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REVIEW

Molecular Effects of C-Peptide in Microvascular Blood Flow Regulation

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■ Abstract

C-Peptide is produced in beta-cells in the pancreas, and secreted into the blood stream in equimolar amounts with insulin. For a long time, C-peptide was considered as an important component in the biosynthesis of insulin, but otherwise believed to possess minimal biological activity. In the recent years, numerous studies demonstrated that lacking C-peptide in type 1 diabetic patients might exert an important role in the development of microvascular complications such as nephropathy or neuropathy. There is increasing evidence that the biological effects of C-peptide are, at least in part, mediated through the modulation of endothelial function and microvascular blood flow. In several tissues, an increase in microvascular and nutritional blood flow could be observed during substitution of physiological amounts of C-peptide. Recent studies confirmed that C-peptide stimulates endothelial NO release by the activation of Ca^{2+} calmodulin-regulated endothelial NO synthase. A restoration of Na^+/K^+-ATPase activity during C-peptide supplementation could be observed in erythrocytes and renal tubular cells. The improvement of erythrocyte Na^+/K^+-ATPase is associated with an increase in erythrocyte deformability, and improved rheological properties. In this article, we consider the role of C-peptide in the context of endothelial function and microvascular blood flow as pathophysiologic components in the development of microvascular complications in patients with diabetes mellitus and loss of beta-cell function.

Keywords: type 1 diabetes · C-peptide · microvascular blood flow · erythrocyte deformability · endothelial nitric oxide · Na+/K+-ATPase · smooth muscle · NF-kappaB · G-protein

Introduction

Patients with diabetes mellitus type 1 present with an excessive risk for such microvascular complications as retinopathy, nephropathy, and peripheral neuropathy. Although hyperglycemia is recognized as a major driver in the development of these diabetic complications, the precise mechanism is not fully understood. In type 1 diabetic patients with good metabolic control, the risk for the development of microvascular complications is reduced but still not avoided.

Insulin treatment of type 1 diabetes is an effective tool for addressing perturbations in glucose metabolism. However, many of the vascular risks associated with the disease persist even in the presence of regular insulin therapy and stable glycemic control. In the DCCT trial, type 1 diabetic patients with sustained C-peptide secretion showed a significant reduction in the risk for microvascular complications compared with those patients totally lacking C-peptide secretion [1]. In this study, even modest beta-cell activity was associated with a decrease in the incidence of mi-

crovascular complications. In a recent cross sectional study, 471 type 1 diabetic patients were followed from 1994 to 2004 [2]. Those patients with the lowest fasting C-peptide levels were found to have the highest rate of microvascular complications. No association was observed between C-peptide levels and macrovascular complications. There is increasing evidence that, in type 1 diabetic patients, the conservation of residual beta-cell function slows microvascular complications, by improving blood glucose control and by the preservation of residual C-peptide secretion.

Abbreviations:
ATP - adenosine triphosphate
BAEC - bovine aortic endothelial cell
Ca^{2+} - bivalent ionic calcium
Cr^{3+} - trivalent ionic chromium
cGMP - cyclic guanosine monophosphate
C-peptide - connecting peptide
DCCT - Diabetes Control and Complications Trial
EDTA - ethylenediaminetetraacetic acid
ERK - extracellular signal-regulated kinase
Fe^{2+} - bivalent iron cation
FMD - flow mediated vasodilatation
GLUT1 - glucosetransporter 1
L-NMA - N(G)-monomethyl-l-arginine
L-NNA - N(G)-nitro-l-arginine
Na^+/K^+-ATPase - natrium, kalium adenosintriphosphatase (also sodium-potassium pump)
NO - nitric oxide
NOS - nitric oxide synthase
eNOS - endothelial nitric oxide synthase
NF-κB - nuclear factor-kappa light-chain enhancer of activated B cells
Pa - Pascal

Several studies were able to show a biological activity of C-peptide in the microcirculation. The observed effects are probably mediated by the release of nitric oxide (NO) from endothelial cells [3-6]. It has been recently shown in a rat model that C-peptide was able to inhibit leukocyte-endothelial interaction in the microcirculation during acute endothelial dysfunction [7], and to exert cardioprotective effects in myocardial ischemia-reperfusion [8]. In hyperglycemic conditions, C-peptide in physiological concentrations was shown to decrease NF-κB-dependent vascular smooth muscle proliferation [9, 10]. However, in insulin-resistant type 2 diabetic patients, unphysiological high levels of C-peptide were found in association with the development of atherosclerotic plaques [11]. Therefore, it seems conceivable that C-peptide in physiological concentrations might evolve vasoprotective effects by improving endothelial function. In contrast, elevated C-peptide plasma levels might interact with the development of atherosclerotic plaques in patients with insulin resistance [12].

