REVIEW

Anti-Inflammatory Properties of C-Peptide

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■ Abstract

C-peptide, historically considered a biologically inactive peptide, has been shown to exert insulin-independent biological effects on a number of cells proving itself as a bioactive peptide with anti-inflammatory properties. Type 1 diabetic patients typically lack C-peptide, and are at increased risk of developing both micro- and macrovascular complications, which account for significant morbidity and mortality in this population. Inflammatory mechanisms play a pivotal role in vascular disease. Inflammation and hyperglycemia are major components in the development of vascular dysfunction in type 1 diabetes. The anti-inflammatory properties of C-peptide discovered to date are at the level of the vascular endothelium, and vascular smooth muscle cells exposed to a variety of insults. Additionally, C-peptide has shown anti-inflammatory properties in models of endotoxic shock and type 1 diabetes-associated encephalopathy. Given the anti-inflammatory properties of C-peptide, one may speculate dual hormone replacement therapy with both insulin and C-peptide in patients with type 1 diabetes may be warranted in the future to decrease morbidity and mortality in this population.

Keywords: type 1 diabetes · C-peptide · endothelial dysfunction · inflammation · vascular smooth muscle cells · NF-κB

Brief introduction to C-peptide

Human C-peptide is a 31 amino acid peptide, a product of proinsulin cleavage, generated in pancreatic beta-cells as part of normal insulin production. C-peptide is released into the bloodstream in equimolar amounts with insulin in response to various stimuli including elevated glucose. In healthy individuals, C-peptide circulates in nanomolar concentrations with a half life of approximately 30 minutes compared to a half life of 4 to 5 minutes for insulin [1, 2]. Historically, C-peptide was considered biologically inactive and necessary only for proinsulin folding within beta-cells, thus a mere byproduct of insulin biosynthesis. In recent years, C-peptide has been shown to exert insulin-independent biological effects on a number of cells, proving itself as a bioactive peptide.

Patients with type 1 diabetes (T1D) are at increased risk of developing both micro- and macrovascular complications, which cause significant morbidity and mortality. In patients with T1D, C-peptide is decreased or absent. Important findings in the current decade were that C-peptide replacement therapy in T1D ameliorates certain complications including peripheral neuropathy and nephropathy [3-8], and that it has anti-inflammatory effects under high glucose conditions [9, 10]. The anti-inflammatory properties of C-peptide that have been discovered to date are at the level of the vascular endothelium and vascular smooth muscle cells exposed to a variety of insults. Additionally, C-peptide has shown anti-

inflammatory properties in models of endotoxic shock and type 1 diabetes-associated encephalopathy [11, 12]. These anti-inflammatory characteristics of C-peptide are discussed in this review.

Origin of vascular disease in type 1 diabetes patients

Endothelial dysfunction: an early lesion in the vasculature of T1D patients

T1D has been recently recognized as an independent risk factor for the development of microvascular disease and atherosclerosis. In T1D, the risk associated with microvascular complications is enormous: approximately, one in three people with diabetes develops aggressive microvascular complications, affecting the small vessels of the eyes, the kidneys, and the peripheral nerves. Over 70% die of atherosclerosis-related disease [13].

The mechanisms of vascular disease in T1D are not fully understood. It has been proposed that the diabetic milieu induces changes in the arterial wall that would render the vessel more susceptible to premature vascular compromise [14]. One of these changes is represented by endothelial dysfunction, which has been recognized as an early event in the pathogenesis of vascular complications in T1D. Endothelial dysfunction, as seen in diabetes, is characterized by impaired flow-mediated vasodilation [15], a low-grade inflammation [16], and increased levels of endothelial cell adhesion molecules, including E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) [16-20]. The upregulation of these molecules on endothelial cells allows attachment and migration of circulating leukocytes into the vessel wall. This is a crucial step in vascular compromise leading to atherosclerotic plaque formation. Following expression at the cell surface, these molecules are shed as soluble forms, and can be detected in the peripheral circulation. Altered levels of circulating endothelial cell adhesion molecules are thus considered biochemical markers of vascular dysfunction in high-risk individuals, such as in T1D patients [19, 21-23]. In these patients, signs of endothelial dysfunction, with increased plasma concentrations of endothelial cell adhesion molecules, have been found shortly after clinical diagnosis of diabetes. This suggests the presence of vascular compromise at an early stage of diabetes, when subjects are still free of any vascular symptoms

[20, 24, 25]. Some other studies also detected signs of endothelial dysfunction in schoolchildren who tested positive for one or more diabetes-associated autoantibodies but are non-diabetic yet [20, 26-29]. The presence of endothelial dysfunction puts subjects at risk of developing premature vascular damage. These subjects could ideally be early recognized, followed over time, and eventually treated to prevent the development of overt vascular complications.

