Mechanisms of C-peptide-mediated rescue of low O$_2$-induced ATP release from erythrocytes of humans with Type 2 diabetes

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Richards JP, Bowles EA, Gordon WR, Ellsworth ML, Stephenson AH, Sprague RS. Mechanisms of C-peptide-mediated rescue of low O$_2$-induced ATP release from erythrocytes of humans with Type 2 diabetes. Am J Physiol Regul Integr Comp Physiol 308: R411–R418, 2015. First published December 31, 2014; doi:10.1152/ajpregu.00420.2014.—The circulating erythrocyte, by virtue of the regulated release of ATP in response to reduced oxygen (O$_2$) tension, plays a key role in maintaining appropriate perfusion distribution to meet tissue needs. Erythrocytes from individuals with Type 2 diabetes (DM2) fail to release ATP in response to this stimulus. However, the administration of C-peptide and insulin at a 1:1 ratio was shown to restore this important physiological response in humans with DM2. To begin to investigate the mechanisms by which C-peptide influences low O$_2$-induced ATP release, erythrocytes from healthy humans and humans with DM2 were exposed to reduced O$_2$ in a thin-film tonometer, and ATP release under these conditions was compared with release during normoxia. We determined that 1) low O$_2$-induced ATP release from DM2 erythrocytes is rescued by C-peptide in the presence and absence of insulin, 2) the signaling pathway activated by C-peptide in human erythrocytes involves PKC, as well as soluble guanylyl cyclase (sGC) and 3) inhibitors of cGMP degradation rescue low O$_2$-induced ATP release from DM2 erythrocytes. These results provide support for the hypothesis that both PKC and sGC are components of a signaling pathway activated by C-peptide in human erythrocytes. In addition, since both C-peptide and phosphodiesterase 5 inhibitors rescue low O$_2$-induced ATP release from erythrocytes of humans with DM2, their administration to humans with DM2 could aid in the treatment and/or prevention of the vascular disease associated with this condition.

THE APPROPRIATE DELIVERY of oxygen and nutrients to skeletal muscle is required for normal physiological function. It has been suggested that the circulating erythrocyte, by virtue of the regulated release of ATP in response to physiological stimuli, plays a key role in maintaining optimal perfusion distribution to meet tissue needs. When erythrocytes pass through areas of skeletal muscle with a decreased P O$_2$, oxygen is released, which initiates a signal transduction pathway, resulting in the release of ATP. This important physiological response contributes to the distribution of perfusion to supply adequate blood flow (O$_2$ supply) to specific regions of skeletal muscle. The signal transduction pathway initiated by oxygen off-loading from hemoglobin involves activation of a heterotrimeric G protein, Gi (35, 36) and subsequent stimulation of adenyl cyclase, which catalyzes the production of cAMP from ATP (6, 14, 39, 41). It is well established that increases in cAMP are critical for stimulation of ATP release in response to low O$_2$. Importantly, in this pathway cAMP levels are regulated by phosphodiesterase 3 (PDE3) (22, 40, 42). Thus, PDE3 activity is required to ensure the localization of this second messenger to that signaling pathway and to limit ATP release (2, 22, 27).

Once released from erythrocytes in the microcirculation, ATP binds to purinergic receptors stimulating production of endothelium-derived vasodilators, including nitric oxide (NO) (9, 30), resulting in localized increases in erythrocyte flux and, consequently, O$_2$ supply rate in areas with decreased tissue P O$_2$. Defects in this mechanism for the regulation of optimal distribution of blood flow (O$_2$ delivery) in skeletal muscle could contribute to the microvascular disease associated with Type 2 diabetes.

In humans with Type 2 diabetes (DM2), erythrocytes fail to release ATP in response to low O$_2$ (43). This defect is associated with a reduction in expression of G$_{O2}$ (44). The loss of this important physiological response would prevent these cells from participating in the regulation of perfusion distribution to meet local O$_2$ need in skeletal muscle (43). This defect may be further complicated when the treatment of diabetes includes administration of insulin at doses that result in supraphysiological concentrations of the peptide to regulate blood glucose levels and overcome insulin resistance.

