Low O2-induced ATP release from erythrocytes of humans with type 2 diabetes is restored by physiological ratios of C-peptide and insulin

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ERYTHROCYTES RELEASE OXYGEN (O2), as well as the potent vasodilator ATP, when exposed to low O2 tension, enabling these circulating cells to respond to the efficient matching of O2 supply to metabolic need in skeletal muscle (5, 11, 13, 14, 30, 33, 43). This release of ATP stimulates local vasodilation, which is conducted upstream to feed arterioles, thereby directing perfusion to localized tissue areas lacking adequate oxygenation (11, 13, 43). However, erythrocytes from humans with type 2 diabetes exhibit an impairment in the ability to release ATP in response to reduced tissue O2 tension, which limits their participation in this important mechanism for the control of perfusion distribution (38, 42, 44).

Type 2 diabetes is associated with an increased incidence of vascular complications, including peripheral vascular disease and impaired wound healing (2, 15, 36). Impairment of low O2-induced ATP release from erythrocytes could contribute to these complications. In support of this hypothesis, it was reported that isolated skeletal muscle arterioles perfused with erythrocytes from healthy humans—cells that respond to low O2 with ATP release—dilate when exposed to reduced, extraluminal O2, a model of increased tissue O2 need (11, 43). In contrast, low O2-induced ATP release from erythrocytes from humans with type 2 diabetes is severely impaired (38, 42, 44), and these cells do not stimulate dilation of isolated, perfused skeletal muscle arterioles under similar conditions (42).

Whereas there is clearly an inherent defect in low O2-induced ATP release from erythrocytes of humans with type 2 diabetes, this can be exacerbated further by the treatment of the disease. Specifically, incubation of healthy human erythrocytes with insulin alone, at concentrations reported to be necessary to regulate blood glucose levels in individuals with insulin resistance, inhibits low O2-induced ATP release (19).

Although insulin alone is administered to humans with type 2 diabetes, in vivo, insulin is released from the pancreas, along with the connecting peptide (C-peptide) (46). The two peptides are released at equimolar concentrations, but due to differing clearance rates, circulating C-peptide levels exceed those of insulin (46). Although initially thought to be inert, recent studies have shown that C-peptide has biological activity (21, 22) and has been suggested to attenuate and perhaps reverse some diabetes-associated microvascular complications (22).

C-Peptide may also have important effects on erythrocytes. It has been shown in humans with type 1 diabetes that C-peptide improves erythrocyte deformability (18, 27). In addition, when coincubated with insulin at physiological ratios and concentrations, C-peptide has been shown to oppose the adverse effects of insulin on low O2-induced ATP release from healthy human erythrocytes (34). Although the signaling pathway(s) initiated by C-peptide are not well characterized, there is strong evidence that C-peptide stimulates a pertussis toxin-sensitive G-protein-coupled receptor (GPCR) (21). Recently, GPR146, an orphan GPCR, was suggested to be a C-peptide receptor on KATOIII cells, based on the loss of C-peptide-sensitive ATP release (19).

METHODS

Isolation of human erythrocytes. Blood was obtained from healthy volunteers (n = 23) or individuals with type 2 diabetes (n = 25) via venipuncture, using a syringe containing heparin (500 U). Blood was centrifuged at 500 g at 4°C for 10 min, and the plasma,uffy coat, and
uppermost layer of erythrocytes were removed by aspiration. The remaining erythrocytes were resuspended and washed three times in a physiological buffer containing (in mM) 21.0 Tris, 4.7 KCl, 2.0 CaCl₂, 140.5 NaCl, 1.2 MgSO₄, 5.5 glucose, and 0.5% BSA with the pH adjusted to 7.4. After the final wash, the hematocrit of the isolated erythrocytes was measured. Erythrocytes were prepared on the day of use. Informed consent was obtained from all subjects, and the protocol for blood removal was approved by the Institutional Review Board of Saint Louis University.

