Synergistic Effects of C-peptide and Insulin on Low O₂-induced ATP Release from Human Erythrocytes.

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Running title: C-peptide and insulin modulate ATP release from human RBCs
Abstract:

Erythrocytes participate in the matching of oxygen (O₂) delivery with local need in skeletal muscle via the release of O₂ and the vasodilator, ATP. It was reported that a concentration of insulin found in humans with insulin resistance inhibits low O₂-induced ATP release. However, in vivo, insulin is co-released with connecting peptide (C-peptide) at equimolar concentrations, but due to shorter insulin half-life, the peptides circulate at ratios of C-peptide to insulin ranging from 1:1 to 6:1.

Here we investigate the hypothesis that C-peptide and insulin work synergistically to maintain low O₂-induced ATP release from human erythrocytes. Using a thin-film tonometer to alter O₂ tension we determined that either C-peptide or insulin alone inhibits low O₂-induced ATP release in a concentration-dependent manner; however, co-administration of the peptides at a 1:1 ratio does not (n=5, p<0.05). Since this ratio of C-peptide to insulin is not present in vivo for extended periods, we also investigated the effect of additional physiological ratios on ATP release. In the presence of insulin concentrations that would be found in fasting humans (0.05 nM), C-peptide to insulin ratios of 4:1 and 6:1 did not adversely affect low O₂-induced ATP release. However, at a concentration of insulin found in the peripheral circulation of humans under post-prandial conditions (0.5 nM), a ratio of C-peptide to insulin of 6:1 inhibited low O₂-induced ATP release (n=5). These findings demonstrate a heretofore unrecognized synergism between C-peptide and insulin that could have physiological importance in the regulation of perfusion distribution in skeletal muscle.

Key words: phosphodiesterase 3, UT-15C, diabetes, microcirculation, adenosine triphosphate
Introduction

The efficient matching of oxygen (O_2) delivery to meet local O_2 need in skeletal muscle requires a mechanism to direct blood flow (O_2 delivery) to areas with reduced tissue oxygen tension (pO_2). The cell responsible for the delivery of O_2, the circulating erythrocyte, is in an ideal position to participate in such a regulatory mechanism. Exposure to reduced pO_2 stimulates the release of O_2 as well as the potent vasodilator adenosine triphosphate (ATP) from erythrocytes. Importantly, erythrocyte-released ATP produces both local vasodilation and dilation that is conducted to upstream feed arterioles resulting in increases in O_2 supply to local areas of O_2 need (1, 4, 7, 39).

Low O_2-induced ATP release from erythrocytes is the result of activation of a well characterized signaling cascade that requires activation of the heterotrimeric G protein, Gi, as well as increases in cAMP that are regulated by the activity of phosphodiesterase 3 (PDE3) (14, 16, 36). Insulin is known to activate PDE3 in several tissues (18, 27, 46), and erythrocytes possess insulin receptors (12, 13, 33). Importantly, incubation of human erythrocytes with insulin was reported to inhibit low O_2-induced ATP release via activation of PDE3 (15, 16). In support of the critical role for PDE3 in this signaling pathway, it was shown that the PDE3 inhibitor, cilostazol, attenuated insulin-induced inhibition of ATP release from healthy human erythrocytes in response to activation of Gi (16). Since low O_2-induced ATP release from erythrocytes is involved in the optimal distribution of blood flow to skeletal muscle and circulating insulin is necessary for the control of blood glucose, some mechanism must exist, \textit{in vivo}, to permit this important physiological response to low O_2 to occur in the presence of insulin. One logical mechanism would be through the action of the connecting peptide that is co-released with insulin (42).
Insulin is formed in beta-cells of the pancreas as proinsulin, a single chain composed of an A-chain, a B-chain, and a thirty-one amino acid connecting peptide (C-peptide) (42). Upon enzymatic cleavage, mature insulin is separated from C-peptide, and both of these peptides are released together into the circulation. However, the half-life of C-peptide is approximately thirty minutes while insulin has a half-life of only three to five minutes (5, 34, 42). Thus, circulating C-peptide levels equal or exceed those of insulin.