Previously, it was reported that insulin, at a concentration that may be achieved in the treatment of DM2 (1 nM), inhibits low O$_2$-induced ATP release from healthy human erythrocytes (21). Insulin is known to stimulate a signaling pathway that results in activation of PDE3, resulting in decreases in cAMP and inhibition of low O$_2$-induced ATP release (21, 22). However, in vivo, insulin is produced from a prohormone and is coreleased with equimolar amounts of C-peptide (45). Since the half-life of C-peptide exceeds that of insulin due to differences in degradation rates, circulating C-peptide levels are greater than those of insulin (23, 26). Recently, we demonstrated that coinubcation of C-peptide with insulin, at physiological concentrations and ratios (1:1 to 5.5:1), prevented the insulin-induced inhibition of low O$_2$-induced ATP release from healthy human erythrocytes (37). More importantly, coinubcation of C-peptide with insulin at ratios of 1:1 or 4:1 rescued low O$_2$-induced ATP release from DM2 erythrocytes (37).

Presently, there is limited insight into the signal transduction pathway stimulated by C-peptide. Evidence suggests that C-peptide initiates signaling via a pertussis toxin-sensitive G protein-coupled receptor (GPCR) and that, in human erythrocytes, a GPCR that C-peptide activates is GPR146 (38). In other cell types, stimulation of PKC has been demonstrated to be a component of a C-peptide signal transduction pathway (3, 28).
Samples in which free hemoglobin increased were not included. was determined by measurement of light absorbance at 405 nm (25). The presence of hemoglobin in the supernatant on low O2-induced ATP release was determined.

Isolation of human erythrocytes. Blood was obtained from healthy volunteers (n = 13) or individuals with DM2 (n = 22) via venipuncture using a syringe containing heparin (500 units). Blood was centrifuged at 500 g at 4°C for 10 min, and the plasma, buffy 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 determined. 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. Briefly, a 200-µl sample of the erythrocyte suspension (0.04% hematocrit) was injected into a cuvette containing 100 µl of firefly lantern extract (10 mg/ml distilled water, FLE 250; Sigma, St. Louis, MO) and 100 µl of d-luciferin solution (10 mg/20 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 × 10⁹ erythrocytes.

Measurement of free hemoglobin. To exclude the possibility that erythrocyte lysis contributed to the observed increases in ATP release, samples used for measuring ATP were centrifuged at 500 g for 10 min at room temperature. The presence of hemoglobin in the supernatant was determined by measurement of light absorbance at 405 nm (25). Samples in which free hemoglobin increased were not included.

Determination of ATP release from DM2 erythrocytes in response to exposure to reduced O₂ tension in the presence and absence of C-peptide or insulin. Isolated DM2 erythrocytes were diluted to a 20% hematocrit in tonometer buffer (4.7 mM KCl, 2.0 mM CaCl₂, 140.5 mM NaCl, 1.2 mM MgSO₄, 5.5 mM glucose, 24.8 mM NaHCO₃, 5.5 mM dextrose, 0.5% BSA, pH 7.4) at 37°C and equilibrated for 30 min in a thin-film tonometer (model DEQ1, Cameron Instrument) with gas containing 15% O₂-6% CO₂-balance N₂ (normoxia, PO₂ = 110 ± 3 mmHg) prior to the addition of 1 nM C-peptide and 1 nM insulin or their vehicle (saline). After 20 min, ATP levels were determined during normoxia and 10 min after exposure to gas containing 0% O₂-6% CO₂-balance N₂ (low O₂, PO₂ = 17 ± 4 mmHg).

Determination of low O₂-induced ATP release from human erythrocytes incubated with C-peptide and insulin in the presence and absence of a PKC inhibitor. Isolated healthy human or DM2 erythrocytes were diluted to a 20% hematocrit in tonometer buffer at 37°C and incubated with a PKC inhibitor, either 10 nM Go6976 or 10 nM Ro-31-8220 or their vehicle (dimethylformamide, DMF). These erythrocyte suspensions were equilibrated for 30 min in a thin-film tonometer with gas containing 15% O₂-6% CO₂-balance N₂ (normoxia, PO₂ = 110 ± 3 mmHg) prior to the addition of 1 nM C-peptide and 1 nM insulin or their vehicle (saline). After 20 min, ATP levels were determined during normoxia and 10 min after exposure to gas containing 0% O₂-6% CO₂-balance N₂ (low O₂, PO₂ = 17 ± 4 mmHg).