Regulation of tissue perfusion is a dynamic process regulated by a complex interaction of several balancing and counterbalancing forces on microvascular blood flow. The purpose of this review is to summarize the recent evidence about the role of human C-peptide in the regulation of endothelial function, and microvascular blood flow.

Endothelial function and microvascular blood flow in type 1 diabetes mellitus

Posterior to the occurrence of beta-cell dysfunction in type 1 diabetes, numerous functional alterations in blood flow can be observed [13, 14]. Early features of type 1 diabetes include endothelial dysfunction, increased leukocyte-endothelial adhesion [15], increased blood viscosity [16, 17], and changes in the hemodynamic properties of red blood cells [18, 19]. All these lead to impaired tissue perfusion, and the development of microvascular complications.

The role of vascular endothelium for the regulation of micro- and macrovascular blood flow has been extensively investigated in the last decade [20, 21]. In addition to serving as a physical barrier between the blood compartment and the underlying smooth muscle cells, the endothelial cell facilitates a complex array of signaling between the vessel wall and blood. There are several transmitters released from endothelial cells like NO, endothelin 1, prostaglandins, thrombin, substance P, bradykinin, serotonin, and others, which impact vascular tone and/or interact with blood cells [22, 23].

NO was identified as the primary vasodilator released from the endothelium [24]. NO is produced in the endothelium cell through the activation of endothelial NO synthase (eNOS). The subsequent release of NO stimulates guanylcyclase in the vascular smooth muscle cell. This in turn, leads to increased cyclic guanosine monophosphate (cGMP) levels in vascular smooth muscle cells, and subsequent vasodilatation [20, 25-27]. In advanced stages of diabetes, cGMP levels are decreased, suggesting that either NO release, or its action on guanylate cyclase, is markedly reduced [28]. On the other hand, NO evokes vasoprotective effects by reducing platelet-vascular wall interactions, reducing the adhesion of circulating mono-

cytes to the endothelial cell, and by the inhibition of vascular smooth muscle proliferation [29].

As shown in Figure 1, eNOS is regulated by a couple of different substrates, both of which are altered in diabetic patients. While insulin and C-peptide activate eNOS, hyperglycemia and oxidative stress are strong inhibitors of the NO-release from the endothelial cell. Early in the course of diabetes mellitus, NO secretion from the endothelial cell is decreased. This entails early endothelial dysfunction, and microcirculation failure to respond to changing perfusion demands in several tissues [12, 30, 31].

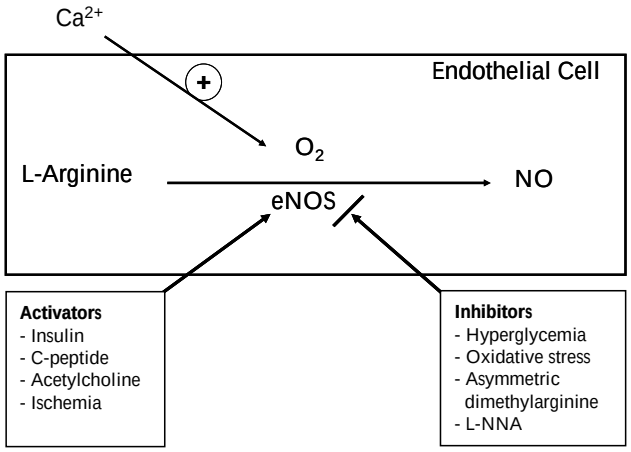

Figure 1. Ilustration of endothelial NO synthesis with activators and deactivators of endothelial nitric oxide synthase (eNOS). L-NNA: N^G-nitro-L-arginine.

flux of Ca^{2+} into endothelial cells. In accordance with these results, C-peptide has been shown to increase Ca^{2+} influx into renal tubular cells [35]. As shown in Figure 2, the release of NO from BAEC following C-peptide stimulation was completely abolished by inhibition of eNOS by L-NNA, or by binding Ca^{2+} in the cell culture medium using ethylenediaminetetraacetic acid (EDTA). These results strengthen the hypotheses that C-peptide is likely to stimulate eNOS activity by facilitating an influx of Ca^{2+} into BAEC.