Abbreviations:

AoSMC - aortic smooth muscle cells
BB/Wor rat - BioBreeding/Worcester rat (animal model of spontaneous autoimmune diabetes)
COX-2 - cyclooxygenase-2
C-peptide - connecting peptide
CD11b - cluster of differentiation molecule 11b (also known as Mac-1)
DCCT/EDIC - Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications
DNA - deoxyribonucleic acid
ERK - extracellular signal-regulated kinase
E-selectin - endothelial selectin (cell adhesion molecule expressed on endothelial cells)
HAEC - human aortic endothelial cells
HEK-293 cells - human embryonic kidney 293 cells
ICAM-1 - inter-cellular adhesion molecule 1
IκB – inhibitor kappa B
IL-1beta – interleukin 1beta (cytokine produced by monocates, inflammatory mediator)
IL-6 - interleukin 6 (secreted by monocytes and endothelial cells, can have inflammatory and anti-inflammatory effects)
IL-8 - interleukin 8 (chemokine of the CXC family, secreted by monocytes and endothelial cells)
L-NAME - N^G-nitro-L-arginine methyl ester
LPS - lipopolysaccharide
Mac-1 - macrophage-1 antigen (also known as CD11b)
MAP - mitogen-activated protein
MAPK - mitogen-activated protein kinase
MCP-1 - monocyte chemotactic protein type 1
NF-κB - nuclear factor-kappa light-chain enhancer of activated B cells
PAI-1 - plasminogen activator inhibitor-1
PDGF - platelet-derived growth factor
PMN - polymorphonuclear
PPARγ - peroxisome proliferator-activated receptor
P-selectin – platelet selectin (cell adhesion molecule expressed on endothelial cells)
RAGE - Receptor for advanced glycation end products
mRNA - messenger ribonucleic acid
ROS - reactive oxygen species
Swiss 3T3 fibroblasts - 3-day transfer, inoculum 3×10^5 fibroblast cells
T1D - type 1 diabetes
T2D - type 2 diabetes
TNF-α - tumor necrosis factor alpha
UASMC - umbilical artery smooth muscle cells
VCAM-1 - vascular cell adhesion molecule-1
VSMC - Vascular smooth muscle cells

Several factors have been reported to induce endothelial cell activation *in vitro*, including abnormal glucose levels, i.e. hyperglycemia [30], glycated proteins [31], and inflammatory cytokines [32]. In this article, we discuss the impact that these factors have in the development of T1D-associated vascular dysfunction.

Importance of high glucose levels in the development of endothelial dysfunction in T1D

Abnormal glucose levels, i.e. hyperglycemia, are a major clinical sign in T1D patients. Hyperglycemia has been recognized to play a crucial role in the development of endothelial dysfunction leading to vascular complications in T1D [33]. Several mechanistic pathways have been proposed in order to elucidate the deleterious effect of high glucose on vasculature function. At the cellular level, hyperglycemia causes an increased vascular tone, and permeability of the endothelial cell monolayer. This is mainly a consequence of impaired production and bioavailability of vasodilators such as nitric oxide, vasoconstrictors such as endothelin-1 [34, 35], and permeability factors such as vascular endothelial growth factor [36, 37]. Exposure of endothelial cells to high glucose increases the production of reactive oxygen species (ROS), mainly through the mitochondrial electron transport chain [38, 39], and by upregulating antioxidant enzymes [40]. The excessive production of ROS caused by hyperglycemia leads to alterations in endothelial cell proliferation and adhesion properties [41]. These alterations contribute to acceleration of the apoptotic process in endothelial cells [42].