Comparison of ATP metabolism to that of a PDE5 inhibitor. Isolated healthy human or DM2 erythrocytes were equilibrated for 30 min in a thin-film tonometer with gas containing 15% O₂-6% CO₂-balance N₂ (normoxia, PO₂ = 110 ± 3 mmHg) prior to the addition of 1 nM C-peptide and 1 nM insulin or their vehicle (saline). After 20 min, ATP levels were determined during normoxia and 10 min after exposure to gas containing 0% O₂-6% CO₂-balance N₂ (low O₂, PO₂ = 17 ± 4 mmHg).

Determination of low O₂-induced ATP release from DM2 erythrocytes in the presence and absence of a PDE5 inhibitor. Isolated DM2 erythrocytes were diluted to a 20% hematocrit in tonometer buffer at 37°C and equilibrated for 30 min in a thin-film tonometer with gas containing 15% O₂-6% CO₂-balance N₂ (normoxia, PO₂ = 110 ± 5 mmHg). ATP levels were determined during normoxia and 10 min after exposure to gas containing 0% O₂-6% CO₂-balance N₂ (low O₂, PO₂ = 15 ± 5 mmHg). The effect of a 30-min pretreatment of erythrocytes with a PDE5 inhibitor, zaprinast (10 µM; Sigma-Aldrich) or tadalafil (10 µM; Eli Lilly) or their vehicle (DMF), on low O₂-induced ATP release was determined.

Statistical analysis. Statistical significance among experiments 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 values < 0.05 were considered statistically significant.

RESULTS

Characteristics of the subjects. Individuals with DM2 were identified by physicians at Saint Louis University in the Endocrinology Clinic, and healthy human volunteers were faculty, staff, and students at Saint Louis University. A medical history was collected for each individual, including a detailed listing of medical conditions, current medications, and the patient’s age. The degree of glycemic control in humans with DM2 was determined by measurement of hemoglobin A1C (HbA1c) at the time of blood removal (A1CNow+, Bayer, Sunnyvale, CA). The subjects studied were healthy human volunteers (n = 13, 8 female, 5 male) and humans with Type 2 diabetes (n = 22, 14 female, 8 male) with a mean age of 39 years (range 24–64 years), and 55 years (range 27–82 years), respectively. The HbA1c of humans with Type 2 diabetes in this study ranged from 5.8% to 13.0% with an average of 8.7%. Patients with DM2 were treated with various combinations of drugs, including aspirin (n = 8), angiotensin-converting enzyme inhibitors, or angiotensin receptor blockers (n = 12), β-adrenergic receptor blockers (n = 3), oral hypoglycemic agents (n = 14), insulin (n = 14), lipid-lowering drugs (n = 9), calcium channel blockers (n = 6), diuretics (n = 4), or a PDE5 inhibitor, (n = 2). Record keeping was in compliance with HIPAA (Health Insurance Portability and Accountability Act) regulations.
Effect of C-peptide or insulin on low O$_2$-induced ATP release from DM2 erythrocytes.

Incubation of C-peptide with DM2 erythrocytes rescued the ability of these cells to release ATP in response to low O$_2$ (Fig. 1, A and B). It is of interest that the amount of C-peptide required varied with HbA1c level. In individuals with a HbA1c below 9%, low O$_2$-induced ATP release was restored in the presence of 0.3 nM C-peptide (Fig. 1A). However, in individuals with a HbA1c above 9%, restoration of low O$_2$-induced ATP release required a greater concentration of 1 nM C-peptide (Fig. 1, A and B). In contrast to C-peptide, incubation with insulin (1 nM) had no effect on low O$_2$-induced ATP release from DM2 erythrocytes irrespective of HbA1c level (Fig. 1C). There were no differences in ATP levels measured during normoxia among the groups (Fig. 1).

Effect of preincubation with PKC$_\alpha$ inhibitors on low O$_2$-induced ATP release from healthy human and DM2 erythrocytes in the presence of C-peptide and insulin.

In the presence of a 1:1 ratio (1 nM each) of C-peptide to insulin, preincubation with either Gö6976 or Ro-31-8220, two chemically dissimilar PKC$_\alpha$ inhibitors, prevented the ability of C-peptide to rescue low O$_2$-induced ATP release from healthy human erythrocytes (Fig. 2, A and B). Similarly, in the presence of a 1:1 ratio of C-peptide to insulin, preincubation with Gö6976 prevented increases in ATP release from DM2 erythrocytes in response to this stimulus (Fig. 3). There were no differences in ATP levels measured during normoxia from DM2 erythrocytes in the absence or presence of Gö6976 (Fig. 3). In four additional studies (data not shown), neither PKC$_\alpha$ inhibitor had any effect on low O$_2$-induced ATP release from healthy human erythrocytes in the absence of C-peptide and insulin.

