Effects of proinsulin C-peptide on nitric oxide, microvascular blood flow and erythrocyte Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in diabetes mellitus type I


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ABSTRACT

This study was conducted to evaluate the influence of proinsulin C-peptide on erythrocyte Na\textsuperscript{+},K\textsuperscript{+}-ATPase and endothelial nitric oxide synthase activities in patients with type I diabetes. In a randomized double-blind study design, ten patients with type I diabetes received intravenous infusions of either human C-peptide or physiological saline on two different occasions. C-peptide was infused at a rate of 3 pmol \(\text{min}^{-1} \text{kg}^{-1}\) for 60 min, and thereafter at 10 pmol \(\text{min}^{-1} \text{kg}^{-1}\) for 60 min. At baseline and after 60 and 120 min, laser Doppler flow (LDF) was measured following acetylcholine iontophoresis or mild thermal stimulation (44 °C), and venous blood samples were collected to determine plasma cGMP levels and erythrocyte membrane Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity.

The LDF response to acetylcholine increased during C-peptide infusion and decreased during saline infusion (18.6 ± 19.2 and 13.2 ± 9.4 arbitrary units respectively; mean ± S.E.M.; \(P < 0.05\)). No significant change in LDF was observed after thermal stimulation. The baseline plasma concentration of cGMP was 5.5 ± 0.6 nmol \(\text{l}^{-1}\); this rose to 6.8 ± 0.9 nmol \(\text{l}^{-1}\) during C-peptide infusion (\(P < 0.05\)). Erythrocyte Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity increased from 140 ± 29 nmol of Pi \(\text{h}^{-1} \text{mg}^{-1}\) in the basal state to 287 ± 5 nmol of Pi \(\text{h}^{-1} \text{mg}^{-1}\) during C-peptide infusion (\(P < 0.01\)). There was a significant linear relationship between plasma C-peptide levels and erythrocyte Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity during the C-peptide infusion (\(r = 0.46, P < 0.01\)). No significant changes in plasma cGMP levels or Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity were observed during saline infusion. This study demonstrates an effect of human proinsulin C-peptide on microvascular function, which might be mediated by an increase in NO production and an activation of the erythrocyte Na\textsuperscript{+},K\textsuperscript{+}-ATPase. These mechanisms are compatible with the previous observed microvascular effects of C-peptide in patients with type I diabetes.

INTRODUCTION

Following the discovery of proinsulin and C-peptide, it has generally been accepted that the main physiological role of C-peptide is to facilitate the folding of the proinsulin molecule in a manner that allows the formation of the disulphide bonds between the cysteine residues of the A- and B-chains of the insulin molecule [1]. However, more recent studies indicate that administration of C-peptide to patients with type I diabetes improves renal and nerve function, and stimulates whole-body glucose uptake [2–5]. Meanwhile, several studies

Key words: cGMP, C-peptide, diabetes mellitus type I, endothelial NO synthase, microvascular blood flow, Na\textsuperscript{+},K\textsuperscript{+}-ATPase.

Abbreviations: a.u., arbitrary units; LDF, laser Doppler flow.

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have now been published focusing on the effects of C-peptide on the microcirculation in diabetes mellitus. In an experimental diabetes model in rats, it has been demonstrated that microvascular dysfunction is reversible following administration of C-peptide [6]. In patients with type I diabetes, increases in muscle blood flow, oxygen uptake, capillary diffusion capacity and nutritive microvascular skin blood flow were observed following the administration of C-peptide [3,5]. In addition, it has been shown that C-peptide increases the resting forearm blood flow and augments the vasoconstrictor effects of neuropeptide Y [7]. A consistent finding in the above studies is that no effect of C-peptide was found in healthy subjects or animals. These findings suggest a saturation of the mechanisms of C-peptide action in healthy subjects.

The cellular mechanisms that form the basis of the vascular effects of C-peptide are not fully understood. In an in vitro study we demonstrated that C-peptide increases calcium influx into endothelial cells, which activates the endothelial nitric oxide synthase and the release of nitric oxide [8]. One of the proposed mechanisms underlying NO-induced vasorelaxation is the stimulation of vascular Na⁺,K⁺-ATPase activity [9].