Although C-peptide was initially considered to be an inert byproduct of insulin biosynthesis, recent studies have demonstrated several biological activities (10, 11, 20, 44), and the role of C-peptide in the microcirculation remains under investigation. It is of interest that, in humans with diabetes, C-peptide levels approaching physiological concentrations have been correlated with a reduced incidence of microvascular complications (2, 41). It has also been reported that C-peptide can oppose diabetes-associated renal and cerebral complications (45). Indeed, the value of therapeutic replacement of C-peptide in type 1 diabetes has been in clinical trials with additional studies in progress at this time (3, 6). In contrast to the beneficial effects of physiological concentrations of C-peptide, the supra-physiological levels reached in humans with insulin resistance have been correlated with the development of atherosclerosis (32, 43). Thus the role of C-peptide in the maintenance of normal vascular function remains controversial. For these reasons, it is critical to understand the effects of physiological concentrations and ratios of C-peptide to insulin on healthy human erythrocytes, cells that participate in the distribution of perfusion in skeletal muscle.

In spite of the fact that insulin and C-peptide are co-released and circulate together, studies that examine the combined effects of the two peptides on the vasculature or on erythrocytes, in particular, are limited. Although it has been shown that C-peptide produces concentration-dependent vasodilation of skeletal muscle arterioles only in the presence of a
small, non-vasodilating concentration of insulin, these studies were performed in the absence of erythrocytes (24). It was also reported that C-peptide stimulates ATP release from erythrocytes; however, those studies used concentrations of C-peptide exceeding those found *in vivo* (25,29,30). In addition, those studies did not determine the effect of C-peptide on a physiological stimulus for ATP release from these cells and did not investigate any relationship between C-peptide and insulin (25, 29, 30).

Here, we investigated the effects of insulin and C-peptide alone and when combined at physiological ratios and concentrations (17, 22) on low O$_2$-induced ATP release from human erythrocytes. The finding of a heretofore unrecognized positive synergistic effect of the peptides on this stimulus for ATP release in the microcirculation would have important implications for the prevention and treatment of peripheral vascular disease.

**Methods**

**Isolation of human erythrocytes**

Blood was obtained from healthy volunteers, 14 females and 9 males with an average age of 43 years (range 20-65 years), via venipuncture using a syringe containing heparin (500 units). Blood was centrifuged at 500 g at 4°C for 10 minutes, 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 CaCl$_2$, 140.5 NaCl, 1.2 MgSO$_4$, 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 of firefly lantern extract (10 mg/mL distilled water, FLE 250; Sigma, St Louis, MO, USA) and 100 µL of D-luciferin solution (10 mg/20 mL distilled water; Research Products International, Mount Prospect, IL, USA). The light emitted from the reaction with ATP was quantified using a luminometer (TD 20/20; Turner Designs). A standard curve was generated for each experiment. ATP values were normalized to the amount released from 4 x10^8 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 800 x g for 10 minutes at room temperature. The presence of hemoglobin in the supernatant was determined by measurement of light absorbance at 405 nm (21). Samples in which free hemoglobin increased were not included resulting in the exclusion of only 12 of 88 experiments.

**Determination of ATP release from erythrocytes in response to exposure to reduced O₂ tension in the presence and absence of C-peptide and/or insulin**