In addition, exposure to high glucose promotes the expression of ICAM-1, VCAM-1, and E-selectin on endothelial cells, a phenomenon observed even after short exposure to high glucose, such as during hyperglycemic spikes after a meal [43-45]. High glucose also stimulates the secretion of the chemokines IL-8 and monocyte chemotactic protein type 1 (MCP-1) by endothelial cells [10, 45, 46]. These inflammatory mediators are crucial in recruiting monocytes to the vessel wall [45, 47]. They are usually found in human atherosclerotic plaques [48]. Simultaneously, the adipocyte itself is recognized to secrete circulating cytokines (adipokines) such as interleukin-6, tumor necrosis factor-alpha, leptin, and PAI-1, exacerbating the ongoing vascular inflammation [13, 49] in the vessel wall of diabetic patients. The prevalence of being overweight in T1D is increasing following the general trend in the population [50].

Rigorous glycemic control may reduce or delay the development of vascular complications, but data from the DCCT/EDIC study demonstrated that even intensive insulin treatment to achieve blood glucose concentrations close to normal could not completely prevent the development of vascular complications in T1D patients [51]. This suggests that factors other than hyperglycemia contribute to the pathogenesis of vascular complications in T1D. Moreover, endogenous insulin secretion (measured as C-peptide) was better sustained by intensive therapy, and was associated with lower risk for retinopathy and microalbuminuria [52]. Thus, C-peptide deficiency may be a contributing factor in the pathogenesis of vascular complications in T1D.

Importance of inflammation in the pathogenesis of T1D-associated vascular complications

Inflammation is considered a major component in the development of T1D-associated vascular dysfunction [19, 53-55]. It has been observed that T1D patients with microvascular complications show increased monocyte production of several inflammatory cytokines such as IL-6, TNF-α, and IL-1β over and above the levels detected in T1D patients without microvascular complications, and healthy control subjects [19, 55]. Plasma levels of other inflammatory biomarkers, such as C-reactive protein, are also elevated in T1D patients with microvascular complications compared to asymptomatic T1D patients [53, 54]. Many of the reported inflammatory changes are detected at the level of monocytes. They show upregulation of the adhesion molecule CD11b (Mac-1) [56], and have aberrant constitutive and lipopolysaccharide(LPS)-stimulated expression of cyclooxygenase (COX)-2, a defect which may predispose to a chronic inflammatory response in T1D [57, 58].

The vascular endothelium is a likely target of the inflammatory response detected in T1D patients. On endothelial cells, inflammatory cytokines induce functional and structural alterations, including oxidative damage or interference with the mechanisms of contraction/relaxation. This results in alterations in vascular integrity, tone, and coagulation. The inflammatory cytokine IL-1β is associated with increased diabetic retinopathy [59]. Certain other cytokines, such as IL-8 and MCP-1, are powerful monocyte attractants during the early stages of endothelial dysfunction. Once

transmigrated into suben-dothelial layers, immune cells produce more inflammatory mediators and cytokines, thus feeding the vicious circle of inflammation that eventually leads to vascular compromise.

Another component of inflammation and endothelial dysfunction is oxidative stress. T1D patients with microvascular complications have accentuated oxidative stress as compared to T1D patients without microvascular complications, as shown by significantly elevated levels of nitrotyrosine, monocyte superoxide anion [55], DNA and protein oxidation [60, 61]. ROS damage endothelial cells directly through cellular injury, and indirectly through inactivation of nitric oxide, or by serving as an endothelium-derived contracting factor [62, 63]. At the molecular level, increased ROS production leads to activation of the nuclear-factor (NF)-κB pathway [39, 64], and ultimately the production of inflammatory mediators [65]. Devaraj *et al.* showed that T1D patients suffering from microvascular complications have increased activation of the NF-κB pathway compared to diabetic patients without complications [55]. To this end, strategies are proposed that target NF-κB pathway activation to reduce inflammatory activity, and prevent vascular dysfunction [66, 67].

