C-peptide protects against hyperglycemic memory and vascular endothelial cell apoptosis

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Abstract

C-peptide exerts protective effects against diabetic complications; however, its role in inhibiting hyperglycemic memory (HGM) has not been elucidated. We investigated the beneficial effect of C-peptide on HGM-induced vascular damage in vitro and in vivo using human umbilical vein endothelial cells and diabetic mice. HGM induced apoptosis by persistent generation of intracellular ROS and sustained formation of \( \text{ONOO}^- \) and nitrotyrosine. These HGM-induced intracellular events were normalized by treatment with C-peptide, but not insulin, in endothelial cells. C-peptide also inhibited persistent upregulation of p53 and activation of mitochondrial adaptor p66\( ^{shc} \) after glucose normalization. Further, C-peptide replacement therapy prevented persistent generation of ROS and \( \text{ONOO}^- \) in the aorta of diabetic mice whose glucose levels were normalized by the administration of insulin. C-peptide, but not insulin, also prevented HGM-induced endothelial apoptosis in the murine diabetic aorta. This study highlights a promising role for C-peptide in preventing HGM-induced intracellular events and diabetic vascular damage.

Keywords

- C-peptide
- endothelial apoptosis
- hyperglycemic memory
- reactive oxygen species
- vasculopathy

Introduction

Diabetic vasculopathy is secondary systemic damage caused by transient or prolonged hyperglycemia (Pirola et al. 2010, Paneni et al. 2012b). Although hyperglycemia can be controlled by insulin or oral anti-hyperglycemic agents, diabetic patients develop long-term vascular complications even after intensive glycemic control (Pirola et al. 2010). Early hyperglycemia (e.g., at the time of diabetes onset) or transient hyperglycemia during anti-hyperglycemic therapy may alter cellular signaling in the vasculature. This altered microenvironment, referred to as metabolic or hyperglycemic memory (HGM), can induce later complications, and therefore, has emerged as...
an underlying mechanism behind diabetic complications (Ceriello et al. 2009b).

Although the molecular mechanism behind HGM is not fully understood, several studies suggest that reactive oxygen species (ROS) play an important role in the development of vascular HGM in diabetes (Ihnat et al. 2007, Giacco & Brownlee 2010, Paneni et al. 2012b). Hyperglycemia generates excessive ROS by promoting a mitochondrial ROS accumulation in response to hyperglycemia, which then stimulates cytosolic ROS generation by the activation of protein kinase C (PKC) and NADPH oxidase (Brownlee 2001, Fadini et al. 2010, Bhatt et al. 2013a). The resulting buildup of ROS is responsible for persistent vascular oxidative stress (Paneni et al. 2012b, Bhatt et al. 2013b). It has also been reported that HGM persists due to epigenetic modifications; specifically, hypermethylation of transcription-activating histone H3 residue Lys4, the de-methylation of transcription-repressive histone H3 residue Lys9 and the acetylation of histone H3 (Cooper & El-Osta 2010, Pirola et al. 2010). These epigenetic changes result in the upregulation of transcription factor p53 and mitochondrial adaptor protein p66SC, both of which play a role in sustained ROS generation, endothelial damage and delayed wound healing in diabetes (Kim et al. 2008, Fadini et al. 2010, Paneni et al. 2012a).

ROS overproduction is the key event in the activation of pathways involved in the pathogenesis of diabetic vasculopathy, and these pathways include polyol and hexoseamine pathway flux, advanced glycation end-product formation and the activation of protein kinase C (Brownlee 2001, Giacco & Brownlee 2010, Paneni et al. 2012b). Cellular nitrosative stress due to peroxynitrite (ONOO−) and nitrotyrosine formation results from hyperglycemia-induced superoxide generation and eNOS uncoupling and can contribute to endothelial cell injury (El-Remessy et al. 2003, Cassuto et al. 2013). Although eNOS-dependent nitric oxide (NO) production promotes vasodilation, the overproduction of O$_2^-$ may quench available NO, leading to increased intracellular ONOO− and nitrotyrosine formation in diabetic vasculopathy (Navarro-Antolin et al. 2001, Pacher et al. 2007).