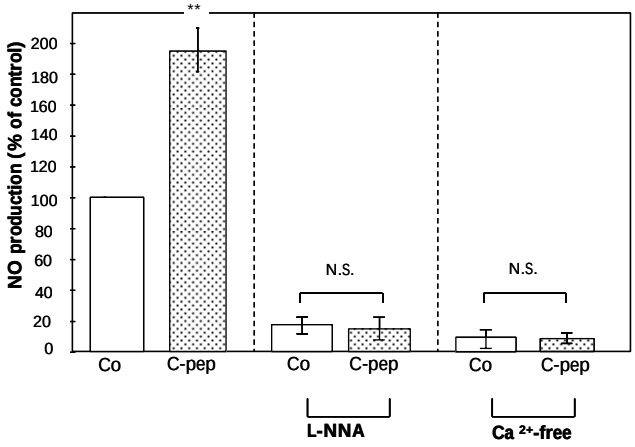

Figure 2. Effect of N^G-nitro-L-arginine (L-NNA) and Ca^{2+}-free Locke's solution on the NO release from non-stimulated and C-peptide-stimulated BAEC. Each column represents the mean ± SEM of 4 experiments. Co: Control. C-pep: C-peptide. NO: nitric oxide. ** $p < 0.01$ vs. control.

Effects of C-peptide on nitric oxide (NO)

C-peptide was shown to enhance the release of NO from bovine aortic endothelial cells (BAEC) [32, 33]. The concentration of C-peptide required for the stimulation of endothelial NO release was found to be in a physiological range of 1-6 nM. These findings are in accordance to the results of Jenssen and Messina, who demonstrated a vasodilatatory effect of C-peptide in isolated rat cremaster muscle arterioles that were sensitive to N(omega)-nitro-l-arginine (L-NNA) inhibition [34]. In contrast to our results, the response to C-peptide was only observed in the presence of insulin. C-peptide also increased the intracellular Ca^{2+} concentration in BAEC. It is likely that C-peptide stimulates NOS III activity by facilitating an in-

In a study by Kitamura *et al*., C-peptide was shown to stimulate NO production by enhancing the mitogen-activated protein-kinase dependent transcription of eNOS in aortic endothelial cells of wistar rats [4]. In their study, C-peptide increased NO release from aortic endothelial cells by enhancing eNOS expression through an extracellular signal-regulated kinase (ERK)-dependent transcriptional pathway.

Acetylcholine is a well characterized pharmacological activator of eNOS. In type 1 diabetic patients, short time intravenous infusion of human C-peptide in physiological concentrations was shown to augment the acetylcholine-induced increase in plasma cGMP levels. This was associated with a 33 % increase in acetylcholine-stimulated microvascular skin blood flow [5].

Effect of C-peptide on red blood cell elasticity

Blood flow in larger vessels is determined by the vessel diameter, blood viscosity, and vessel length. Whereas, blood flow in the nutritive capillary bed is predominantly determined by blood viscosity and the elastic properties of the cellular compartments of the blood, especially if the lumen of the vessel is below the diameter of the erythrocytes. Thus, reduced erythrocyte elasticity will limit microvascular blood flow, if the capillary diameter and blood pressure remain constant [36]. In patients with diabetes mellitus, several factors such as decreased erythrocyte deformability, increased erythrocyte aggregation, and increased erythrocyte membrane viscosity contribute to erythrocyte dysfunction, and might affect microvascular blood flow and tissue nutrition [16, 18, 19, 37-39]. Impaired erythrocyte function in patients with diabetes mellitus was attributed to the modification of proteins and lipids by advanced glycation endproducts, to the generation of free oxygen radicals, and to changes in ion homeostasis attributed to hyperglycemia [40].

The effect of C-peptide on erythrocytes obtained from diabetic and non-diabetic subjects was investigated under physiological (0.3 to 10 Pa) and supraphysiological (>10 Pa) shear stress rates by means of laser diffractoscopy [33]. Glucose levels were matched in both subject groups to exclude glycemia-related effects on erythrocyte deformability. In type 1 diabetic patients, the erythrocyte deformability was significantly reduced, when compared to the erythrocytes obtained from the non-diabetic controls. Incubation of the erythrocytes with different concentrations of C-peptide restored erythrocyte deformability in type 1 diabetic patients, but had no effect on erythrocytes obtained from non-diabetic controls.