C-peptide and endothelial function

C-peptide is now recognized as a molecule displaying potential beneficial effects on the dysfunctional endothelium, as observed in several *in vivo* and *in vitro* models of inflammation-mediated vascular injury. In T1D, a decrease (or lack) of circulating C-peptide levels as a result of the autoimmune attack on the pancreatic beta-cells, has been considered an important factor in the development of vascular dysfunction. In fact, in a clinic-based cohort of T1D, higher fasting C-peptide levels were found to have a protective effect on microvascular
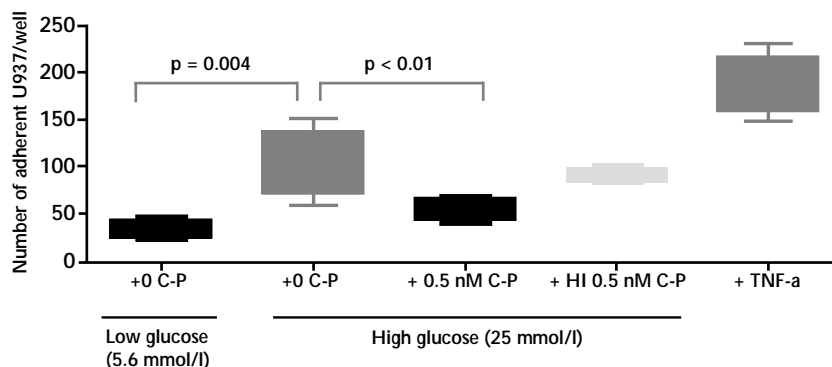

Figure 1. Physiologic concentrations of C-peptide reduce adherence of U-937 to HAEC under high-glucose conditions. HAEC were cultured in 25 mmol/l glucose in the presence or absence of 0.5 nmol/l C-peptide (C-P) for 4 hours. U-937 were added for 1 hour then counted. Boxplot graphs show the median values (limits of the lines are 5th and 95th centiles) of number of adherent U-937 per well. High glucose (25 mmol/l) increased the number of adherent U-937 compared to low glucose (5.6 mmol/l) (p = 0.004). Addition of C-peptide reduced the number of adherent U-937 compared to 25 mmol/l glucose alone (p < 0.01). Heat-inactivated (HI) C-peptide did not significantly alter adherence of U-937. TNF-α, used as a positive control, produced more than a five-fold increase in adherent U-937 in comparison to normal glucose. Modified with kind permission from Springer Science+Business Media, Figure 4A [10].

complications [68]. Results from clinical trials of C-peptide replacement therapy in T1D patients with microvascular complications have demonstrated significant improvement of signs and symptoms of peripheral neuropathy and kidney function [4, 5]. An improvement of myocardial blood flow in T1D patients has also been observed in similar studies [69].

Although the mechanisms by which C-peptide exerts its cytoprotective effects on the endothelium are not entirely understood, it has been reported that C-peptide can influence activation of different signaling pathways that ultimately modulate or shut down inflammatory responses. In the sections hereafter, we present the current knowledge on this topic.

Effect of C-peptide on leukocyte-endothelium interactions: modulation of adhesion molecule expression and cytokine secretion

The adhesion and migration of circulating monocytes into the subendothelial space is one of the key events in the early stages of atherogenesis [70]. Evidence supports the idea that C-peptide affects interactions of circulating monocytes and
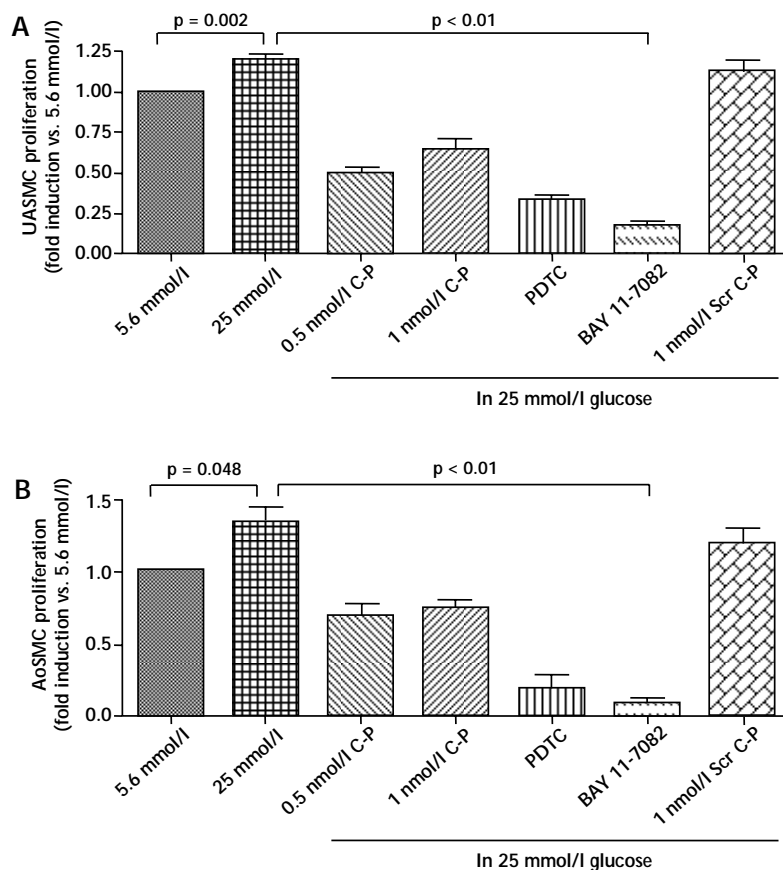
Figure 2. In VSMC, both physiologic concentrations of C-peptide and NF-κB inhibitors reduce high glucose-induced proliferation. VSMC were incubated with 25 mmol/l glucose in the presence or absence of C-peptide (C-P) for 48 hours, and assayed for proliferation by measuring the nuclear incorporation of BrdU (DNA synthesis). In UASMC, BrdU incorporation showed cellular proliferation in high glucose (vs. 5.6 mmol/l, p = 0.002). C-peptide reduced high glucose-induced UASMC proliferation (p < 0.01), while addition of scrambled C-peptide (Scr C-P) did not have a significant effect. Addition of the NF-κB inhibitors PDTC (20 µM) and Bay-11-7082 (1 µM) also showed a decrease in proliferation (vs. 25mmol/l glucose, p < 0.01). In AoSMC, BrdU incorporation showed cellular proliferation in high glucose (vs. 5.6 mmol/l glucose, p = 0.048). C-peptide reduced high glucose-induced AoSMC proliferation (p < 0.01), while addition of scrambled C-peptide (Scr C-P) did not have a significant effect. Addition of the NF-κB inhibitors PDTC (20 µM) and Bay-11-7082 (1 µM) also showed a decrease in proliferation (vs. 25 mmol/l glucose, p < 0.01). Values are mean ± SD of 10 different experiments run in triplicate. Modified with kind permission from Elsevier, Figure 1 [9].

