Title: Pannexin 1 is the Conduit for Low Oxygen Tension-Induced ATP Release from Human Erythrocytes

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Abstract

Erythrocytes release ATP in response to exposure to the physiological stimulus of lowered oxygen (O₂) tension as well as pharmacological activation of the prostacyclin receptor (IPR). ATP release in response to these stimuli requires the activation of adenylyl cyclase, accumulation of cAMP and activation of protein kinase A. The mechanism by which ATP, a highly charged anion, exits the erythrocyte in response to lowered O₂ tension or receptor-mediated IPR activation by iloprost is unknown. It was demonstrated previously that inhibiting pannexin 1 with carbenoxolone inhibits hypotonically-induced ATP release from human erythrocytes. Here we demonstrate that three structurally dissimilar compounds known to inhibit pannexin 1 prevent ATP release in response to lowered O₂ tension but not to iloprost-induced ATP release. These results suggest that pannexin 1 is the conduit for ATP release from erythrocytes in response to lowered O₂ tension. However, the identity of the conduit for iloprost-induced ATP release remains unknown.

Key Words: iloprost, carbenoxolone, probenecid, red blood cell, CFTR
Introduction

Erythrocytes contribute to the regulation of vascular caliber by virtue of their ability to release adenosine triphosphate (ATP) (14-17, 48, 50). ATP released from erythrocytes binds to purinergic receptors on the vascular endothelium which leads to the local formation of endothelium-derived vasodilators such as nitric oxide, prostaglandins, and endothelium derived hyperpolarizing factor (20, 46, 50).

Erythrocytes release ATP in response to mechanical deformation, exposure to lowered oxygen (O_2) tension, and in response to incubation with pharmacological agents such as the prostacyclin analog, iloprost (5, 44, 45). ATP release in response to these various stimuli requires the activation of adenylyl cyclase, accumulation of cAMP, and activation of PKA (3, 43, 47). Although several components of the signaling pathway for ATP release from erythrocytes have been investigated, the identity of the conduit by which ATP exits that cell is not fully characterized. In other cell types, several membrane channels, including connexin hemichannels, voltage dependent anion channels, volume regulated anion channels, and ATP binding cassette proteins have been implicated as conduits for ATP release (2, 27, 29, 37, 40, 41). In addition, the cystic fibrosis transmembrane conductance regulator (CFTR), an ATP binding cassette protein required for ATP release from erythrocytes in response to mechanical deformation (25, 45), was once considered to be a possible ATP conduit in cells. However, more recent studies demonstrate that CFTR is not likely to serve as an ATP conduit itself but rather that it regulates other channels that serve that role (1, 7, 19, 24, 51).
Recently, the protein family of pannexins, orthologs of the invertebrate innexins, has been implicated as ATP conduits in many cell types including astrocytes (23), airway epithelial cells (36), and erythrocytes (28). Locovei et al. demonstrated that pannexin 1 is present in erythrocytes and that ATP release in response to hypotonic stress can be inhibited by the pannexin 1 inhibitor, carbenoxolone (28). In addition, that group was able to detect erythrocyte pannexin 1-like channel activity when the erythrocyte was depolarized (28). Here we extend the former observation and show that treating erythrocytes with three pannexin 1 inhibitors prevents low O$_2$ tension-induced ATP release. In contrast, these inhibitors had no effect on ATP release in response to receptor-mediated activation of the prostacyclin receptor (IPR) with iloprost. In addition we demonstrate that, similar to mechanical deformation-induced ATP release, CFTR is required for low O$_2$ tension- and iloprost-induced ATP release. These results demonstrate that although CFTR is required for both low-O$_2$ tension and iloprost induced ATP release, the final conduits responsible for that ATP release are different.
Methods

Isolation of Healthy Human Erythrocytes

Human blood was collected from 12 males and 17 females (average age: 34.7 ± 2.3 years; range: 23-60 years) by venipuncture using a syringe containing heparin (500 units) and centrifuged at 500 x g at 4 °C for 10 min. The plasma, buffy coat, and uppermost erythrocytes were removed by aspiration and discarded. The remaining erythrocytes were washed three times in buffer containing 21.0 mM tris(hydroxymethyl)aminomethane, 4.7 mM KCl, 2.0 mM CaCl₂, 140.5 mM NaCl, 1.2 mM MgSO₄, 5.5 mM glucose, and 0.5% bovine albumin fraction V, final pH 7.4. Erythrocytes isolated in this fashion contain less than 1 leukocyte per 50 high power fields (approximately 8–10 leukocytes per mm³) and are devoid of platelets (21). Cells were prepared on the day of use.