Isolated erythrocytes were diluted to a 20% hematocrit in a Ringers buffer containing bicarbonate (4.7 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. Erythrocyte suspensions were equilibrated for 30 minutes in a thin-film tonometer (model DEQ1, Cameron Instrument Company) with gas containing 15% O₂, 6% CO₂, balance N₂ (normoxia, pO₂ = 111 ± 6 mmHg) and ATP levels were determined. The erythrocytes were then exposed to reduced O₂ (0% O₂, 6% CO₂, balance N₂, pO₂ = 16 ± 6 mmHg) for 10 minutes with ATP levels again determined.
The effect of a 20 minute pretreatment of erythrocytes with insulin (0.05, 0.1, or 0.5 nM) (Humalog U 100®, Eli Lilly) or C-peptide (0.3 and 1 nM) (human, amino acid sequence 55-89, Sigma, 97.4% pure by HPLC analysis) on low O₂-induced ATP release was determined. In separate studies, the effect of co-administration of C-peptide and insulin at various concentrations and ratios of C-peptide to insulin (1:1 to 6:1) on low O₂-induced ATP release was also determined.

**Determination of ATP release from erythrocytes in response to incubation with a prostacyclin analog in the presence and absence of insulin or C-peptide**

Isolated erythrocytes (20% hematocrit) were incubated with the prostacyclin analog, UT-15C (1 µM) (United Therapeutics), in the presence and absence of a 25 minute pretreatment at room temperature with either 1 nM insulin or 1 nM C-peptide. UT-15C-induced ATP release was determined at 5, 10 and 15 minutes after addition of the prostacyclin analog. The maximum response is reported.

**Statistical analysis**

Statistical significance among experiments was determined using an ANOVA. In the event that the F-ratio indicated that a change had occurred, a Fisher’s LSD test was performed to identify individual differences between groups. Results are reported as means ± SE. P values of 0.05 or less were considered to indicate a significant difference.

**Results**

**Effect of Insulin on Low O₂-induced ATP Release from Erythrocytes**

Erythrocytes release O₂ and the vasodilator ATP in response to exposure to low O₂ via a signaling pathway that requires increases in intracellular cAMP that are regulated by phosphodiesterase 3 (PDE3) (14, 16, 36). Insulin, at levels reported to be present in humans
with insulin resistance, increases PDE3 activity resulting in inhibition of ATP release (15, 16). Here, we determined the effect of insulin at concentrations that represent those observed in healthy humans on this physiological response (17, 22). Insulin at a concentration of 0.05 nM had no effect on low O_2-induced ATP release. However, at concentrations of 0.1 nM and 0.5 nM, insulin inhibited the response to low O_2 (Figure 1). These data establish that insulin-induced inhibition of low O_2-induced ATP release is concentration-dependent.

**Effect of C-peptide on Low O_2-induced ATP Release from Erythrocytes**

We next determined the effect of C-peptide in the absence of insulin on low O_2-induced ATP release from human erythrocytes. At 1 nM, a concentration of C-peptide reached under postprandial conditions in healthy humans (17, 22), low O_2-induced ATP release was inhibited (Figure 2). However, when erythrocytes were incubated with 0.3 nM C-peptide, a concentration found during fasting, low O_2-induced ATP release was not prevented (Figure 2). Thus, similar to insulin, C-peptide-induced inhibition of low O_2-induced ATP release is concentration dependent.

**Effect of Insulin or C-peptide on UT-15C-induced ATP Release from Erythrocytes**

In addition to exposure to low O_2, activation of erythrocyte prostacyclin receptors results in ATP release via a signaling pathway that differs from that initiated by exposure to low O_2 (37, 40). We determined that pretreatment of erythrocytes with either 1 nM insulin or 1 nM C-peptide had no effect on ATP release stimulated by exposure of these cells to the prostacyclin analog, UT-15C (Figure 3, A and B). Thus, the adverse effects of insulin and C-peptide on low O_2-induced ATP release are not the result of a general inhibition of all signaling pathways for release of ATP from these cells.
Effect of Co-administration of C-peptide and Insulin at Various Ratios and Concentrations on Low O₂-induced ATP Release from Erythrocytes

Although administration of either C-peptide or insulin alone at postprandial concentrations had inhibitory effects on low O₂-induced ATP release, this is not representative of the in vivo relationship between these two peptides. These peptides are co-released from the pancreas in equimolar amounts, but due to differing degradation rates, a range of C-peptide to insulin ratios is present physiologically with C-peptide levels equaling or exceeding those of insulin (17, 22). We examined the effect of co-administration of C-peptide and insulin at a 1:1 ratio using concentrations (1 nM each) that inhibited low O₂-induced ATP release when the peptides were administered alone (Figures 1 and 2). Importantly, co-administration of the peptides at this concentration did not inhibit low O₂-induced ATP release (Figure 4).