Scalia and co-workers. They examined pretreatment with C-peptide to rats injected with the inflammatory agents thrombin or N^G-nitro-L-arginine methyl ester (L-NAME), which cause acute endothelial dysfunction in these animals. It emerged that the expression of the endothelial cell adhesion molecule ICAM-1 and P-selectin on the mesenteric microvascular endothelium was attenuated [71]. As a consequence, the number of rolling, adhering, and transmigrating leukocytes also decreased upon C-peptide administration to the animals.

In another model of vascular injury, C-peptide was able to decrease polymorphonuclear leukocyte (PMN) infiltration in isolated rat hearts following ischemia-reperfusion injury [72]. PMN infiltration induced endothelial and myocardial injury by releasing cytotoxic substances such as oxygen-derived free radicals, inflammatory cytokines, and proteolytic enzymes. By reducing PMN infiltration to the myocardium, C-peptide restored cardiac contractile function and postreperfusion coronary heart flow [72]. These findings have been recently recapitulated *in vitro* in a model of high glucose-induced endothelial dysfunction, in which adhesion of the monocytic cell line U-937 to high glucose-stimulated human aortic endothelial cells (HAEC) *in vitro* decreased by 50% after addition of physiological concentrations of C-peptide. This effect was not detected when C-peptide was heat-inactivated (Figure 1) [10]. The effect was likely the consequence of a decreased expression of the adhesion molecule VCAM-1 on HAEC by C-peptide, an effect demonstrated both at the level of mRNA and protein. In the same

other leukocytes to endothelial cells by reducing the upregulation of critical endothelial cell adhesion molecules typically observed under inflammatory conditions and exposure to high glucose. The first who described this C-peptide effect were

model, full-length, native C-peptide, but not heat-inactivated C-peptide, was demonstrated to reduce high glucose-induced secretion of IL-8 and MCP-1 by HAEC to basal levels measured under normal glucose concentrations [10]. These two chemokines are essential to promote leukocyte adhesion to endothelial cells. C-peptide did not seem to have any apparent effect on endothelial cells under normal glucose levels. Thus, the most meaningful biological effects of C-peptide on the endothelium are realized in conditions of vascular insult or damage.