**Measurement of ATP.** ATP was measured using the luciferin-luciferase assay. A 200-μl sample of the erythrocyte suspension (0.04% hematocrit) was injected into a cuvette containing 100 μl firefly lantern extract (10 mg/ml distilled water, FLE-250; Sigma, St. Louis, MO) and 100 μl D-luciferin solution (10 mg/ml distilled water; Research Products International, Mount Prospect, IL). The light emitted from the reaction with ATP was quantified using a luminometer (TD-20-20; Turner Designs, Sunnyvale, CA). A standard curve was generated for each experiment. ATP values were normalized to the amount released from 4 × 10⁶ cells.

**Measurement of free hemoglobin.** To exclude the possibility that amounts of ATP measured were the result of erythrocyte lysis, samples used for measuring ATP were centrifuged at 500 g for 10 min at room temperature. The presence of hemoglobin in the supernatant was measured by measurement of light absorbance at 405 nm (23). Samples in which free hemoglobin increased were not included.

**Purification of erythrocyte membranes.** Washed erythrocytes were diluted 1:100 with ice-cold hypotonic buffer containing 5 mM Tris-HCl, 2 mM EDTA, pH 7.4, and stirred vigorously at 4°C for 20 min. The lysate was centrifuged at 23,000 g for 15 min at 4°C. The supernatant was removed and discarded. The pellet was washed with ice-cold buffer and centrifuged, and the procedure was repeated a second time. The membranes were frozen at −80°C. Membrane protein concentrations were determined using the BCA Protein Assay (Pierce, Thermo Fisher Scientific, Rockford, IL).

**Western analysis.** Erythrocyte membranes were suspended in buffer containing 150 mM NaCl, 50 mM Tris, pH 8.0, 1% Nonidet P-40, followed by centrifugation to remove insoluble debris. The resulting supernatant was loaded on a 4–20% gradient Tris-glycine precast gel (Bio-Rad Laboratories, Hercules, CA) at the same protein concentration. Electrophoresis at 100 V was carried out for 45 min in Towbin’s transfer buffer [25 mM Tris, 192 mM glycine, 0.05% Tween 20]. Membrane protein concentrations were determined using the BCA Protein Assay (Pierce, Thermo Fisher Scientific, Rockford, IL).

**Formalin-fixed, paraffin-embedded renal sections.** A decrease in the RCTT indicates an increase in erythrocyte deformability. Measurement of erythrocyte deformability was measured using the St. George’s blood filtermeter (Carri-Med, Dorking, UK) (39–41). This device develops a calibrated pressure gradient across a vertically mounted, 13-mm diameter polycarbonate filter (Nucleopore) with 9.53 mm exposed surface diameter and average pore size of 5 μm. Proximal to the filter, the inlet tube was filled with either buffer [in mM; 21.0 Tris(hydroxymethyl)aminomethane, 4.7 KCl, 2.0 CaCl₂, 140.5 NaCl, 1.2 MgSO₄, 5.5 glucose, and 0.5% BSA, pH adjusted to 7.4 at 37°C] alone or buffer containing erythrocytes, diluted to a 10% hematocrit. The erythrocyte suspension or buffer alone was then passed through the calibrated filter, and the transmittance was recorded. The ratio between the rate at which the erythrocyte suspension traversed the filter relative to that of buffer alone was used to determine the red (blood) cell transit time (RCTT). If average filter pore size and hematocrit are kept constant, then RCTT is a unitless index of the degree of deformability of the erythrocytes. A decrease in the RCTT indicates an increase in erythrocyte deformability. Measurements of erythrocyte deformability were made after a 20-min incubation with C-peptide (1 nM), insulin (1 nM), or a 1:1 ratio of C-peptide and insulin (1 nM each) or vehicle, saline.

**Statistical analysis.** Statistical significance was determined using ANOVA. In the event that the F-ratio indicated that a change had occurred, a Fisher’s least significant difference test was performed to
identify individual differences between groups. Results are reported as means ± SE. \( P < 0.05 \) was considered statistically significant.