It is noteworthy that, under diabetic conditions, Na⁺,K⁺-ATPase activity is decreased in different cell types and might be involved in the pathogenesis of various diabetic complications [10–13]. In a recent study conducted in patients with type II diabetes mellitus, a positive linear relationship was observed between residual plasma C-peptide levels and erythrocyte Na⁺,K⁺-ATPase activity [14]. Administration of C-peptide to renal tubular cells has been shown to increase Na⁺,K⁺-ATPase activity, an effect which was enhanced in the presence of neuropeptide Y [15]. A decrease in Na⁺,K⁺-ATPase activity is known to be associated with a decrease in the deformability of erythrocytes, which results in an increase in blood viscosity [16]. Furthermore, we have recently shown with the use of laser diffractoscopy that C-peptide under in vitro conditions is able to restore the deformability of erythrocytes from patients with type I diabetes [17].

These considerations prompted us to conduct an in vivo study with patients with type I diabetes, with the aim of investigating the effects of C-peptide on microvascular and haemorheological parameters, such as acetylcholine-induced microvascular blood flow, the microvascular blood flow response to thermal stimulation, plasma cGMP levels and erythrocyte Na⁺,K⁺-ATPase activity.

**METHODS**

**Subjects**

Microvascular and haemorheological blood flow parameters were measured in a placebo-controlled, double-blind, crossover investigation in 10 patients with type I diabetes (five male, five female; six non-smokers, four smokers). The mean age (+ S.E.M.) was 34.6 ± 1.9 years (range 26–44 years), and the mean duration of diabetes was 11.2 ± 2.4 years (range 3–22 years). The mean HbA1c (glycated haemoglobin) level was 7.2 ± 0.5 % (range 5.7–10.3 %; normal range 4.5–6.5 %). All patients were clinically free from peripheral macrovascular disease, as evaluated by palpable pedal pulses and segmental blood pressure measurements. Subjects were not included if they were on vasoactive drugs or on medication known to influence microvascular blood flow. None of the patients exhibited a blood glucose level below 4 mmol·l⁻¹ at the study onset, or suffered from hypoglycaemia before or during the investigation. All subjects refrained from smoking for 2 h prior to the study. The study was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association. All subjects gave their informed consent to the study protocol, which had been approved by the local Ethics Committee.

**Assessment of microvascular skin blood flow**

The technique of laser Doppler flowmetry (MBF 3D; Moor Instruments, Axminster, Devon, U.K.) was used to measure total skin blood flow at the dorsum of the foot. Laser Doppler flow (LDF) measurements were performed in a room with an ambient temperature of 21–24 °C. LDF readings are represented in arbitrary units (a.u.). The subjects were allowed to lie down and to acclimatize for a period of about 20 min before the start of measurements.

A battery-powered iontophoresis controller (MIC 1; Moor Instruments) with an indirect ion chamber (ION 3; Moor Instruments) was used to investigate the LDF response to acetylcholine on the dorsum of the right foot [18–20], without changing the position of the iontophoresis chamber during the entire investigation. A solution of 1 % (w/v) acetylcholine (Miochol E; Ciba Vision, Wessling, Germany) was delivered using an anodal current of 200 μA for a period of 60 s. LDF was measured immediately before the start of iontophoresis, and the response to acetylcholine was recorded for a period of 5 min. The total increase in LDF was calculated for the comparison between the different groups.

In addition, the microvascular response to a mild thermal stimulus was studied using a thermostatically controlled LDF probe. The skin probe was attached to the dorsum of the right foot using an adhesive ring, and the position of the probe remained unchanged throughout the whole investigation. After recording the LDF at a probe temperature of 37 °C for a period of 5 min, the probe was heated to 44 °C by a heater incorporated into the skin electrode, and the LDF was again recorded for 5 min [21,22]. The total increase in LDF was calculated for the comparison between the different groups.
Other clinical investigations

The skin temperature of the dorsum of the foot was measured with an electronic thermometer (Digimed H11S; Medizin-Messtechnik, Waldkirch, Germany). Systemic blood pressure and heart rate were measured in the right arm.