Anti-inflammatory effects of C-peptide on smooth muscle cells, and intracellular signaling activities of C-peptide at the basis of anti-inflammatory function

Vascular smooth muscle cells (VSMC) play an integral role in vascular disease and atherosclerotic plaque formation. They serve as a primary source of collagens, elastic fibers, and several proteoglycans, all components of the extracellular matrix [73]. In response to growth factors such as platelet-derived growth factor (PDGF), VSMC migrate and proliferate, and inflammatory stimuli become intermixed in the area of inflammation [74]. VSMC serve as a critical component of intermediate lesion formation, and with continued inflammation and remodeling, for the formation of advanced, complicated lesions of macroangiopathy [74]. Increased proliferation of VSMC, increased migration into the intima, altered matrix components, increased matrix degradation, and increased nonenzymatic collagen glycation, all have been implicated in diabetic macrovascular disease [75]. Specifically, under high glucose conditions, human, porcine, and rat VSMC proliferate and migrate from the media to the subendothelial space, contributing to early atherosclerotic lesions [76-78].

NF-κB regulates the transcription of a number of genes that are involved in inflammation and proliferation. In the unstimulated state, NF-κB exists as a heterodimer in the cytoplasm, and is composed of p50 and p65 subunits bound to IκB [79]. In response to any number of stimuli, IκB is phosphorylated and degraded causing a release of the p50/p65

subunit, which subsequently translocates to the nucleus, and initiates the transcription of different genes [79]. Evidence suggests the NF-κB pathway is involved in atherosclerosis by acting at various pathophysiological levels during atherogenesis [80]. The activated p65 subunit has been found in the fibrotic thickened intima-media and atheromatous areas of the atherosclerotic lesion, and also in macrophages, endothelial cells, and smooth muscle cells [80].

High glucose initiates activation of the NF-κB pathway in VSMC [81]. Administration of p65 antisense oligonucleotides inhibits VSMC proliferation, which further demonstrates the critical role of NF-κB in VSMC physiology [82]. Other studies implicate NF-κB as a modulator of apoptosis and inflammatory signaling in VSMC rather than proliferation [83]. Dragomir *et al.* found that high glucose upregulates MCP-1, and fractalkine, key inflammatory chemokines, through upstream involvement of the mitogen-activated protein kinase (MAPK) signaling pathway in NF-κB, and activator protein-1 (AP-1) activation [84]. MCP-1 and fractalkine expression was reduced when high glucose-exposed VSMC were treated with peroxisome proliferator-activated receptors alpha (PPARα), by reducing the activation of the MAPK pathway [84]. Nonetheless, NF-κB activation in VSMC represents a key mechanism for the accelerated vascular disease observed in diabetes. Strategies targeting the NF-κB pathway activation for the prevention and/or treatment of cardiovascular disease are becoming important [66, 67, 85, 86].
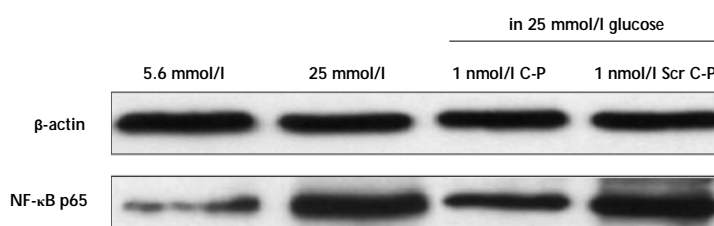

Figure 3. Expression of p65 subunits of NF-κB in AoSMC cultured in high glucose in the presence or absence of physiologic amounts of C-peptide. Representative immunoblot depicting the 65-kDa band of the p65 subunit in AoSMC cultured in 5.6 mmol/l or 25 mmol/l glucose in the presence or absence of C-peptide (C-P) for 48 hours. In high glucose, AoSMC in the presence of C-peptide showed a decreased NF-κB nuclear translocation as compared to high glucose alone. Scrambled C-peptide (Scr C-P) did not have significant effect. To show equal loading of the gel, staining for β-actin is also shown. Modified with kind permission from Elsevier, Figure 5 [9].