RESULTS

Characteristics of the subjects. Individuals with type 2 diabetes were identified by physicians at Saint Louis University in the Endocrinology Clinic. A patient history was collected for each individual, including a detailed listing of current medications and the patient’s age. The degree of glycemic control in humans with type 2 diabetes was determined by measurement of hemoglobin A1C (HbA1c) at the time of blood removal (AICNow+ device; Bayer, Sunnyvale, CA). The patients were not fasted before blood withdrawal. The subjects studied were healthy human volunteers (n = 23; 15 women, 8 men) and humans with type 2 diabetes (n = 25; 12 women, 13 men) with a mean age of 36 yr (range 18–64 yr) and 59 yr (range 31–82 yr), respectively. The average HbA1c of humans with type 2 diabetes in this study was 8.6% (70 mmol/mol) with a range from 5.6% to 15.3% (38–144 mmol/mol). Patients with type 2 diabetes were treated with multiple drugs in various combinations, including aspirin (n = 13), angiotension-converting enzyme inhibitors or angiotension receptor blockers (n = 16), \( \beta \)-adrenergic receptor blockers (n = 8), oral hypoglycemic agents (n = 14), insulin (n = 18), lipid-lowering drugs (n = 13), calcium channel blockers (n = 4), diuretics (n = 5), and the phosphodiesterase 3 (PDE3) inhibitor, cilostazol (n = 2). The nature of the patient’s illness precluded discontinuation of medications. Record-keeping was in compliance with Health Insurance Portability and Accountability Act regulations.

Western analysis of a C-peptide receptor. With the use of an antibody directed against an intracellular domain of human GPR146, a band of \( \sim 36 \) kDa, consistent with the molecular weight of the receptor, was identified in membranes of healthy human erythrocytes, as well as in the human erythroleukemia cell line, TF-1, and the human gastric tumor cell line, KATOIII (positive controls; Fig. 1A) (50). A similar band was detected in membranes of erythrocytes of humans with type 2 diabetes with HbA1c levels ranging from 5.6 to 15.3 (Fig. 1B). Additionally, immunohistochemistry, using an antibody directed to an external epitope of GPR146, demonstrated immunoreactivity confined to erythrocytes in tissue sections of several organs, including human kidney (Fig. 1C). No reactivity was observed in an isotype-matched control (Fig. 1D).

Effect of preincubation with a GPR146 antibody on low \( O_2 \)-induced ATP release in the presence of C-peptide or insulin. Preincubation of healthy human erythrocytes with the GPR146 antibody used in immunohistochemistry studies, which is directed to an external epitope of the receptor, attenuated C-peptide-mediated inhibition of ATP release in response to low \( O_2 \) in a concentration-dependent manner (Fig. 2A). In contrast, the antibody had no effect on insulin-induced inhibition of low \( O_2 \)-induced ATP release (Fig. 2B).

Effect of physiological ratios of C-peptide and insulin on low \( O_2 \)-induced ATP release from erythrocytes of humans with type 2 diabetes. As depicted in Fig. 3, coincubation of erythrocytes of humans with type 2 diabetes, with a 1:1 or 4:1 ratio of C-peptide to insulin, restored the ability of these cells to release ATP in response to low \( O_2 \). However, at a ratio of 6:1 (6 nM C-peptide to 1 nM insulin), low \( O_2 \)-induced ATP release was inhibited, as was reported previously with healthy human erythrocytes (34). ATP levels at normoxia did not differ among groups.

Residual C-peptide in humans with type 2 diabetes is insufficient to prevent the adverse effect of insulin on low \( O_2 \)-induced ATP release from erythrocytes. Individuals with type 2 diabetes who require insulin therapy have been reported to have circulating C-peptide levels of \( \sim 0.4 \) nM (9, 10, 20). We

Fig. 1. Western analysis and immunohistochemistry demonstrate the presence of GPR146, a C-peptide receptor, on erythrocytes. Membranes of erythrocytes from healthy humans (HH) and humans with type 2 diabetes (DM2) were incubated with a GPR146 polyclonal antibody, generated against an internal epitope of GPR146. A: for comparison, solubilized membranes from KATOIII and TF-1 cells (a human erythroid precursor) were included. The presence of GPR146 was observed in samples from both HH (A) and humans with DM2 over a range of hemoglobin A1C (HbA1c) values (B). C: immunostaining with a rabbit polyclonal antibody to the extracellular domain of GPR146 demonstrated intense immunoreactivity (brown) for the membrane of red blood cells (RBCs) within tissue sections of multiple organs (kidney is shown). D: an isotype-matched control was negative.
determined that 0.4 nM C-peptide is insufficient to restore low O2-induced ATP release from erythrocytes of humans with type 2 diabetes in the presence of 1 nM insulin, a concentration of insulin that may be required for the treatment of type 2 diabetes (Fig. 4).