Assays

Measurement of NO in vivo is difficult because of its very short half-life. cGMP is the second messenger of NO, and much more stable. Therefore we measured the plasma levels of cGMP as a parameter of NO activity using a radioimmunoassay (Immuno Biological Laboratories, Hamburg, Germany) [23,24]. Venous blood samples for the determination of cGMP were collected in EDTA tubes and immediately placed on ice. After separation, EDTA-plasma was stored at −20°C until subsequent analysis.

Venous blood samples were collected on sodium citrate for the examination of erythrocyte Na⁺,K⁺-ATPase activity. Erythrocytes were separated immediately by centrifugation at 350 g for 15 min at 4°C. After carefully removing the supernatant, the erythrocytes were diluted with a Tris buffer (0.011 M Tris, pH 7.4) and stored at 4°C until further analysis. Na⁺,K⁺-ATPase activity was assayed in isolated erythrocyte membranes and expressed as the difference between PNa⁺,K⁺-ATPase activity, as described elsewhere [10,14]. For technical reasons erythrocyte Na⁺,K⁺-ATPase activity was not measured in two patients. Therefore data for Na⁺,K⁺-ATPase were obtained in only eight patients with type I diabetes. In addition, venous blood samples were taken for the assessment of C-peptide, insulin and blood glucose levels. Blood glucose was measured with a Yellow Springs Glucose Analyser. C-peptide, insulin and cGMP levels, LDF and erythrocyte Na⁺,K⁺-ATPase activity were measured at baseline. No significant difference in the LDF response induced by iontophoresis of acetylcholine increased by about 10 pmol min⁻¹ kg⁻¹ for another 60 min. Another catheter was inserted into an antecubital vein in the left arm for blood sampling.

Baseline measurements of LDF, systemic blood pressure, heart rate, blood sugar, insulin, cGMP, erythrocyte Na⁺,K⁺-ATPase activity and plasma C-peptide levels were obtained in the resting period of 20 min. After starting C-peptide/saline infusion, these parameters were again measured after 60 and 120 min.

Statistical analysis

Values are given as means ± S.E.M. Statistical analysis was performed using the Wilcoxon signed-rank test, and the Mann–Whitney U test was used to test differences between the groups. A value of P < 0.05 was considered statistically significant. Linear regression analysis was performed to compare plasma C-peptide levels, cGMP levels, LDF and erythrocyte Na⁺,K⁺-ATPase activity

RESULTS

C-peptide levels

During the C-peptide infusion study, plasma C-peptide levels increased continuously, reaching a maximum of 3.5 ± 0.3 nmol l⁻¹ after 120 min (P < 0.005). In contrast, plasma C-peptide levels remained below 0.05 nmol l⁻¹ during saline infusion (Figure 1).

Microvascular blood flow measurements (LDF)

Following C-peptide infusion, the LDF response induced by iontophoresis of acetylcholine increased by about 27.3 ± 22.9 a.u. during the infusion of 3 pmol min⁻¹ kg⁻¹ and by about 18.6 ± 19.2 a.u. during the infusion of 10 pmol min⁻¹ kg⁻¹. This was in contrast with findings observed on saline infusion, during which the LDF response to acetylcholine decreased by about 8.1 ± 15.1 a.u. during the first 60 min and by about 13.2 ± 9.4 a.u. during the second 60 min. A significant difference in the LDF responses to acetylcholine was found between C-peptide infusion and saline infusion at 120 min after baseline. No significant difference in the LDF response to acetylcholine was observed when comparing infusions of different C-peptide concentrations. In our series, no direct association was demonstrable with the use of linear regression analysis between cGMP levels and the LDF response, or between erythrocyte Na⁺,K⁺-ATPase activity and the LDF response.

No significant change in the LDF response to thermal stimulation was observed during infusion of C-peptide or saline.

cGMP levels

Plasma levels of cGMP increased gradually during C-peptide infusion, by 0.42 ± 0.13 nmol l⁻¹ (not significant)
Figure 1 C-peptide plasma levels during infusion of C-peptide or saline

- ■, C-peptide infusion; □, NaCl infusion. Results are means ± S.E.M.; *P < 0.005 compared with baseline.

Figure 2 Percentage increases in the serum cGMP concentration after 60 and 120 min of C-peptide or NaCl infusion

The basal cGMP concentration is set at 100%. Results are means ± S.E.M. n.s., not significant.