We next examined the effects of a range of C-peptide to insulin ratios on low O₂-induced ATP release from human erythrocytes with the concentration of insulin kept constant at 0.5 nM. Under these conditions, C-peptide to insulin ratios of 3:1, 4:1, 5:1, and 5.5:1 did not inhibit low O₂-induced ATP release (Figure 5). However, at a ratio of 6:1 (3 nM C-peptide and 0.5 nM insulin), low O₂-induced ATP release was inhibited similarly to that seen when C-peptide was administered alone (Figures 3 and 6).

Finally, we determined if the concentration of the two peptides, as well as their ratio, was critical for maintenance of low O₂-induced ATP release. In these studies, the concentration of insulin was reduced ten-fold (0.05 nM) to values that represent those found during fasting. At both the higher (Figure 5) and lower (Figure 6a) concentrations of insulin, a 4:1 ratio of the peptides did not inhibit low O₂-induced ATP release. However, although a 6:1 ratio of the two peptides inhibited ATP release at the higher concentrations (0.5 nM insulin, Figure 5), at the lower concentrations that same ratio did not (0.05 nM, Figure 6, B). These results provide support for the hypothesis that both the ratios and concentrations of C-peptide
and insulin are critical in modulating the effect of these peptides on low $O_2$-induced ATP release from human erythrocytes.

Discussion

It is well recognized that erythrocytes are far more than simple $O_2$ carriers, but are, in fact, complex cells that contribute actively to the control of local vascular caliber (1, 4, 7, 9, 39). We and others have shown that erythrocytes of healthy humans stimulate vasodilation in the skeletal muscle microcirculation via their ability to release ATP in response to exposure to reduced $O_2$ tension (1, 4, 8, 35, 38). This erythrocyte-released ATP then binds to purinergic receptors on the endothelium (23, 26, 31) inducing both local vasodilation as well as dilation that is conducted to feed arterioles resulting in increased blood flow and $O_2$ delivery (1, 4, 7, 39). Thus, in skeletal muscle, the ability of erythrocytes to release both $O_2$ and ATP in response to exposure to low $O_2$ permits these circulating cells to participate in the regulation of blood flow to provide optimal delivery of $O_2$ and nutrients.

The release of ATP from erythrocytes exposed to low $O_2$ requires activation of a signaling pathway that is inhibited in the presence of insulin at a concentration found in humans with insulin resistance (15). Here we report that, in the absence of C-peptide, concentrations of insulin that are present in humans in the postprandial period (17, 22) also inhibit low $O_2$-induced ATP release from erythrocytes (Figure 1). Studies in which insulin alone is administered ignore a potential synergistic role for C-peptide in the microcirculation.

Although C-peptide was previously considered to be an inactive by-product of insulin synthesis and release, more recently this peptide has been reported to have biological activity (10, 11, 20, 44). The potential importance of physiological levels of C-peptide in circulatory control is suggested by reports that in type 1 and type 2 diabetes higher residual endogenous
C-peptide levels correlate negatively with the incidence of neuropathy, nephropathy, and retinopathy (2, 41). In contrast, C-peptide may not be beneficial at supra-physiological concentrations such as those observed in prediabetes (32, 43). In insulin resistant individuals, elevated C-peptide has been associated with increased levels of the proinflammatory cytokine, IL-6 (19) and C-peptide has been found in arteriosclerotic lesions in humans with diabetes (28). However, these studies do not address the relationship between C-peptide and insulin in circulatory control.