**Effect of acute exposure to high glucose in the presence of C-peptide and insulin on low O2-induced ATP release from erythrocytes.** It was previously suggested that C-peptide-mediated increases in glucose entry into erythrocytes result in increased ATP release (28, 29). We determined that when the concentration of glucose is elevated threefold (16.5 mM, 300 mg/dl), a level that can be found in humans with type 2 diabetes, erythrocytes from healthy humans, and humans with type 2 diabetes demonstrated no change in low O2-induced ATP release compared with that observed at a more physiological glucose concentration (5.5 mM, 100 mg/dl). Values are means ± SE. *P < 0.05, significant increase from normoxia; †P < 0.01, significant increase from normoxia.

Influence of C-peptide and/or insulin on deformability of erythrocytes. Incubation of erythrocytes from humans with type 1 diabetes with insulin or C-peptide has been shown to enhance erythrocyte deformability (12, 18). We determined that in our series of patients with type 2 diabetes, erythrocyte deformability was not decreased. Moreover, neither insulin nor C-peptide, either alone or in combination, had any effect on deformability of erythrocytes from healthy humans or humans with type 2 diabetes (Table 1).

**DISCUSSION**

The release of both O2 and the potent vasodilator ATP from erythrocytes exposed to reduced O2 tension contributes to the appropriate distribution of perfusion required for adequate tissue oxygenation in skeletal muscle (5, 11, 13, 43). Importantly, it has been reported that low O2-induced ATP release from erythrocytes of humans with type 2 diabetes is severely impaired (38, 42, 44). Moreover, these cells do not stimulate dilation of isolated, perfused skeletal muscle arterioles exposed to reduced extravascular O2, a model of increased tissue O2.

**Fig. 2.** Effect of a GPR146 antibody on low oxygen (O2)-induced ATP release in the presence of C-peptide or insulin. Erythrocytes isolated from HH were preincubated with a GPR146 antibody (10 μL, n = 5 or 30 μL, n = 5) for 30 min before a 20-min incubation with 1 nM C-peptide (A) or 1 nM insulin (n = 5; B) while exposed to a gas mixture containing 15% O2, 6% CO2, and balance N2 (normoxia). ATP release was determined during normoxia and after a 10-min exposure to 0% O2, 6% CO2, and balance N2 (low O2). Values are means ± SE. *P < 0.05, significant increase from normoxia; †P < 0.01, significant increase from normoxia.

**Fig. 3.** Effect of physiological ratios of C-peptide to insulin on low O2-induced ATP release. Erythrocytes isolated from HH were incubated with saline (control), while erythrocytes from humans with DM2 were incubated with saline (control) or various ratios of C-peptide (1, 4, or 6 nM) to insulin (1 nM) for 20 min while exposed to a gas mixture containing 15% O2, 6% CO2, and balance N2 (normoxia). ATP release was determined during normoxia and after a 10-min exposure to 0% O2, 6% CO2, and balance N2 (low O2). Values are means ± SE. *P < 0.05, significant increase from normoxia; †P < 0.01, significant increase from normoxia.

**Fig. 4.** Effect of C-peptide (0.4 nM) and insulin (1 nM) on low O2-induced ATP release from erythrocytes of humans with DM2. Isolated erythrocytes were incubated with saline (control) or 0.4 nM C-peptide and 1 nM insulin (n = 5) for 20 min while exposed to a gas mixture containing 15% O2, 6% CO2, and balance N2 (normoxia). ATP release was determined during normoxia and after a 10-min exposure to 0% O2, 6% CO2, and balance N2 (low O2). Values are means ± SE.
Fig. 5. Effect of high glucose in the presence of a 1:1 ratio of C-peptide to insulin on low O2-induced ATP release from erythrocytes. Isolated erythrocytes from HH (n = 5) or humans with DM2 (n = 5) were incubated with saline (control) or 1 nM C-peptide and 1 nM insulin in buffer with normal glucose (5.5 mM, 100 mg/dl) or with high glucose (16.5 mM, ~300 mg/dl) for 20 min while exposed to a gas mixture containing 15% O2, 6% CO2, and balance N2 (normoxia). ATP release was determined during normoxia and after a 10-min exposure to 0% O2, 6% CO2, and balance N2 (low O2). Values are means ± SE. †P < 0.01, significant increase from normoxia.

need (42). Restoration of ATP release from these erythrocytes in response to this physiologically important stimulus could provide a novel approach for the prevention and treatment of the microvascular disease associated with type 2 diabetes.