Evidence from large clinical trials reveals that therapeutic approaches toward intensive glycemic control alone have not been entirely successful in reducing deaths due to adverse cardiovascular events (Gerstein et al. 2008, Brown et al. 2010). The strong association between HGM and cardiovascular complications in diabetes suggests that early aggressive treatment targeted at glycemic control together with supplementation with agents that may prevent the generation of intracellular reactive oxygen and nitrogen species may be beneficial for preventing long-term cardiovascular complications associated with diabetes (Ihnat et al. 2007, Ceriello et al. 2009a,b). C-peptide is one such potential agent, due to its ability to inhibit ROS-mediated intracellular events, including mitochondrial dysfunction, transglutaminase 2 activation and endothelial cell apoptosis (Bhatt et al. 2013a,b).

Human C-peptide is a 31-amino acid polypeptide cleaved from pro-insulin and secreted into the bloodstream by pancreatic β-cells in equimolar concentrations with insulin (Wahren et al. 2012, Bhatt et al. 2014). C-peptide and insulin deficiencies are typical comorbidities of type 1 diabetes mellitus (DM) and the later stages of type 2 DM (Bhatt et al. 2014). C-peptide replacement therapy ameliorates several diabetic complications, including neuropathy, nephropathy, vascular dysfunction and damage, retinopathy and impaired wound healing, both in animal models and in patients with type 1 DM (Ido et al. 1997, Hills et al. 2010, Wahren et al. 2012, Bhatt et al. 2013a,b, Lim et al. 2014, 2015). We previously showed a protective effect of C-peptide against vascular endothelial growth factor-induced microvascular permeability in the retinas of diabetic mice (Lim et al. 2014), and we also recently showed that C-peptide replacement therapy reduces impaired wound healing by activating angiogenesis and inhibiting inflammation (Lim et al. 2015). However, whether C-peptide replacement therapy can inhibit HGM and thus positively affect health outcomes in diabetic patients is unclear.

In this study, we aimed to characterize the physiological role of C-peptide against diabetes-associated HGM, hypothesizing that C-peptide inhibits HGM through its ability to inhibit hyperglycemia-induced intracellular events, including hyperglycemia-induced persistent ROS generation and nitrosative stress and vascular endothelial apoptosis (Ceriello et al. 2009a, Bhatt et al. 2013b). We investigated the specific mechanism via which C-peptide prevents HGM persistence both in vitro and in vivo and evaluated it as a therapeutic candidate for preventing HGM.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins according to the Declaration of Helsinki as described previously.
(Bhatt et al. 2013a). Cells from several batches were maintained in M199 culture media supplemented with 20% FBS, 3 ng/mL bFGF, 5 U/mL heparin, 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells in passages three to seven were incubated overnight in low-serum medium (M199 supplemented as described previously, but with 5% FBS and 1 ng/mL bFGF) before their use in experiments. This study was approved by the Institutional Review Board of Kangwon National University Hospital.

Measurement of intracellular ROS and NO levels
Intracellular ROS and NO levels were measured using H₂DCFDA and DAF-FM diacetate (Molecular Probes, Eugene, OR), respectively, as described previously (Bhatt et al. 2013b). To determine the hyperglycemic effect, cells mounted on 2% gelatin-coated coverslips were exposed to 33 mM high glucose (HG) for 48 h in the presence or absence of 10 μM C-peptide or 1 nM insulin. To characterize hyperglycemic memory, cells were incubated with 33 mM glucose for 48 h followed by 48 h of treatment with 33 mM glucose (HGHG) or 5.5 mM glucose (HGM) in the presence or absence of 10 nM C-peptide or insulin.