**Fig. 1.** Effect of C-peptide or insulin on low O$_2$-induced ATP release from erythrocytes of humans with Type 2 diabetes (DM2). Isolated DM2 erythrocytes were incubated with saline (control) or C-peptide at 0.3 nM (A) or 1 nM (B) for 20 min while exposed to a gas mixture containing 15% O$_2$, 6% CO$_2$, balance N$_2$ (normoxia) prior to measurement of ATP release. This was followed by exposure to 0% O$_2$, 6% CO$_2$, balance N$_2$ (low O$_2$) for 10 min prior to a second measurement of ATP release. C: isolated DM2 erythrocytes were incubated with saline (control) or 1 nM insulin ($n=4$) for 20 min, while exposed to normoxia prior to measuring ATP release. This was followed by exposure to low O$_2$ for 10 min before a second measurement of ATP release. Baseline values measured during normoxia did not differ and were as follows (in nM per 4×10$^8$ erythrocytes): saline = 25.9 ± 3.2, 0.3 nM C-peptide = 20.5 ± 2.7, 1.0 nM C-peptide = 18.0 ± 5.0 and 1.0 nM insulin = 27.6 ± 5.0. Values are expressed as means ± SE. *Significant increase from normoxia ($P < 0.05$). †Significant increase from normoxia ($P < 0.01$).

**Fig. 2.** PKC$_\alpha$ inhibitors prevent C-peptide-mediated rescue of insulin-induced inhibition of low O$_2$-induced ATP release from healthy human erythrocytes. Erythrocytes isolated from healthy volunteers were preincubated with either 10 nM Gö6976 ($n=4$; A) or 10 nM Ro 31–8220 ($n=4$; B), two chemically distinct PKC$_\alpha$ inhibitors, for 10 min prior to a 20-min incubation with 1 nM C-peptide and 1 nM insulin (1:1) were initially exposed to a gas mixture containing 15% O$_2$, 6% CO$_2$, balance N$_2$ (normoxia). ATP release was determined during normoxia and after a 10-min exposure to 0% O$_2$, 6% CO$_2$, balance N$_2$ (low O$_2$). Values are expressed as means ± SE. †Significant increase from normoxia ($P < 0.01$).

**Effect of C-peptide or insulin on low O$_2$-induced ATP release from DM2 erythrocytes.** Incubation of C-peptide with DM2 erythrocytes rescued the ability of these cells to release ATP in response to low O$_2$ (Fig. 1, A and B). It is of interest that the amount of C-peptide required varied with HbA1c level.
Initially exposed to a gas mixture containing 15% O₂-6% CO₂-balance N₂ to a 20-min incubation with 1 nM C-peptide and 1 nM insulin (1:1) were preincubated with 10 nMforexposed to a gas mixture containing 15% O₂-6% CO₂-balance N₂ (normoxia). ATP release was determined during normoxia and after a 10-min exposure to 0% O₂-6% CO₂-balance N₂ (low O₂). Baseline values measured during normoxia did not differ and were as follows (in nM per 4 × 10⁸ erythrocytes): C-peptide and insulin alone (1:1) = 13.8 ± 4.4 and in the presence of Go6976 = 16.0 ± 2.0. Values are expressed as means ± SE. *Significant increase from normoxia (P < 0.05).

Effect of preincubation with the soluble guanylyl cyclase inhibitor, ODQ, on low O₂-induced ATP release from healthy human or DM2 erythrocytes in the presence of C-peptide and insulin. Preincubation of either healthy human (Fig. 4) or DM2 erythrocytes (Fig. 5) with the soluble guanylyl cyclase inhibitor, ODQ, prior to the addition of a 1:1 ratio (1 nM each) of C-peptide to insulin, inhibited low O₂-induced ATP release. There were no differences in ATP levels measured during normoxia from healthy human (Fig. 4) and DM2 (Fig. 5) erythrocytes in the absence or presence of ODQ. In four additional studies (data not shown), ODQ had no effect on low O₂-induced ATP release from healthy human erythrocytes in the absence of C-peptide and insulin.