Table 1. Amino acid structure of C-peptide and C-peptide fragments used in the study on erythrocyte deformability

Structure	Amino acid structure	Position
C-peptide	EAEDLQV-GQVELGGGPGAGSLQPLALEGSLQ	(1-31)
MF	ELGGGPGAG	(11-19)
HP	LEGSLQ	(26-31)
PP	EGSLQ	(27-31)

Legend: MF: middle fragment. HP: C-terminal hexapeptide. PP: C-terminal pentapeptide.

The entire C-peptide molecule, and also the middle and C-terminal fragments of the peptide, were all shown to exert physiological effects in several tissues and cell types [41, 42]. Therefore, the physiological effects of C-peptide, and several C-terminal fragments, were studied in a complementary investigation on erythrocyte deformability. The fragments used in this study are presented in Table 1.

Again, erythrocyte deformability was significantly decreased in type 1 diabetic patients, compared with healthy controls at physiological and supraphysiological shear stress levels. In the physiological shear stress range of 0.3-10 Pa, the difference between diabetic patients and healthy controls were found in a range of 18-25%. Incubation of the red blood cells from type 1 diabetes patients with C-peptide completely restored the erythrocyte deformability at all shear stress ranges [43]. Incubation with the C-terminal penta- and hexapeptide, also improved erythrocyte deformability, which was not significantly different from that obtained with the entire peptide. In contrast, the middle fragment and the scrambled C-peptide failed to exert any effect on erythrocyte deformability.

Additional efforts were made to elucidate the signal transduction pathway of C-peptide and its fragments in the erythrocytes. To investigate the role of pertussis toxin sensitive G-proteins in the intracellular transmission of the C-peptide signal, erythrocytes were preincubated with ouabain, EDTA, and pertussis toxin. Ouabain inhibited Na^+/K^+-ATPase, and EDTA eliminated extracellular Ca^{2+}. Preincubation with oubain or EDTA, completly abrogated the effect of C-peptide, or its fragments, on erythrocyte deformability. In this experimental approach, exposure of the erythrocytes to pertussis toxin resulted in alteration of red cell deformability, which interfered with the interpretation of the C-peptide effects on a G-protein coupled receptor. Nevertheless, these data indicate a Ca^{2+}-dependent stimulation of erythrocyte Na^+/K^+-ATPase. This is analogous to the previously established signal transduction pathway for C-peptide in renal tubular cells [44].

In a recent investigation, C-peptide was shown to increase the release of ATP from erythrocytes due to an activation of GLUT1 [45]. ATP is a known stimulus of NO production in the endothelium. The ability of C-peptide to release increased levels of ATP from erythrocytes might be an important mechanism in facilitating the path of erythrocytes to the nutritive capillaries. In this

investigation, the ATP releasing effects of C-peptide were only obtained when C-peptide was complex with metal ions such as Fe^{2+} or Cr^{3+}.

Figure 3 presents the effect of the different C-peptide fragments on erythrocyte deformability, and the inhibition with oubain or EDTA at a shear stress rate of 1.2 Pa. In a type 2 diabetes rat model, Zn^{2+}-activated C-peptide was shown to exert less ATP release from erythrocytes, as compared with controls [46]. Pretreatment of the type 2 diabetic erythrocytes with metformin restored the C-peptide-stimulated release of ATP from the erythrocyte.

Therefore, it needs to be considered that the inhibitory effect of EDTA on numerous biological effects of C-peptide might be explained by deactivation of the C-peptide molecule, due to the lack of metal ions necessary to facilitate its biological activity.

Effects of C-peptide on erythrocyte Na^+/K^+-ATPase

It has been shown that hyperglycemia inhibits Na^+/K^+-ATPase activity by an endothelium dependent mechanism [47]. Na^+/K^+-ATPase activity is involved in vascular regulation based on a complex interaction between Na^+K^+-pump-activity and an endothelium dependent increase of NO [48, 49]. NO and cyclic-GMP have been shown to increase vascular Na^+/K^+-ATPase activity, with subsequent vasorelaxation [50, 51].

In a recent study, erythrocyte Na^+/K^+-ATPase activity was found to be reduced in type 1 diabetic patients. Whilst in type 2 diabetic patients a wide range of individual Na^+/K^+-ATPase activity was observed, including some patients with very low Na^+/K^+-ATPase activity, and others with normal Na^+/K^+-ATPase activity [52]. It appeared that erythrocyte Na^+/K^+-ATPase activity was significantly lower in type 2 diabetic patients treated with insulin compared with those on oral treatment. In insulin-treated type 2 diabetic patients, Na^+/K^+-ATPase activity was comparable to those in type 1 diabetic patients.