To measure the intracellular ROS levels, cells were stained with 10 μM H₂DCFDA for 10 min in serum- and phenol red-free media. To measure the intracellular NO levels, cells were stained with 2 μM DAF-FM diacetate for 30 min in low-serum medium. Labeled cells were analyzed immediately using confocal microscopy (Olympus, Fluoview-300, Japan). Single-cell fluorescence intensities were determined for 30 cells per experiment. Intracellular ROS and NO levels were determined by comparing the fluorescence intensities of treated cells with those of control cells (fold difference).

Measurement of intracellular peroxynitrite (ONOO⁻) levels and nitrotyrosine formation
Intracellular peroxynitrite (ONOO⁻) levels were measured using dihydrorhodamine 123 fluorescent probe as described previously (Pacher et al. 2007). Briefly, cells were stained with 5 μM dihydrorhodamine 123 (Molecular Probes) for 30 min. Stained cells were then observed using confocal microscopy, and intracellular ONOO⁻ levels were determined by comparing the fluorescence intensities of treated cells with those of control cells (fold).

Nitrotyrosine formation was measured by immunostaining using an anti-nitrotyrosine antibody. Briefly, cells were fixed with an ethanol:acetic acid (95:5, v/v) solution and incubated with rabbit polyclonal anti-nitrotyrosine antibody in 1% BSA for 2 h followed by an incubation with TRITC-conjugated anti-rabbit IgG antibody for 1 h. Nitrotyrosine levels were determined by comparing the fluorescence intensities of treated cells with those of control cells (fold difference).

Measurement of apoptosis
Cell death (apoptosis) was assessed via 3,3′-dihexyloxacarbocyanine iodide (DiOC₆) / propidium iodide (PI) double staining as described previously (Yoo et al. 2011). Briefly, cells were incubated with 33 mM glucose for 72 h followed by an additional 72 h of incubation with 33 mM glucose (HGHG) or 5.5 mM glucose (HGM) in the presence of 1 nM C-peptide and/or insulin. Live cells were stained with 50 nM DiOC₆ (green) and 10 μg/mL PI (red) for 20 min and analyzed using confocal microscopy. Cells were differentiated as viable cells (DiOC₆ bright/PI negative), early apoptotic cells (DiOC₆ dim/PI negative) and late apoptotic cells (DiOC₆ dim/PI positive). Single-cell fluorescence intensities of approximately 300 cells per experiment were determined using the Fluoview 300 software. The abundances of early apoptotic cells, late apoptotic cells and total cell death (sum of early and late apoptotic cells) were expressed as the percentage of total cells.

Western blot analysis
Cellular proteins were extracted from HUVECs using a lysis buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, 150 mM sodium chloride, 1% Triton X-100, 10 μg/mL aprotenin, 10 μg/mL leupeptin, 0.1 mM phenylmethanesulfonyl fluoride, 25 mM β-glycerophosphate and 2 mM sodium orthovanadate. Protein extracts were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were incubated with monoclonal antibodies against phospho-p66shc (Abcam), β-actin (Sigma-Aldrich, St Louis, MO) and p53 (Calbiochem) followed by incubation with HRP-conjugated secondary antibodies. Protein bands were visualized with a chemiluminescence reagent (Pierce).

Generation of the diabetic mouse model
Six-week-old male C57BL/6 mice were obtained from KOATECH (Pyungtaek, Korea). Diabetic mice were generated by a single intraperitoneal injection of...
streptozotocin (150 mg/kg body weight) as described previously (Bhatt et al. 2013a). Sufficient hyperglycemia was observed 2 days after injection, as determined by measuring blood glucose and glycosuria. Mice with nonfasting blood glucose levels greater than 16 mM, polyuria and glycosuria were considered diabetic. All animal experiments conformed to the NIH guidelines (Guide for the Care and Use of Laboratory Animals) and were approved by the Kangwon Institutional Animal Care and Use Ethics Committee.