Effect of preincubation with the soluble guanylyl cyclase inhibitor, ODQ, on low O₂-induced ATP release from healthy human or DM2 erythrocytes in the presence of C-peptide and insulin. Preincubation of either healthy human (Fig. 4) or DM2 erythrocytes with either zaprinast or tadalafil, two chemically dissimilar PDE5 inhibitors (10 μM), rescued low O₂-induced ATP release. The PDE5 inhibitors had no effect on values for ATP determined under normoxic conditions (Fig. 6).

DISCUSSION

Erythrocytes have been shown not only to deliver O₂, but also to influence the distribution of microvascular perfusion in skeletal muscle via the low O₂-initiated release of ATP (11, 13, 18). However, the release of ATP in response to low O₂ is defective in DM2 erythrocytes (43). This defect may be exacerbated further by the administration of insulin that results in supraphysiological concentrations in the absence of C-peptide in the treatment of DM2. Although C-peptide was initially thought to be a biologically inert by-product of insulin production, more recently C-peptide has been shown to have physiological function in a variety of tissues (3, 16, 24, 45). Importantly, coincubation of C-peptide and insulin at physiological ratios and concentrations rescues insulin-induced inhibition of low O₂-induced ATP release from healthy human erythrocytes, resulting in compensation for the inherent defect in the low O₂ pathway in DM2 (37).

Since insulin-induced stimulation of PDE3 inhibited low O₂-induced ATP release from healthy human erythrocytes (21, 22), it was not unexpected that insulin alone did not rescue low O₂-induced ATP release from DM2 erythrocytes. In a previous study, incubation with cilostazol, an inhibitor of PDE3, prevented the adverse effects of insulin on healthy human erythrocytes and rescued ATP release from DM2 erythrocytes in response to low O₂ (22, 40). Here, we report that C-peptide, like cilostazol, when administered to DM2 erythrocytes in the absence of insulin, rescues low O₂-induced ATP release (Fig. 1). Interestingly, the concentration of C-peptide required varied on the basis of glycemic control, as measured by HbA1c. Low

![Fig. 3](http://ajpregu.physiology.org/Downloadedfrom) - The PKCα inhibitor Go6976 prevents C-peptide-mediated rescue of low O₂-induced ATP release from erythrocytes of humans with DM2 in the presence of insulin. Erythrocytes isolated from volunteers with DM2 were preincubated with 10 nM Go6976 (n = 4), a PKCα inhibitor, for 10 min prior to a 20-min incubation with 1 nM C-peptide and 1 nM insulin (1:1) were initially exposed to a gas mixture containing 15% O₂-6% CO₂-balance N₂ (normoxia). ATP release was determined during normoxia and after a 10-min exposure to 0% O₂-6% CO₂-balance N₂ (low O₂). Baseline values measured during normoxia did not differ and were as follows (in nM per 4 × 10⁸ erythrocytes): C-peptide and insulin alone (1:1) = 13.8 ± 4.4 and in the presence of Go6976 = 16.0 ± 2.0. Values are expressed as means ± SE. *Significant increase from normoxia (P < 0.05).

![Fig. 4](http://ajpregu.physiology.org/Downloadedfrom) - The soluble guanylyl cyclase inhibitor, ODQ, prevents C-peptide-mediated rescue of low O₂-induced ATP release from healthy human erythrocytes. Erythrocytes isolated from healthy volunteers with DM2 were preincubated with 10 μM ODQ (n = 5), a soluble guanylyl cyclase inhibitor, for 10 min prior to a 20-min incubation with 1 nM C-peptide and 1 nM insulin (1:1) were initially exposed to a gas mixture containing 15% O₂-6% CO₂-balance N₂ (normoxia). ATP release was determined during normoxia and after a 10-min exposure to 0% O₂-6% CO₂-balance N₂ (low O₂). Baseline values measured during normoxia did not differ and were as follows (in nM per 4 × 10⁸ erythrocytes): C-peptide and insulin alone (1:1) = 21.4 ± 3.6 and in the presence of ODQ = 24.5 ± 5.3. Values are expressed as means ± SE. †Significant increase from normoxia (P < 0.01).