Erythrocyte Na⁺,K⁺-ATPase activity

Levels of erythrocyte Na⁺,K⁺-ATPase activity increased during C-peptide infusion, by 83.3 ± 34.1 nmol of P_i h⁻¹ mg⁻¹ protein (not significant) during the low infusion rate (3 pmol·min⁻¹·kg⁻¹) and by 146.4 ± 26.9 nmol of P_i h⁻¹ mg⁻¹ protein (P < 0.01) during the high infusion rate (10 pmol·min⁻¹·kg⁻¹). However, there was also a slight (although statistically insignificant) increase in erythrocyte Na⁺,K⁺-ATPase activity during saline infusion: by 43.3 ± 56.9 nmol of P_i h⁻¹ mg⁻¹ protein during the first 60 min and by 90.9 ± 48.9 nmol of P_i h⁻¹ mg⁻¹ protein during the second 60 min. The percentage increases in Na⁺,K⁺-ATPase activity during both investigation arms are shown in Figure 3. No significant change in erythrocyte Na⁺,K⁺-ATPase activity was found between the two different concentrations of C-peptide infused. Linear regression analysis revealed a direct association between plasma C-peptide levels and erythrocyte Na⁺,K⁺-ATPase activity (r = 0.46; P < 0.01; Figure 4).

Insulin, blood sugar, blood pressure and heart rate

Blood pressure, heart rate, blood glucose levels, plasma insulin levels and skin temperature measurements at the
dorsum of the foot are given in Table 1. Throughout the investigation, plasma insulin levels, heart rate and skin temperature at the dorsum of the foot declined significantly. Nevertheless, with respect to these parameters, there was no significant difference between the two investigation arms (C-peptide and saline) throughout the study.

**DISCUSSION**

Although the prognosis for diabetes patients has improved greatly since the advent of insulin treatment, the complications of diabetes mellitus are still a major threat to both quality of life and life expectancy. A variety of microvascular disturbances may occur soon after the diagnosis of diabetes mellitus which are involved in the pathogenesis of different long-term diabetic complications, such as retinopathy [26,27], nephropathy [26], heart disease [28,29], neuropathy [30] and the development of diabetic foot ulceration [31,32]. The aetiology of diabetic microvascular dysfunction is not fully understood, but several mechanisms have been proposed which appear to act synergistically as pathogenetic factors in the setting of microvascular blood flow regulation. In addition to functional abnormalities of the microvasculature, such as disturbed neurovascular responses [33,34], alterations in endothelial function [35] and an increased intracapillary pressure [36], several haemorheological disturbances have been described for diabetes mellitus. An increase in leucocyte–endothelial interactions [37], increased blood viscosity [38,39] and changes in the rheological properties of erythrocytes [16,40] may contribute to the observed microvascular alterations in blood flow. These early microvascular and haemorheological abnormalities may precede structural vascular changes, which occur later in the course of the microvascular disease [41].

Although these microvascular and haemorheological disturbances may be ameliorated by treatment with insulin, many well-controlled diabetic subjects develop vascular complications during the onset of their illness [42]. Residual β-cell function in patients with type I diabetes has been suggested to be associated with a slower progression of microvascular complications [43–45]. There is increasing evidence that, in addition to better control of blood sugar, maintenance of C-peptide secretion may contribute to the beneficial effects of residual β-cell function on microvascular complications. C-peptide has been shown to increase forearm muscle blood flow [7], to enhance oxygen uptake and capillary diffusion capacity in the exercising forearm [3] and to redistribute microvascular skin blood flow [46] in C-peptide-negative patients with type I diabetes, while no effect was found in healthy subjects. In addition, microvascular complications such as diabetic nephropathy and diabetic neuropathy have been shown to improve during treatment with C-peptide [2,4].

One of the most interesting questions arising from the observed effects of C-peptide in patients with type I diabetes regards the underlying molecular mechanisms. In a recent in vitro study we showed that C-peptide activates endothelial NO secretion in a calcium-dependent fashion, and that this effect is completely abolished in the presence of l-nitro-arginine, a specific inhibitor of endothelial NO synthase [8,47]. In the previous investigation, C-peptide infusion into patients with type I diabetes resulted in plasma C-peptide levels which were within the physiological range of those in non-diabetic subjects [22,48].