Supplementation of mice with C-peptide and insulin using osmotic pumps

Four weeks after streptozotocin injection, a group of diabetic mice (DM+CP) were anesthetized with an intraperitoneal injection of 2.5% Tribromoethanol (240 mg/kg, Sigma-Aldrich) and supplemented with human C-peptide (American Peptide Company, Inc, Sunnyvale, CA, USA) using an Alzet Mini-Osmotic Pump 2004 (DURECT, Cupertino, CA, USA) with a delivery rate of 35 pmol/min/kg. Anesthesia was monitored via the pain reflex reaction (lack of response to hind limb toe pinch). A second group of diabetic mice (DM+Ins; HGM) was supplied with human recombinant insulin (Sigma-Aldrich), with a delivery rate of 58.4 pmol/min/kg to mimic hyperglycemic memory. To assess the effect of C-peptide treatment on HGM, a third group of diabetic mice (DM+Ins+CP; HGM+CP) was supplemented with a mixture of C-peptide and insulin, also via the osmotic pump. Diabetic (DM) and control mice groups also underwent sham operations. The lack of mouse C-peptide in appropriate groups was measured using a C-peptide Enzyme Immunoassay Kit (RayBiotech, Norcross, GA, USA). Supplemented human C-peptide and/or insulin levels in serum were measured using ELISA kits specific for each (Millipore). The vital statistics of each mouse were recorded throughout the experiment.

Measurement of ROS generation, ONOO⁻ formation and apoptosis in mouse aorta endothelial layers

After killing mice via cervical dislocation, aortas from control (n = 14), diabetic (DM, n = 13), C-peptide-supplemented

Figure 1

C-peptide, but not insulin, prevents hyperglycemia-induced formation of intracellular ROS, ONOO⁻ and nitrotyrosine in endothelial cells. HUVECs were incubated for 48h with high glucose (HG, 33 mM) and/or 5.5 mM normal glucose (control) in the presence of 1 nM C-peptide (CP) or indicated concentrations (A) or 1 nM (B–D) of insulin (Ins). Endothelial cells were also treated with normal glucose (control), low-glucose (LG, 33 mM), C-peptide or insulin for 48 h. Intracellular ROS and ONOO⁻ levels and nitrotyrosine formation were determined by confocal microscopy as described in the ‘Materials and methods’ section. Representative images of control, high glucose, high glucose and C-peptide (HG+CP) and high glucose and insulin (HG+Ins) are shown for the measurement of endothelial ROS levels (A and B), ONOO⁻ levels (C) and nitrotyrosine levels (D). Bar, 60 μm. (A–B) C-peptide, but not insulin, prevents HG-induced intracellular ROS formation. (C–D) C-peptide, but not insulin, prevents HG-induced formation of intracellular ONOO⁻ (C) and nitrotyrosine (D). Data are expressed as the mean ± s.d. from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-16-0349.
diabetic (DM+CP, n=10), insulin-supplemented diabetic (DM+ins, n=11) and both insulin- and C-peptide-supplemented diabetic (DM+ins+CP, n=14) mice were dissected and cut longitudinally to expose the endothelium for immediate staining. To measure the intracellular levels, aortic segments were quickly transferred to serum-free M199 media and incubated at 37°C with 10μM H2DCFDA for 10 min. Stained aortic segments were then observed using confocal microscopy. ONOO− production in aortic endothelial layers was measured by immunostaining 1% paraformaldehyde-fixed aortic segments as described previously for measurements of peroxynitrite formation in HUVECs. Aortic endothelial apoptosis was measured by a TUNEL assay as described previously (Bhatt et al. 2013a). The number of TUNEL-positive cells per field (40×) in the confocal images was counted from at least 10 aortic segments in each group and was compared with that in the control group.

Statistical analyses

Data processing was performed using Origin 6.1 software (OriginLab, Northampton, MA, USA). Differences between treatment groups were assessed using analysis of variance (ANOVA). A P value <0.05 was considered statistically significant, and data were expressed as the mean±s.d. of at least three independent experiments.