Previous studies investigating the effects of C-peptide on erythrocytes reported that the peptide directly promoted ATP release (25, 29, 30). However, those studies used either supra-physiological concentrations of C-peptide (17) and/or did not address either a physiological stimulus for ATP release or a time course for ATP release that is relevant for the moment-to-moment regulation of blood flow and oxygen delivery in vivo (25, 29, 30). We report here that C-peptide alone, at a concentration reported to be present in humans during the postprandial period (1 nM) (17), inhibits low O$_2$-induced ATP release from human erythrocytes (Figure 2). Although studies in which C-peptide is administered in the absence of insulin are interesting, they do not replicate the physiological relationship between the two peptides. In contrast, insulin is administered to humans with diabetes in the absence of C-peptide. Therefore we investigated the effect of several concentrations of insulin alone on low O$_2$-induced ATP release (Figure 1). We determined that both C-peptide and insulin, given alone, inhibit low O$_2$-induced ATP release in a concentration-dependent manner (Figures 1 and 2). It is important to note that this inhibition of low O$_2$-induced ATP release is not due to a general failure to release ATP from erythrocytes since the peptides do not inhibit ATP release in response to prostacyclin receptor activation which initiates a distinct signaling pathway for ATP release from erythrocytes (Figure 3) (37, 40).
Although incubation with either C-peptide or insulin alone can inhibit low O₂-induced ATP release from erythrocytes, it is important to recognize that these peptides are co-released and circulate together. Therefore, it is critical to determine if C-peptide and insulin act synergistically with respect to this important physiological response. In support of such a relationship, it has been reported that C-peptide stimulates concentration-dependent vasodilation of isolated arteries only when a small, non-vasodilating concentration of insulin is present (24). In that study, co-administration of insulin with increasing concentrations of C-peptide resulted in greater and more uniform increases in vasodilation than was seen with C-peptide alone. Although this result demonstrates a synergistic effect of C-peptide and insulin directly on arterioles, no previous studies have addressed the effect of physiological concentrations and ratios of C-peptide to insulin on ATP release from erythrocytes. Here, we report that at a ratio of C-peptide to insulin of 1:1 and at a concentration of each peptide of 1 nM, a concentration that has been measured in the portal vein (22), these peptides do not inhibit low O₂-induced ATP release from human erythrocytes (Figure 4). However, although insulin is co-released with C-peptide in vivo, due to differing clearance rates, circulating C-peptide levels exceed those of insulin (17, 22, 42).

To evaluate further the synergistic relationship between C-peptide and insulin on low O₂-induced ATP release from healthy human erythrocytes, we measured ATP release in the absence and presence of varying ratios of the two peptides. In these studies, the concentration of insulin was held constant at 0.5 nM, a concentration that can be achieved in healthy humans under postprandial conditions (17, 22). When C-peptide and insulin were co-administered at ratios of 1:1 to 5.5:1 (C-peptide to insulin) low O₂-induced ATP release from healthy human erythrocytes was maintained (Figure 5). However, when the ratio of C-peptide to 0.5 nM insulin was increased to 6:1, low O₂-induced ATP release is compromised (Figure
5). A ratio of C-peptide to insulin of 6:1 with an insulin concentration of 0.5 nM could be
anticipated to be present in humans under postprandial conditions (17, 22). In contrast, low
O₂-induced ATP release was not inhibited at the same ratio of the two peptides (6:1) when the
concentration of insulin was reduced to represent values that are present in the fasting state
(0.05 nM) as shown in Figure 6B (17, 22). Thus, both the ratio and concentration of C-peptide
and insulin are critical factors that determine the effect of these peptides on low O₂-induced
ATP release from human erythrocytes.