Laser Doppler flowmetry is a technique that has increased our understanding of skin microvascular blood flow disturbances associated with diabetes mellitus [49,50]. Several stimulation techniques have been developed to investigate skin microvascular blood flow with this method. While the microvascular blood flow response to a heat stimulus gives an estimate of peripheral neurovascular control [21,51], the microvascular response to acetylcholine is affected by different
mechanisms [52]: first by direct stimulation of the vascular endothelium and the release of vasodilatory substances such as NO [18,53,54], and secondly by activation of an axon reflex arc and initiation of neurogenic inflammation [19,55,56]. In our present investigation, C-peptide has been shown to increase the microvascular response to iontophoresis of acetylcholine without affecting the response to thermal stimulation. This finding underscores the effect of human C-peptide on endothelium-mediated microvascular blood flow regulation and release of NO.

Because it is very difficult to measure NO directly in human studies in vivo, we measured the second messenger cGMP for the determination of endothelial NO synthase activity. Our study exhibited a gradual increase in plasma cGMP levels during C-peptide infusion, while no significant change in plasma cGMP levels was observed during saline infusion. Hence these in vivo experiments corroborate the hypothesis of a NO-mediated mechanism underlying the observed effects of C-peptide in patients with type I diabetes. In our limited sample size, no direct relationship was observed by linear regression analysis between plasma cGMP levels and the microvascular response to acetylcholine.

Na⁺,K⁺-ATPase activity has been found to be attenuated in various cell types under diabetic conditions [12,13,57]. Na⁺,K⁺-ATPase activity is involved in vascular modulation, based on a complex interaction between Na⁺/K⁺-pump activity and the endothelium-dependent increase in cGMP levels [58]. On the other hand, NO and cGMP have been shown to increase vascular Na⁺,K⁺-ATPase activity, with subsequent vasorelaxation [9,59]. Impaired Na⁺,K⁺-ATPase activity may contribute to the observed decrease in erythrocyte deformability by increasing the intracellular sodium concentration, leading to an increase in blood viscosity [60,61]. Thus one may speculate that the diabetes-induced decrease in Na⁺,K⁺-ATPase activity may compromise microvascular blood flow via two mechanisms: first by affecting microvascular regulation, and secondly by increasing blood viscosity. Animal studies have shown that C-peptide treatment of diabetic rats leads to an increase in Na⁺,K⁺-ATPase activity in rat renal tubule segments [15,62]. In a recent study, an association was found between plasma C-peptide levels and erythrocyte Na⁺,K⁺-ATPase activity in patients with type II diabetes [14]. Furthermore, the ability of C-peptide to restore erythrocyte deformability to levels similar to those observed in normal subjects has been demonstrated by laser diffractoscopy. This effect of human C-peptide on erythrocyte deformability was completely abolished by pretreatment with ouabain, which confirms the link between erythrocyte Na⁺,K⁺-ATPase activity and erythrocyte deformability [17]. The results of the present study confirm the increase in erythrocyte Na⁺,K⁺-ATPase activity following restoration of human C-peptide in patients with type I diabetes. In addition, a direct relationship was found between plasma C-peptide levels and erythrocyte Na⁺,K⁺-ATPase activity during C-peptide infusion.

During our investigation, significant decreases in plasma insulin levels, heart rate and skin temperature were found in both study arms (C-peptide and saline infusion), which could be interpreted in part to be a consequence of diminished insulin action since the last injection, a gradual decrease in anxiety during the investigation, and cooling of the skin during the procedure. However, these changes seem to have no influence on the observed effects of C-peptide on microvascular blood flow and erythrocyte Na⁺,K⁺-ATPase activity, since there was no significant difference in the course of the changes between the two investigation arms.

In conclusion, the present study corroborates the hypothesis that C-peptide exerts effects on microvascular and haemorheological function in humans. The present investigation is the first in vivo study to confirm an effect of human C-peptide on endothelial NO metabolism and erythrocyte Na⁺,K⁺-ATPase activity, which may indeed mediate the beneficial effects of human C-peptide on microvascular complications. Additional studies will be required to further elucidate the possibility that C-peptide administration may be of benefit in the treatment and prevention of microvascular diabetic complications in C-peptide-negative diabetic patients.

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