Results

C-peptide, but not insulin, prevents high glucose-induced generation of intracellular ROS, ONOO− and nitrotyrosine in HUVECs

We first investigated the effects of C-peptide and insulin on high glucose (HG, 33 mM)-induced generation of intracellular ROS, ONOO− and nitrotyrosine in HUVECs. High glucose elevated intracellular ROS level, which was inhibited by treatment with C-peptide; however, insulin did not affect high glucose-induced ROS generation at any concentrations tested up to 100 nM (Fig. 1A and B). Osmotic control L-glucose, insulin or C-peptide alone had no effect on ROS levels. Heat-inactivated C-peptide had no effect on high glucose-induced ROS generation (Fig. 1B). These results demonstrate that high glucose-induced ROS generation is specifically inhibited by C-peptide, but not by insulin.

Intracellular ONOO− generation and subsequent protein nitrotyrosine formation are stimulated by ROS in the presence of available NO (Navarro-Antolin et al. 2001).

Figure 2

C-peptide prevents hyperglycemic memory (HGM)-induced formation of intracellular ROS, ONOO− and nitrotyrosine in endothelial cells. HUVECs were incubated for 48 h with high glucose and further treated for additional 48 h with high glucose (HGHG) or 5.5 mM normal glucose (HGM) in the presence of 1 nM C-peptide (CP) and/or insulin (Ins). Endothelial cells were also treated with normal glucose (control), L-glucose (LG, 33 mM), C-peptide or insulin for 96 h. Intracellular ROS (A) and ONOO− (B) levels and nitrotyrosine formation (C) were determined by confocal microscopy as described in the ‘Materials and methods’ section. Representative images of control, HGM, HGM and C-peptide (HGM+CP) and HGM and insulin (HGM+Ins) are shown for the measurement of endothelial ROS levels (A), ONOO− levels (B) and nitrotyrosine levels (C). Data are expressed as mean±s.d. of from three independent experiments. *P<0.05, **P<0.01, ***P<0.001. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-16-0349.
Therefore, we investigated the effects of C-peptide and insulin on high glucose-induced formation of ONOO− and protein nitrotyrosine in endothelial cells. High glucose-induced ONOO− and nitrotyrosine formation were abolished by treatment with C-peptide, but not by insulin or by heat-inactivated C-peptide (Fig. 1C and D). Neither ONOO− levels nor protein nitrotyrosine formation were affected by insulin or C-peptide alone, or by l-glucose. These results demonstrate that high glucose induces ROS generation and subsequent ONOO− and nitrotyrosine formation and that these high glucose-induced intracellular events are inhibited by C-peptide.

C-peptide prevents persistent elevation of intracellular ROS, ONOO− and nitrotyrosine levels after glucose normalization

To investigate the effects of C-peptide and insulin on HGM, we determined intracellular ROS, ONOO− and nitrotyrosine levels in HUVECs after treatment with high glucose (33 mM) for 48 h followed by normal glucose (HGM) or high glucose (HGHG) for an additional 48 h. Elevated intracellular ROS levels (P<0.001) were detected in HUVECs under HGM conditions after glucose normalization. These levels were similar to those observed in cells under HGHG conditions (Fig. 2A). The persistent ROS generation was inhibited by C-peptide, but not by insulin or heat-inactivated C-peptide.

We then measured intracellular ONOO− and nitrotyrosine formation in HGM HUVECs after glucose normalization. HGM conditions induced sustained elevation of intracellular ONOO− and nitrotyrosine (P<0.01), and these levels were normalized by C-peptide treatment in the presence or the absence of insulin (Fig. 2B and C). HGHG conditions also induced persistent formation of ONOO− and nitrotyrosine. These results demonstrate that HGM activates persistent generation of intracellular ROS and subsequently sustained formation of ONOO− and nitrotyrosine and that these HGM-induced intracellular events are normalized by C-peptide but not by insulin.