C-peptide is emerging as a potential therapeutic option; it reduces high glucose-induced proliferation of vascular smooth muscle cells [9, 87]. Rat aortic smooth muscle cells cultured in high-glucose conditions with 1 to 100 nmol/l of C-peptide show a dose-dependent reduction in proliferation, which is mediated through inhibited phosphorylation of p42/p44 MAP kinases, and inhibited PDGF-β receptor expression [87]. In human umbilical artery smooth muscle cells (UASMC) and human aortic smooth muscle cells (AoSMC), physiologic concentrations of C-peptide (as well as NF-κB inhibitors) decrease high glucose-induced proliferation (Figure 2) [9]. Furthermore, C-peptide reduced high glucose-induced nuclear translocation of NF-κB p65 and p50 in both UASMC (Figure 3) and AoSMC, and reduced high glucose-induced phosphorylation of IκBα in VSMC [9]. The latter is an upstream signaling event that regulates NF-κB translocation from the cytoplasm to the nucleus. Thus, physiologic concentrations of C-peptide appear to reduce high glucose-induced VSMC proliferation via suppression of NF-κB activation. These findings underscore a role of C-peptide in VSMC functions, especially in conditions of diabetic insult to the vasculature.

There are also conflicting data on C-peptide's role in VSMC proliferation. *In vitro* studies by Walcher *et al.* revealed human and rat VSMC proliferation upon stimulation with the respective C-peptide in a dose-dependent manner, with a maximal induction at 10 nmol/l human C-peptide or 0.5 nmol/l rat C-peptide [88]. It is possible that C-peptide in excessive or non-physiologic quantities may promote lesion development, for example in patients with type 2 diabetes (T2D) and insulin resistance, while preventing lesion development in patients with T1D and insulin deficiency. Further studies are needed to delineate the exact role of C-peptide in lesion development.

Other studies also demonstrate the importance of C-peptide in intracellular signaling and inflammation in the central nervous system. T1D patients may suffer impairments in learning, memory, problem solving, and mental and motor speed with primary diabetic encephalopathy recognized as a late complication of T1D [11]. In the type 1 BB/Wor rat (rat model of human T1D), duration-related cognitive impairment is associated with duration-related apoptosis-induced neuronal loss in the hippocampus [89]. C-peptide replacement reduced several apoptotic mechanisms, and significantly prevented the cognitive dysfunction and hippocampal neuronal loss [90].

Furthermore, the expression of NF-κB was significantly increased in diabetic hippocampi. This phenomenon was associated with increased expression of RAGE, and the downstream activation of pro-inflammatory TNF-α, IL-1β, IL-2, and IL-6 [11]. The upregulation of RAGE and activation of NF-κB, TNF-α, IL-1β, IL-2, and IL-6 were significantly reduced when the diabetic rats received C-peptide replacement, which was also associated with the prevention of astrocyte proliferation [11]. In addition to stimulating NF-κB activation, RAGE plays a role in innate immune responses [11]. Thus, both NF-κB and RAGE activation contribute to the eventual apoptotic cell death and cognitive dysfunction in T1D, all of which may be prevented by C-peptide replacement [11].

Anti-inflammatory effects of C-peptide in endotoxic shock

Sepsis, defined as acute systemic inflammatory response to infection, is the most common cause of death in inten-
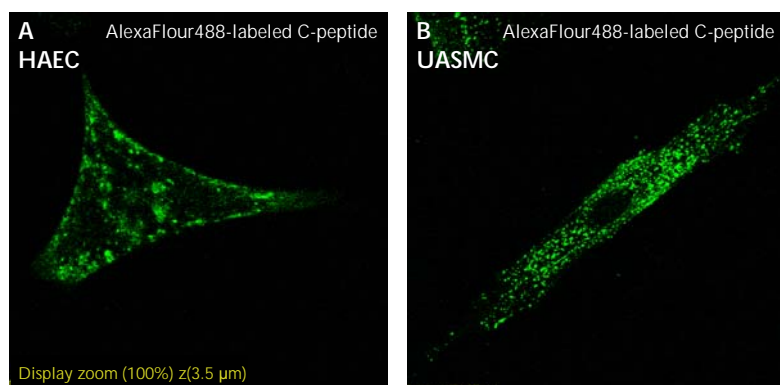

Figure 4. C-peptide internalization in human aortic endothelial cells (HAEC) and umbilical artery smooth muscle cells (UASMC). In HAEC and UASMC, C-peptide internalizes as punctuate structures. HAEC (**A**) and UASMC (**B**) were incubated for 30 minutes with 1 mmol/l Alexa Fluor 488-labeled C-peptide at 37°C, washed with medium, and imaged by confocal microscopy. The C-peptide probe localized at the periphery of the cell and in the cytoplasm, represented by the green punctuate staining. The figure shows a representative z-section across one cell. Modified with kind permission from Springer Science+Business Media, Figure 1 [100].