![Fig. 5](http://ajpregu.physiology.org/Downloadedfrom) - ODQ prevents C-peptide-mediated rescue of low O₂-induced ATP release from erythrocytes of humans with DM2 in the presence of insulin. Erythrocytes isolated from volunteers with DM2 were preincubated with 10 μM ODQ for 10 min prior to a 20-min incubation with 1 nM C-peptide and 1 nM insulin (1:1) were initially exposed to a gas mixture containing 15% O₂-6% CO₂-balance N₂ (normoxia). ATP release was determined during normoxia and after a 10-min exposure to 0% O₂-6% CO₂-balance N₂ (low O₂). Baseline values measured during normoxia did not differ and were as follows (in nM per 4 × 10⁸ erythrocytes): control = 20.9 ± 3.3, C-peptide and insulin alone (1:1) = 14.8 ± 4.2 and 1:1 in the presence of ODQ = 23.8 ± 3.1. Values are expressed as means ± SE. †Significant increase from normoxia (P < 0.01).
O₂-induced ATP release from DM2 erythrocytes with a HbA1c below 9% was rescued with 0.3 nM C-peptide; however, the concentration of C-peptide had to be increased to 1 nM in erythrocytes of humans with a HbA1c above 9%. It is noteworthy that a HbA1c above 9% is recognized by the National Committee for Quality Assurance’s Healthcare Effectiveness Data and Information Set to indicate poor glycemic control in DM2 (7, 28). The requirement of a higher concentration of C-peptide to rescue low O₂-induced ATP release from individuals with a HbA1c above 9% may be indicative of the development of C-peptide resistance or some additional impairment of ATP release that is present as glycemic control worsens.

Although the signal transduction pathway for C-peptide is not fully characterized, several components of the pathway have been proposed (Fig. 7). It is clear that C-peptide signals through a pertussis toxin-sensitive GPCR (4, 24). Recently, GPR146, an orphan GPCR, was demonstrated to be present on KATO III cells, TF-1 cells (an erythroid precursor cell line) and both healthy human and DM2 erythrocytes (49, 38). In support of the role of GPR146 as a C-peptide receptor, it was reported that knockdown of GPR146 prevented C-peptide-stimulated c-Fos expression in KATO III cells (49), and preincubation with an antibody to an external epitope of GPR146 blocked the effect of C-peptide on low O₂-induced ATP release from healthy human erythrocytes (38). Thus, these data strongly support the hypothesis that GPR146 is a functional C-peptide receptor on human erythrocytes.

One of the downstream effectors reported to be a component of a C-peptide-mediated signaling pathway in other cells is PKC. Indeed, multiple isoforms of PKC have been implicated in C-peptide signaling, including PKCα, PKCε, and PKCδ (3, 17, 50). Importantly, only PKCα is known to be present in human erythrocytes (19). Preincubation of healthy human erythrocytes with either Gö6976 or Ro-31-8220, two chemically dissimilar PKCα inhibitors, prevented the C-peptide-mediated rescue of insulin-induced inhibition of low O₂-induced ATP release (Fig. 2, A and B). In addition, preincubation of DM2 erythrocytes with Gö6976 also prevented the C-peptide-mediated rescue of ATP release (Fig. 3). These results are consistent with the hypothesis that PKCα is a component of a C-peptide-mediated signaling pathway that interacts with the pathway for low O₂-induced ATP release from human erythrocytes. Although PKC phosphorylation can activate several downstream targets, two possible components of particular...
interest in the erythrocyte are Na\(^+\)/K\(^+\)-ATPase and soluble guanylyl cyclase (17, 32, 46).

C-peptide has been shown to stimulate the Na\(^+\)/K\(^+\)-ATPase via phosphorylation by PKC and enhance Na\(^+\)/K\(^+\)-ATPase activity in neurons, renal cells, and erythrocytes of humans with Type 1 diabetes (DM1) (10, 17, 31, 34, 46). A reduction in Na\(^+\)/K\(^+\)-ATPase activity has been observed in DM1, which reportedly correlates with impaired erythrocyte deformability (10, 29). Incubation of DM1 erythrocytes with either C-peptide or insulin has been shown to improve both Na\(^+\)/K\(^+\)-ATPase activity and erythrocyte deformability (20, 29, 31). In DM2, impaired erythrocyte deformability remains controversial, as several groups have observed reduced deformability, while other reports do not confirm this finding (8, 48). Although coadministration of C-peptide with insulin at a 1:1 ratio (1 nM of each peptide) rescues low O\(_2\)-induced ATP release from DM2 erythrocytes, the 1:1 ratio had no effect on DM2 erythrocyte deformability (38). This finding suggests that a C-peptide-mediated increase in Na\(^+\)/K\(^+\)-ATPase activity resulting in increased erythrocyte deformability is not responsible for the restoration of low O\(_2\)-induced ATP release from DM2 erythrocytes.