Recently, it was shown that incubation of healthy human erythrocytes with either C-peptide or insulin alone inhibits low O2-induced ATP release (34). In contrast, coincubation of C-peptide with insulin, at physiological ratios and concentrations of the two peptides, did not inhibit this important physiological response (34). Since C-peptide and insulin are co-released in vivo, neither peptide would be anticipated to circulate alone in healthy humans (46). However, humans with type 2 diabetes who do not release sufficient insulin to regulate blood glucose are treated with insulin but not with C-peptide. Therefore, erythrocytes of humans with type 2 diabetes have both an inherent defect in low O2-induced ATP release (38, 42, 44) and are exposed to levels of insulin that in the absence of C-peptide, inhibit ATP release in response to this stimulus (19).

Human erythrocytes clearly possess insulin receptors (17, 35). However, the identification of a receptor for C-peptide has not been described previously on these cells. Evidence from multiple cell lines suggests that C-peptide-mediated effects result from stimulation of a pertussis toxin-sensitive GPCR (1, 21). Recently, it was demonstrated that C-peptide stimulates cFos expression in the human erythroleukemia cell line, TF-1, and the human gastric tumor cell line, KATOIII. Both cell types express the GPCR GPR146 (50), and knockdown of GPR146 with siRNA prevented C-peptide-induced cFos expression (50). These findings suggest that GPR146 is a receptor for C-peptide on KATOIII cells. Knockdown studies cannot be performed with erythrocytes, since these cells lack a nucleus and protein-synthesizing capacity. However, with the use of Western analysis, we qualitatively determined that GPR146 is present on both an erythrocyte precursor cell line (TF-1) as well as mature erythrocytes of healthy humans and humans with type 2 diabetes (Fig. 1, A and B). The erythroleukemia cell line, TF-1, which has been shown to be of proerythrocyte lineage, not only produced GPR146 protein (Fig. 1A) but also produced GPR146 mRNA (50). Finally, immunohistochemistry provided further evidence of the presence of GPR146 on human erythrocytes (Fig. 1, C and D). Although these data demonstrate the presence of GPR146 on erythrocytes, this does not establish a relationship between GPR146 and C-peptide-mediated effects on low O2-induced ATP release from erythrocytes.

To determine that GPR146 is responsible for C-peptide-mediated effects on low O2-induced ATP release from erythrocytes, we preincubated healthy human erythrocytes with an antibody directed to an external epitope of GPR146 with the intent of blocking the interaction between C-peptide and the receptor. Antibodies directed against an extracellular receptor domain to block activity have been used successfully with other receptors (6, 26, 31). Here, we show that preincubation of healthy human erythrocytes with the GPR146 antibody attenuates C-peptide-mediated inhibition of low O2-induced ATP release in a concentration-dependent manner (Fig. 2). Importantly, preincubation with the antibody had no effect on insulin-induced inhibition of low O2-induced ATP release, indicating that the antibody did not act in a nonspecific manner to stimulate low O2-induced ATP release. Taken together, these data provide strong evidence that GPR146 is a receptor responsible for C-peptide-mediated effects on low O2-induced ATP release from erythrocytes.

As reported previously, coincubation of healthy human erythrocytes with C-peptide and insulin at physiological ratios and concentrations does not interfere with low O2-induced ATP release (34). Here, we report that coincubation of erythrocytes from humans with type 2 diabetes with physiological ratios of 1:1 and 4:1 (C-peptide to insulin) rescues low O2-induced ATP release (Fig. 3). However, coincubation of erythrocytes from humans with type 2 diabetes with a 6:1 ratio of C-peptide to insulin does not. Thus the maintenance of physiological ratios and concentrations of these peptides is critical for the rescue of low O2-induced ATP release from erythrocytes of humans with type 2 diabetes. It must be recognized that although the patients were on a variety of treatment regimens, including in two cases, the PDE3 inhibitor cilostazol, the inability to release ATP in response to low O2 and the rescue of that function by C-peptide were seen in all type 2 diabetes patients, regardless of their medications.

