normal; $n = 6$ per group). Retinas were visualized by confocal microscopy as described in Methods. (A) Representative fluorescent images of the retinas from Normal, Diabetic, and Diabetic + C-peptide eyes. The square areas are displayed as magnified images at the bottom of each image. Scale bar, 100 $\mu$m. (B) Retina permeability was quantified by measuring the fluorescence intensities of whole retina tissues ($n = 6$). **$P < 0.01$. (C) Increased number of infiltrated leucocytes was quantified by counting CD11b$^+$ cells of retina tissues ($n = 8$). **$P < 0.01$. 

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Figure 6 Possible signalling pathway involved in the C-peptide-mediated prevention of VEGF-induced vascular permeability. C-peptide prevents vascular permeability via inhibiting VEGF-induced ROS generation.

necessary to prevent the VEGF-induced disassembly of VE-cadherin. Thus, C-peptide prevents VEGF-induced vascular permeability in endothelial cells by inhibiting the disassembly of adherens junctions via mechanism(s) involving intracellular ROS generation and stress fibre formation.

VEGF also has the ability to produce NO in endothelial cells. VEGF-induced elevation of both ROS and NO causes peroxynitrite flux, which can enhance vascular permeability. C-peptide also activates NO production without affecting eNOS expression. However, C-peptide inhibition of VEGF-induced ROS generation may prevent VEGF-induced peroxynitrite formation. Therefore, it appears likely that C-peptide inhibition of ROS generation may also prevent VEGF-induced vascular permeability by inhibiting peroxynitrite production.

In terms of clinical relevance, our study proposes potential roles of C-peptide in protecting against VEGF-induced microvascular permeability as demonstrated in the skin and retina of streptozotocin diabetic mice. Additionally, infiltration of leucocytes was significantly inhibited by intravitreal injection of C-peptide in the retinas of diabetic mice. The protection afforded by C-peptide against the deleterious effect of VEGF-induced microvascular permeability could prevent further development and progression of macular oedema and progression of proliferative angiogenesis in the late stages of diabetes. These beneficial roles of C-peptide revealed by our study are similar to those of angiopoietin-1, which also shows protection against vascular permeability via the inhibition of VE-cadherin disruption. However, the protective effect of C-peptide against vascular permeability is mediated by the inhibition of ROS production, and thereby the inhibition of stress fibre formation and subsequent disruption of VE-cadherin at the endothelial junctions, whereas ROS is not involved in the action mechanism of angiopoietin-1. Thus, C-peptide exhibits beneficial effects against the development of destructive outcomes via distinctive signalling from angiopoietin-1.

The development and progression of diabetic retinopathy is also influenced by the ROS-induced apoptotic loss of retinal pericytes and endothelial cells in diabetes. In this regard, C-peptide has an anti-apoptotic effect on endothelial cells by inhibiting hyperglycaemia-induced ROS generation. Therefore, one can speculate that the lack of C-peptide may provide a favourable physiological environment to develop and progress diabetic complications via increased pathological stimuli, such as the generation of intracellular ROS, induction of apoptosis, and dysregulation of vascular permeability. On the other hand, these multiple beneficial effects of C-peptide indicate promising preventive roles against diabetes-induced pathologies, providing potential replacement therapies along with insulin treatment for type 1 DM as well as late-stage type 2 DM.

In conclusion, our data suggest that C-peptide can protect microvascular leakage in diabetic mice by preventing the potentially harmful effects of VEGF as demonstrated by inhibiting ROS generation, stress fibre formation, VE-cadherin disruption, and endothelial permeability. Thus, C-peptide replacement is a promising therapeutic strategy for the prevention of VEGF-induced diabetic retinopathy through the inhibition of ROS-mediated vascular permeability.

Conflict of interest: none declared.

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