In addition to stimulation of Na\(^+\)/K\(^+\)-ATPase activity, PKC activation has also been reported to stimulate soluble guanylyl cyclase (sGC) activity (32). The enzyme sGC catalyzes the conversion of GTP to cGMP. Notably, increases in cGMP levels were reported in human plasma and RFL-6 cells following the administration of C-peptide (15, 47). Intracellular cGMP is an endogenous inhibitor of PDE3-mediated cAMP hydrolysis (33). Therefore, in the low O\(_2\) pathway for ATP release from erythrocytes, elevated intracellular cGMP levels could limit cAMP degradation by inhibition of PDE3. Importantly, this mechanism would counter-balance the increase in PDE3 activity observed when human erythrocytes are incubated with insulin (21, 22). When healthy human or DM2 erythrocytes were preincubated with the sGC inhibitor, ODQ, C-peptide-mediated rescue of the insulin-induced inhibition of low O\(_2\)-induced ATP release was prevented (Figs. 4 and 5, respectively). These results are consistent with the hypothesis that in human erythrocytes, the C-peptide-mediated signal transduction pathway includes stimulation of cGMP production via sGC activation. Increases in cGMP would provide a mechanism for inhibition of PDE3 activity resulting in the rescue of low O\(_2\)-induced ATP release from DM2 erythrocytes (22, 40).

In human erythrocytes, levels of intracellular cGMP are regulated by PDE5 (1). We hypothesized that preventing the hydrolysis of cGMP by administration of a PDE5 inhibitor would rescue low O\(_2\)-induced ATP release from DM2 erythrocytes similar to either the PDE3 inhibitor, cilostazol, or C-peptide. When either 10 \(\mu\)M tadalafil or 10 \(\mu\)M zaprinast, two chemically distinct PDE5 inhibitors, was incubated with DM2 erythrocytes, ATP release in response to low O\(_2\) was restored (Fig. 6). This finding could be of particular importance as oral PDE5 inhibitors are already clinically available and could readily be used in the treatment of DM2. It must be recognized that although the patients were on a variety of treatment regimens, including, in two cases, a PDE5 inhibitor, the inability to release ATP in response to low O\(_2\) and the rescue of that function by C-peptide was seen in all DM2 patients, regardless of their medications. Although control subjects did have a lower average age, we found no age differences with respect to impairment of ATP release or in the response to C-peptide and insulin or PDE5 inhibitors in erythrocytes of humans with DM2.

In summary, we provided evidence that in human erythrocytes C-peptide initiates a signal transduction pathway via stimulation of a GPCR, GPR146, which results in activation of PKC\(_\alpha\), as well as sGC (Fig. 7). Elevation of cGMP via sGC provides a mechanism by which C-peptide can inhibit PDE3-mediated hydrolysis of cAMP. This is consistent with the hypothesis that C-peptide-mediated effects on low O\(_2\)-induced ATP release are a result of PDE3 inhibition.

**Perspectives and Significance**

The reported defects in the distribution of O\(_2\) supply in skeletal muscle in DM2 could be further exacerbated by the failure of DM2 erythrocytes to release ATP in response to low O\(_2\) (5, 12, 43), a defect that can be overcome by the administration of C-peptide. These findings are similar to results obtained with the PDE3 inhibitor cilostazol, suggesting that C-peptide may be acting through a similar mechanism (22, 40). C-peptide, via activation of PKC and sGC, would increase levels of the endogenous PDE3 inhibitor, cGMP. Rescue of low O\(_2\)-induced ATP release from DM2 erythrocytes by preventing cGMP hydrolysis with a PDE5 inhibitor suggests that inhibition of PDE3 may be the mechanism common among C-peptide-, cilostazol-, and PDE5 inhibitor-mediated rescue of low O\(_2\)-induced ATP release from DM2 erythrocytes. The use of C-peptide and/or clinically available PDE5 inhibitors in the treatment of DM2 may provide a novel approach for the treatment or prevention of the microvascular complications associated with DM2 in humans.

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**


**REFERENCES**

C-PEPTIDE SIGNALING IN HUMAN RBCS