C-peptide prevents HGM-induced endothelial cell apoptosis

Persistent elevation of intracellular ROS, ONOO− and nitrotyrosine can contribute to vascular damage.
and nitrotyrosine generation, we determined whether C-peptide had any effect on HGM-induced endothelial apoptosis using DiOC<sub>4</sub> or PI double staining and confocal microscopy (Fig. 3A and B). HGM induced endothelial cell apoptosis (P<0.001), which was inhibited by C-peptide but not by insulin (Fig. 3C). Similarly, HGHG conditions induced a significant increase in endothelial cell apoptosis (P<0.001). In contrast, endothelial cell apoptosis was not affected by l-glucose or heat-inactivated C-peptide. These results indicate that C-peptide, but not insulin, inhibits HGM-induced endothelial cell apoptosis induced by persistent ROS generation and subsequent ONOO<sup>−</sup> and nitrotyrosine formation.

**C-peptide prevents HGM-induced p53 expression and p66<sub>shc</sub> activation**

Because upregulation of transcription factor p53 and activation of mitochondrial adaptor protein p66<sub>shc</sub> play essential roles in persistent ROS generation and endothelial apoptosis (Schisano et al. 2011, Paneni et al. 2012a,b), we next investigated the effects of C-peptide on p53 expression and p66<sub>shc</sub> phosphorylation in HGM HUVECs. In HGM HUVECs, persistent p53 upregulation was observed after glucose normalization (P<0.001), and these elevated levels were normalized by C-peptide (Fig. 4A). HGM conditions additionally induced persistent p66<sub>shc</sub> phosphorylation (P<0.05), which was also inhibited by C-peptide (Fig. 4B). Interestingly, insulin also prevented HGM-induced p53 upregulation and p66<sub>shc</sub> phosphorylation, probably via a different mechanism from that used by C-peptide because insulin had no effect on persistent generation of HGM-induced ROS, ONOO<sup>−</sup> or nitrotyrosine. These results suggest that HGM induces persistent generation of intracellular ROS and subsequent ONOO<sup>−</sup> and nitrotyrosine formation, possibly via sustained p53 upregulation and p66<sub>shc</sub> activation, resulting in endothelial cell apoptosis. Additionally, these HGM-induced intracellular events are inhibited by C-peptide.

**C-peptide supplementation prevents HGM-induced vasculopathy in diabetic mice**

Compared with nondiabetic controls (Con; n=14), diabetic mice (DM; n=13) had increased food and water consumption and severe hyperglycemia (30.04 mmol/L) with glycosuria (data not shown). These parameters were improved in diabetic mice supplemented with insulin.
for 4 weeks (DM+Ins; n = 11); due to normalized glucose levels, these mice were designated as experiencing HGM. In contrast, food and water consumption and hyperglycemia were not improved in diabetic mice supplemented with C-peptide for 4 weeks (DM+CP; n = 10) but returned to levels similar to those observed in control mice when supplemented with both insulin and C-peptide (HGM+CP; n = 14). Additionally, C-peptide levels significantly decreased in untreated diabetic (<0.1 nmol/L) or insulin-supplemented diabetic mice (<0.1 nmol/L) and were fully restored to normal ranges after treatment with C-peptide alone or C-peptide with insulin (Table 1).

<table>
<thead>
<tr>
<th>Observations</th>
<th>Control</th>
<th>Diabetes (DM)</th>
<th>Diabetes+Ins (HGM)</th>
<th>Diabetes+CP (DM+CP)</th>
<th>Diabetes+Ins+CP (HGM+CP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice (n)</td>
<td>14</td>
<td>13</td>
<td>11</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Nonfasting blood sugar (mmol/L)</td>
<td>9.16±0.85</td>
<td>30.04±2.96***</td>
<td>9.29±3.18</td>
<td>29.11±2.71***</td>
<td>10.35±3.07</td>
</tr>
<tr>
<td>Serum C-peptide (nmol/L)</td>
<td>1.31±0.85</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>1.73±0.57</td>
<td>1.51±0.67</td>
</tr>
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To confirm our in vitro findings, we investigated the preventive effect of C-peptide supplementation on the persistent HGM-induced elevation of intracellular ROS and ONOO− in mouse aortic endothelium. Intracellular ROS levels increased in the aortic endothelium of diabetic mice compared with those in controls (DM; P<0.001), and insulin supplementation had no effect on endothelial ROS generation (HGM; P<0.01; Fig. 5A and B). However, C-peptide prevented endothelial ROS generation both in diabetic and HGM mice (P<0.01).