In an *in vitro* study by Djemli-Shiplolye *et al.*, incubation of erythrocytes from type 1 diabetic patients with C-peptide normalized erythrocyte Na^+/K^+-ATPase activity [53]. Also, intravenous infusion of C-peptide in type 1 diabetic patients was found to improve erythrocyte Na^+/K^+-ATPase activity [5].

Na^+/K^+-ATPase controls many essential cellular functions, e.g. cell volume, free calcium concentrations, and membrane potential [54]. Although there are tissue-specific differences in the regulations of Na^+/K^+-ATPase activity, hyperglycemia and diabetes are predominantly characterized by a decrease in ouabain-sensitive Na^+/K^+-ATPase activity.

Effects of C-peptide on microvascular blood flow

In a study by Lindstrom *et al.*, C-peptide supplementation was shown to increase microvascular blood flow in isolated kidneys of the rat due to increased recruitment of capillaries [55]. In another study, C-peptide evoked arterial dilatation in skeletal muscle arterioles isolated from rat cremaster muscles, which was further reinforced by the addition of low (but not by high) insulin concentrations [34]. Addition of the NOS inhibitor N(G)-monomethyl-l-arginine (L-NMA) completely

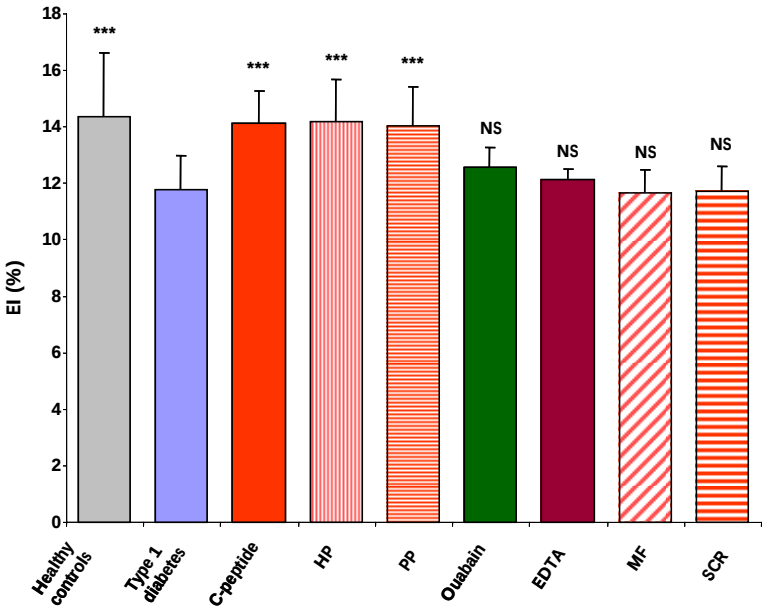

Figure 3. Erythrocyte deformability at a representative physiological shear stress level of 1.2 Pa. EI (%) ± SD. HP: C-terminal hexapeptide. PP: C-terminal pentapeptide. MF: middle fragment of C-peptide. SCR: scrambled peptide. *** $p < 0.001$ vs. erythrocytes of type 1 diabetic patients.

abolished the vasodilating activity of both peptides. This confirmed the NO-dependent signaling of this pathway.

In diabetic rats, biosynthetic human C-peptide given twice daily for 5 weeks, normalized blood flow in the anterior uvea, retina, and sciatic nerve [42]. In addition, C-peptide reduced ^{125}I-labeled albumin permeation in retinal and nerve tissue. These microvascular effects in neural blood flow were accompanied by an increase in caudal motor nerve conduction velocity. In healthy control rats, no effect of C-peptide could be observed either on microvascular blood flow, or on motor nerve conduction velocity. In accordance with these findings, Cotter *et al*. observed an increase in sciatic endoneurial blood flow in streptozotocin diabetic rats, following supplementation with physiological C-peptide concentrations [56]. In their study, the improvement in endoneurial microvascular blood flow was followed by an improvement of nerve fiber function. Both effects of C-peptide were blunted after blocking eNOS using L-NMA.

Johansson *et al*. investigated the effect of C-peptides on skeletal muscle blood flow in type 1 diabetic patients and in healthy controls during exercise [57]. C-peptide increased forearm blood flow and capillary diffusion capacity to levels similar to those observed in healthy controls. In accordance with the increase in muscle blood flow, forearm oxygen and glucose uptake increased after C-peptide administration.