Perspectives and Significance

Our data are consistent with the hypothesis that co-release of C-peptide and insulin in vivo prevents adverse effects of insulin alone on low O₂-induced ATP release from erythrocytes permitting these cells to participate fully in the optimal matching of O₂ delivery with need in skeletal muscle. These findings also suggest that administration of the combination of C-peptide with insulin, at appropriate physiological ratios, could aid in the prevention and treatment of the peripheral vascular disease associated with diabetes. In light of the use of C-peptide in clinical trials for the treatment of humans with diabetes, it becomes increasingly important to understand the relationship between C-peptide and insulin in erythrocyte physiology.

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References:


Figure Legends:

Figure 1: Effect of insulin on low O₂-induced ATP release from erythrocytes. Isolated erythrocytes were incubated with vehicle (saline, control) or insulin at various concentrations for 20 minutes while exposed to a gas mixture containing 15% O₂, 6% CO₂, balance N₂ (normoxia) prior to measurement of ATP levels. This was followed by exposure to 0% O₂, 6% CO₂, balance N₂ (low O₂) for 10 minutes prior to a second measurement of ATP levels. Values are mean ± SE. * = significant increase from normoxia (P<0.05).

Figure 2: Effect of C-peptide on low O₂-induced ATP release from erythrocytes. Isolated erythrocytes were incubated with saline (control) or C-peptide at 0.3 nM (n=4) or 1 nM (n=6) for 20 minutes while exposed to a gas mixture containing 15% O₂, 6% CO₂, balance N₂ (normoxia) prior to measurement of ATP release. This was followed by exposure to 0% O₂, 6% CO₂, balance N₂ (low O₂) for 10 minutes prior to a second measurement of ATP release. Values are mean ± SE. * = significant increase from normoxia (P<0.05).

Figure 3: Effect of insulin or C-peptide on UT-15C-induced ATP release from erythrocytes. Isolated erythrocytes were incubated with 1 μM UT-15C in the absence or presence of a 25 minute pre-incubation with 1 nM insulin (Panel A, n=5) or 1 nM C-peptide (Panel B, n=5). The maximum ATP release stimulated by UT-15C treatment was reported. Values are mean ± SE. * = significant increase from control (P<0.05).

Figure 4: Effect of a 1:1 ratio of C-peptide to insulin on low O₂-induced ATP release from erythrocytes. Isolated erythrocytes were incubated with saline (control) or 1 nM C-peptide and 1 nM insulin (n=5) for 20 minutes while 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). Values are mean ± SE. * = significant increase from normoxia (P<0.05).

**Figure 5: Effect of physiological ratios of C-peptide to insulin on low O₂-induced ATP release from erythrocytes.** Isolated erythrocytes were incubated with various ratios of C-peptide (1.5, 2.0, 2.5, 2.75, or 3.0 nM) to insulin (0.5 nM) for 20 minutes while 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). Values are mean ± SE. * = significant increase from normoxia (P<0.05).

**Figure 6: Effect of physiological ratios of C-peptide to insulin at lower concentrations on low O₂-induced ATP release from erythrocytes.** Isolated erythrocytes were incubated with saline (control), 0.20 nM C-peptide and 0.05 nM insulin (4:1 ratio, n=4, panel A), or 0.30 nM C-peptide and 0.05 nM insulin (6:1 ratio, n=4, panel B) for 20 minutes while 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). Values are mean ± SE. † = significant increase from normoxia (P<0.01).
Figure 1:
Figure 2:

% Increase in ATP Release

Control  0.30 nM  1 nM

C-Peptide

*
Figure 3:

A) ATP (mM) per 4x10^6 erythrocytes in control and UT-15C (1 μM) conditions with and without insulin.

B) ATP (mM) per 4x10^6 erythrocytes in control and UT-15C (1 μM) conditions with and without C-peptide.
Figure 4:
Figure 5:

% Increase in ATP Release
egin{align*}
\text{Ratio of C-peptide to Insulin} \\
3:1 & \quad n=6 \\
4:1 & \quad n=7 \\
5:1 & \quad n=7 \\
5.5:1 & \quad n=5 \\
6:1 & \quad n=5
\end{align*}
Figure 6:

A)

B)