![Figure 5](http://dx.doi.org/10.1530/JOE-16-0349)
insulin supplementation ($P<0.001$; Fig. 5C and D). Hyperglycemia- and HGM-induced ONOO$^-$ generation was inhibited by treatment with C-peptide. These results suggest that C-peptide, but not insulin, effectively prevents persistent HGM-induced ROS and ONOO$^-$ generation in the aortic endothelium of diabetic mice, confirming in vitro results.

We then studied whether C-peptide has an inhibitory effect on HGM-induced endothelial apoptosis in diabetic mice aortic endothelium (Fig. 6A and B). Aortic endothelial apoptosis increased in both diabetic and HGM mice, and C-peptide supplementation inhibited hyperglycemia- and HGM-induced endothelial apoptosis ($P<0.05$). Taken together, our results demonstrate that C-peptide supplementation prevents HGM-induced endothelial apoptosis by inhibiting persistent ROS generation and subsequent ONOO$^-$ formation in the aorta of diabetic mice. Additionally, although insulin normalizes glucose levels in diabetic mice, it does not inhibit HGM-induced vascular endothelial cell apoptosis.

**Discussion**

Therapeutic management of hyperglycemia is the primary intervention for preventing diabetic complications and is generally achieved by regular insulin administration in type 1 DM, treatment with oral hypoglycemic agents in the early stages of type 2 DM and administration of insulin together with hypoglycemic agents in the later stages of type 2 DM (Massi-Benedetti & Orsini-Federici 2008, Bhatt et al. 2014). Nevertheless, significant prevention and control of diabetic complications has not yet been achieved using intensive insulin therapy (Ceriello et al. 2009a, Pirola et al. 2010). Chronic or transient hyperglycemia-induced ROS generation plays a crucial role in the pathogenesis of diabetic vasculopathy through nitrosative stress and endothelial apoptosis (Bhatt et al. 2014). These processes have not been successfully prevented or controlled even after maintaining normoglycemia, and additional antioxidant therapies have shown limited beneficial outcomes (Beckman et al. 2003). Here, we confirm the hypothesis that endogenous C-peptide can be used as a frontline therapeutic supplement in diabetic patients, both in vitro using HUVECs and in vivo using mouse aortas. Specifically, C-peptide is a particularly promising therapeutic candidate due to its ability to inhibit HGM-associated adverse cellular events by reducing ROS, ONOO$^-$ and nitrotyrosine formation.

Previous studies have provided insights into the molecular mechanisms behind HGM-induced progression of diabetic vascular complications despite glycemic control, demonstrating that persistent ROS production promotes HGM-induced complications.
We investigated hyperglycemia-induced persistent ROS overproduction after glucose normalization in human endothelial cells and in the aortas of diabetic mice, as HGM-induced persistent ROS overproduction is described as a key metabolic memory event in the activation of endothelial apoptosis during diabetic vasculopathy (Giacco & Brownlee 2010, Paneni et al. 2012b). To our knowledge, we report for the first time the potential therapeutic use of C-peptide against HGM-induced diabetic vasculopathy. C-peptide prevented HGM-induced ROS generation, ONOO− and nitrotyrosine formation and subsequent apoptosis in cultured HUVECs. C-peptide supplementation also consistently inhibited HGM-induced ROS and ONOO− generation and endothelial apoptosis in the aortas of diabetic mice (Fig. 6C).