sive care units. It has been calculated that 10% of all deaths in the United States are attributable to sepsis [91]. Systemic inflammation, with increased circulatory levels of inflammatory cytokines, is a pathogenetic component of severe sepsis and of other critical illnesses such as major trauma, burns, and pancreatitis, and can occur after major surgery [92]. To date, the conventional therapies, combined with circulatory and respiratory support, are not always sufficient to reverse systemic inflammation [12, 93]. A recent report showed that injections of C-peptide *in vivo* to lipopolysaccharide (LPS)-treated mice, an animal model of endotoxic shock, drastically improved the survival rate of the animals compared to vehicle-treated mice. This effect is associated with reduced plasma levels of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α) and MCP-1, and a decreased overall inflammatory response in the lung [12]. In the lung of endotoxin-treated mice, C-peptide inhibited phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, followed by upregulation of nuclear expression and DNA binding of the nuclear transcription factor proliferator-activated-receptor gamma (PPAR-γ), which plays an important role in the modulation of inflammation [12].

Internalization of C-peptide in target cells

C-peptide has been shown to exert biological effects on a variety of cells. However, little is known about the C-peptide receptor, and how C-peptide achieves its intracellular effects in target cells. It was initially thought that C-peptide works via nonchiral mechanisms rather than by stereospecific receptors or binding sites [94]; though, earlier reports showed specific binding of C-peptide to cultured B cells from a transplantable rat islet cell tumor [95]. Using sensitive techniques (fluorescence correlation spectroscopy), Rigler *et al.* confirmed specific binding of human C-peptide to membrane-bound receptors in several human cell types, including renal tubular cells, skin fibroblasts, and saphenous vein endothelial cells [96, 97]. Furthermore, C-peptide binding reached full saturation at 0.9 nM; thus in healthy subjects, receptor saturation is achieved at physiologic levels [96]. Pretreatment with pertussis toxin prevented C-peptide binding, suggesting C-peptide binds to specific G-protein coupled receptors on human cell membranes (96). C-peptide binding to cells may be displaced by its C-terminal pentapeptide with Glu27, which is particularly important for specific binding to human renal tubular cells (96, 98).

In addition to specific binding to cell membranes, C-peptide internalization has recently also been verified. In HEK-293 cells and Swiss 3T3 fibroblasts, C-peptide was shown to bind and cross the plasma membrane, localizing in the cytoplasm where it was detected up to 1 hour after its uptake [99]. Nuclear localization of C-peptide in HEK-293 cells and Swiss 3T3 fibroblasts was also demonstrated [99]. C-peptide internalization was also explored in HAEC and UASMC. In these cells, C-peptide internalizes to punctate structures localized at the level of the cellular membrane and in the cytoplasm (Figure 4) [100]. Internalization of C-peptide was minimal after 5 minutes, clearly detectable after 10 minutes, resulted in bright staining after 30 minutes, and internalization was completed by 1 hour [100]. Furthermore, C-peptide co-localized with endosomes, and was eventually trafficked to lysosomes [100]. Based on these findings, signaling from putative C-peptide-receptor complexes might be initiated at the plasma membrane, continue from early endosomes, and terminate at lysosomes. Endosomes, classically regarded as sorting stations for internalized activated receptor-peptide complexes on their way to lysosomal degradation, are emerging as crucial players in intracellular signaling [101]. This trafficking provides a possible platform for the intracellular signaling events initiated by C-peptide in target cells.

Concluding remarks

Contrary to past belief, recent years have exposed C-peptide as a biologically active peptide, independent of insulin. C-peptide circulates in nanomolar concentrations in healthy individuals, but is reduced or absent in individuals with T1D, a population with inherent increased risk for vascular sequelae. C-peptide also seems to be present in too large quantities in T2D. Though DCCT clearly demonstrated rigorous glycemic control may reduce or delay the onset of vascular complications in T1D, these complications could not be completely prevented. Thus, other factors must be playing a role. C-peptide, in physiologic amounts, is emerging as a molecule displaying potential beneficial effects on the dysfunctional endothelium, as observed in several *in vivo* and *in vitro* models of inflammation-mediated vascular injury. Although further studies are needed, one may speculate dual hormone replacement therapy with both insulin and C-peptide in patients with T1D may be warranted to combat cardiovascular morbidity and mortality in this population.

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