Skin blood flow is affected early after the diagnosis of diabetes mellitus. While in early stages of diabetic microvascular dysfunction, total capillary blood flow might be increased, nutritive capillary blood flow is significantly reduced in type 1 diabetic patients compared to non-diabetic subjects [58-60]. Short term infusion of C-peptide in type 1 diabetic patients improved microvascular skin blood flow by a redistribution of microvascular blood flow from the subpapillary thermoregulatory blood flow into the nutritive capillary bed [6]. Thirty minutes after termination of the C-peptide supplementation, capillary skin blood flow returned to baseline levels, as observed before the start of C-peptide supplementation. No effect of C-peptide on microvascular skin blood flow could be observed in non-diabetic subjects. In a study by Delaney *et al*., transdermal iontophoresis of C-peptide resulted in a dose-related increase in microvascular skin blood flow, which was comparable to that observed after the iontophoresis of insulin [3].

Fernqvist-Forbes *et al*. studied the effect of C-peptide on flow mediated vasodilatation (FMD) in type 1 diabetic patients [61]. When compared with healthy controls, type 1 diabetic patients revealed a lower FMD, which was increased by approximately 35% following supplementation with human C-peptide. In contrast, Polska *et al*. found no effect of C-peptide supplementation on retinal blood flow in type 1 diabetic patients [62]. Therefore, it seems conceivable that C-peptide affects microvascular blood flow in a specific manner according to tissue type.

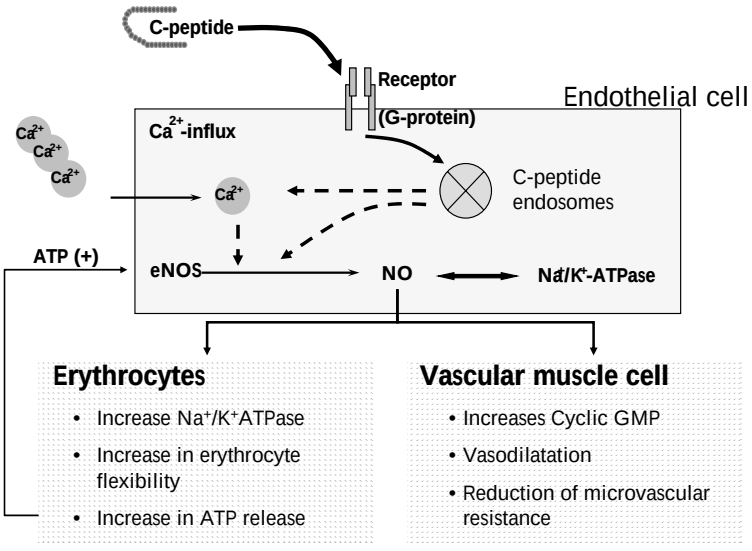

Figure 4. Illustration of the molecular effects of C-peptide on endothelial cells, vascular smooth muscle cells, and erythrocytes.

Conclusions

C-peptide affects microvascular blood flow by interfering with several signaling pathways. Recent data suggest that C-peptide binds to a G-protein coupled receptor, increasing Ca^{2+} influx, with subsequent activation of Ca^{2+}-calmodulin. In human aortic endothelial cells, and in umbilical artery smooth muscle cells, C-peptide was shown to be internalized in the cell by endocytosis [63]. Binding of C-peptide to a specific membrane

receptor with consecutive endocytosis might represent a conceivable signaling pathway to achieve the stimulation of eNOS or Na^+/K^+-ATPase in erythrocytes and endothelial cells. Increasing activity of eNOS, and the release of NO from the endothelial cell, interacts with vascular smooth muscle cells and with cellular components in the blood.

In the vessel wall, NO increases cGMP levels, and leads to vasodilatation. Many studies suggest a link between endothelial NO release and erythrocyte deformability [5, 64, 65]. At physiological concentrations (~10^{-7}M), NO was found to increase erythrocyte deformability, and to improve the hemorheological properties, especially in the nutritive capillary bed [65]. Thereby, C-peptide promotes several potent mechanisms in the regulation of nutritive microvascular blood flow. The failure of C-peptide secretion might have important consequences for the development of microvascular complications in patients with type 1 diabetes mellitus.

Figure 4 summarizes recent understanding of the molecular effects of C-peptide in microvascular blood flow regulation.

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