Interestingly, insulin prevented HGM-induced p53 upregulation and p66shc phosphorylation, whereas it had no inhibitory effect on the persistent generation of HGM-induced ROS, ONOO− and nitrotyrosine. Hyperglycemia generates excessive ROS via a vicious cycle between mitochondrial and cytosolic ROS, which is responsible for persistent vascular oxidative stress (Paneni et al. 2012b, Bhatt et al. 2013b). Mitochondrial superoxide is generated in response to hyperglycemia through respiratory chain overflow, which results in the activation of PKC, polyol and hexoseamine pathways and increased formation of advanced glycation end products (Giacco & Brownlee 2010). PKC activation, in turn, induces ROS generation by activating NADPH oxidase. Hyperglycemia also produces mitochondrial ROS by phosphorylating p66shc, regulated by SIRT1 and p53 (Paneni et al. 2012b). Thus, it is likely that hyperglycemia induces persistent vascular oxidative stress by sustaining ROS generation through electron transport chain, PKC-NADPH oxidase pathway and SIRT1-p53-p66shc pathway. In our study, C-peptide inhibited hyperglycemia-induced persistent ROS generation in endothelial cells and in the aorta of diabetic mice. We also previously reported that C-peptide inhibited hyperglycemia-induced generation of cytosolic and mitochondrial ROS (Bhatt et al. 2013b). However, insulin had no inhibitory effect on hyperglycemia-induced ROS generation, persistent oxidative stress and subsequent apoptosis in endothelial cells and the aorta of diabetic mice, though it inhibited p53 upregulation and p66shc phosphorylation (Figs 1 and 4). These results suggest that inhibition of p53 upregulation and p66shc phosphorylation by insulin may not be sufficient to inhibit hyperglycemia-induced persistent oxidative stress and endothelial cell apoptosis. Alternatively, insulin might inhibit p53 upregulation and p66shc phosphorylation via a different mechanism from that used by C-peptide, including AMP-activated protein kinase and protein phosphatase-1 (Gamble & Lopaschuk 1997, Brady & Saltiel 2001, Ruderman et al. 2013, Bhatt et al. 2014). However, it is necessary to elucidate the molecular mechanisms behind C-peptide and insulin-induced p53 upregulation and p66shc phosphorylation in endothelial cells.

Clinical approaches targeting glycemic control have been unsuccessful in preventing diabetic complications (Gerstein et al. 2008). Thus, an additional therapeutic strategy is required for preventing diabetic vasculopathy and organ-specific complications. It has recently been shown that transient exposure to hyperglycemia after insulin therapy results in long-lasting epigenetic modifications that mediate persistent metabolic characteristics in diabetes (El-Osta et al. 2008, Gerstein et al. 2008, Pirola et al. 2010). In this study, we demonstrated that C-peptide consistently prevented HGM-induced intracellular events. Thus, we suggest that combinatory therapy with C-peptide and insulin may be beneficial for treating type 1 DM and the later stages of type 2 DM. This seems particularly likely given that insulin can control hyperglycemic states, whereas C-peptide normalizes hyperglycemia and HGM-induced vascular damage. This combinatory therapy may provide a new approach for diabetes treatments and may significantly improve outcomes for diabetics.

In conclusion, we investigated the beneficial effect of C-peptide on HGM-induced vascular endothelial cell apoptosis in diabetes. C-peptide inhibited persistent p53 upregulation and p66shc activation, ROS generation, nitrosative stress and subsequent endothelial apoptosis in human endothelial cells and in the murine aortas. These studies highlight C-peptide as a potential therapeutic candidate for treating HGM-induced diabetic vascular damage.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
M P B designed and performed experiments, analyzed the data and wrote this manuscript. Y-J L and S-H J designed experiments and acquired data. Y H K designed experiments and synthesized C-peptide. J Y H, E-T H, W S P, S-H H and Y-M K designed experiments and interpreted the data. K-S H conceptualized the study, designed experiments, analyzed and interpreted the data, and critically revised this manuscript. All authors have approved the final version of the manuscript.

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