Although pancreatic release of insulin and C-peptide can be compromised severely in type 2 diabetes, some individuals...
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 retain modest beta-cell activity, allowing for the production of low levels of C-peptide (9, 10, 20). In a study analyzing patient tertiles based on C-peptide levels, the incidence of retinopathy, nephropathy, and neuropathy decreased from the first (C-peptide ≤ 0.47 nM) to the third tertile (C-peptide ≥ 0.80 nM) (7). In humans with type 2 diabetes, several reports suggest that a basal level of C-peptide of ~0.4 nM is often present, with minimal increases occurring after meals due to some residual beta-cell activity (9, 10, 20). Here, we report that in the presence of 1 nM insulin—a concentration of insulin that may be present in insulin-treated humans with type 2 diabetes—0.4 nM C-peptide is insufficient to restore low O2-induced ATP release from erythrocytes of humans with type 2 diabetes (Fig. 4). These data suggest a previously unrecognized mechanism contributing to microvascular complications in individuals with reduced C-peptide levels.

C-Peptide has been suggested to stimulate the uptake of glucose into erythrocytes, resulting in increased ATP release (28, 29). However, glucose entry into the erythrocyte is not the rate-limiting step in ATP formation via glycolysis (48). Moreover, increased ATP levels inhibit glycolysis in these cells due to end-product inhibition (3). Importantly, we have shown that erythrocytes of humans with type 2 diabetes with markedly increased HbA1c levels, indicating chronic increases in plasma glucose, do not exhibit differences in intracellular ATP when compared with healthy humans (42). Moreover, the impairment in low O2-induced ATP release seen in erythrocytes of humans with type 2 diabetes is not related to HbA1c level (42). As shown in Fig. 5, elevated glucose concentration (16.5 mM, 300 mg/dl) had no effect on low O2-induced ATP release from erythrocytes of healthy humans or humans with type 2 diabetes in the presence of a 1:1 ratio of C-peptide and insulin.

Humans with type 1 diabetes are reported to have reduced erythrocyte deformability and increased blood viscosity attributed to a reduction in erythrocyte Na+–K+–ATPase activity (10, 25, 47). Interestingly, C-peptide has been reported to stimulate Na+–K+–ATPase activity in these erythrocytes, resulting in increased deformability (12, 16, 27). However, studies of the deformability of erythrocytes from humans with type 2 diabetes have yielded conflicting results (4, 8, 37, 49). This is important, because we and others have demonstrated that reduced erythrocyte deformability can inhibit low O2-induced ATP release (45). Here, we examined the deformability of erythrocytes of healthy humans and humans with type 2 diabetes in the absence and presence of insulin and C-peptide. As depicted in Table 1, there were no differences in RCTT, a measure of erythrocyte deformability, among the various groups. Thus it is unlikely that restoration of low O2-induced ATP release from erythrocytes of humans with type 2 diabetes by insulin and C-peptide is a direct result of altered erythrocyte deformability. Clearly, further study will be necessary to elucidate the signaling mechanisms involved in the rescue of this important physiological response.

Perspectives and Significance

Restoration of low O2-induced ATP release from erythrocytes from humans with type 2 diabetes would re-establish an important contributor to the local control of perfusion distribution, resulting in the adequate delivery of O2 to skeletal muscle. This hypothesis is supported by reports that confusion of C-peptide with even a subphysiological concentration of insulin (1 μU/ml) induced concentration-dependent vasodilation in isolated skeletal muscle arterioles and returned coronary flow to normal levels in a type 1 diabetes rat model (24, 32). The results presented here suggest that the addition of C-peptide to the treatment regimen for type 2 diabetes could have beneficial effects on blood flow distribution in skeletal muscle (11, 34), which could be useful in the prevention and/or treatment of the peripheral vascular disease associated with type 2 diabetes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

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