Determination of ATP release from Erythrocytes in Response to Exposure to Reduced O₂ Tension

Washed erythrocytes were diluted to a 20% hematocrit in a buffer containing bicarbonate (4.7 KCl, 2.0 mM CaCl₂, 140.5 mM NaCl, 1.2 mM MgSO₄, 11 mM glucose, 23.8 mM NaHCO₃, 0.2% dextrose, 0.5% BSA, pH 7.4) at 37 °C. Erythrocytes were equilibrated for 30 min in a thin film blood tonometer (Dual Equilibrator model DEQ1, Cameron Instrument Company)(9) with a gas mixture containing 15% O₂, 6% CO₂, balance N₂ (normoxia, pO₂=110.8±1.7 mmHg). The erythrocytes were then exposed sequentially to gases with compositions of 4.5 % O₂, 6% CO₂, balance N₂ and 0% O₂, 6% CO₂, balance N₂. The pH, pO₂, and pCO₂ were determined after a 10 min
exposure to each gas mixture using a blood gas analyzer (model pHox, Nova Biomedical). Exposure of erythrocytes sequentially to gases with compositions of 4.5 % O₂, 6% CO₂, balance N₂ and 0% O₂, 6% CO₂, balance N₂ for ten min each, resulted in a pO₂ of 34.1±0.59 and 12.3±1.0 mmHg of the erythrocyte suspension, respectively. The amount of ATP released from erythrocytes was determined during normoxia and following the 10 min exposure to each gas mixture.

**Determination of ATP release from Erythrocytes in Response to Exposure to Reduced O₂ Tension in the Absence and Presence of a CFTR Inhibitor**

Experiments in which erythrocytes were exposed to lowered O₂ tension, identical to those described above, were performed in the presence or absence of glybenclamide (10 µM, Sigma) or its vehicle (DMF) for 30 min. Inhibition of ATP release from erythrocytes was studied using the 0% O₂, 6% CO₂, balance N₂ gas composition because that is when maximal low O₂-induced ATP release occurred. The concentration of glybenclamide was chosen based on previous studies (45).

**Determination of ATP release from Erythrocytes in Response to Exposure to Iloprost in the Absence and Presence of a CFTR Inhibitor**

Isolated erythrocytes, exposed to room air and temperature, were diluted to a hematocrit of 20% in a buffer containing 21.0 mM tris(hydroxymethyl)aminomethane, 4.7 mM KCl, 2.0 mM CaCl₂, 140.5 mM NaCl, 1.2 mM MgSO₄, 5.5 mM glucose, and 0.5% bovine albumin fraction V, final pH 7.4. The erythrocyte suspension was then preincubated with glybenclamide (10µM) or its vehicle (DMF) for 30 min. Erythrocytes
were then incubated with iloprost (ILO, 1 μM, Cayman) and ATP release was measured after 5, 10, and 15 min. The maximal response to ILO is reported.

**Determination of ATP release from Erythrocytes in Response to Exposure to Reduced O₂ Tension in the Absence and Presence of Pannexin 1 Inhibitors**

Experiments in which erythrocytes were exposed to lowered O₂ tension, identical to those described above, were performed in the presence or absence of carbenoxolone (100 μM, Sigma), probenecid (100 μM, Sigma), ¹⁰panx1 peptide (200 μM, Applied Biosystems) or their vehicles (DMF or phosphate buffered saline (PBS)) for 30 min (20 min for experiments performed in the presence of ¹⁰panx1 peptide). Concentrations of the various inhibitors were chosen based on other studies as well as published IC₅₀ values (28, 34, 42).

**Incubation of Erythrocytes with Iloprost (1μM) in the Absence and Presence of Pannexin 1 Inhibitors**

Experiments in which erythrocytes were exposed to iloprost, identical to those described above, were performed using erythrocytes which were preincubated with either carbenoxolone (100 μM), probenecid (100 μM), or ¹⁰panx1 peptide (200 μM) or their vehicles (DMF or PBS). Erythrocytes were then incubated with iloprost (ILO, 1 μM) and ATP release was measured after 5, 10, and 15 min. The maximal response to ILO is reported.
Measurement of ATP

ATP was measured using the luciferin–luciferase assay as described previously (45, 49). A 200 μL sample of erythrocyte suspension (0.04% hematocrit) was injected into a cuvette containing 100 μL of firefly lantern extract (10 mg/mL; Sigma) and 100 μL of a solution of synthetic D–luciferin (50 mg/100 mL; RPI). The light emitted was detected using a luminometer (Turner Designs). A standard curve for ATP (CalBioChem) was obtained for each experiment. Cell counts were obtained from the suspension of erythrocytes and amounts of ATP measured were normalized to 4x10^8 cells/mL.

Measurement of Total Intracellular ATP of Erythrocytes

A known number of erythrocytes determined by counting were lysed in distilled water. ATP was measured as described above. Values were normalized to ATP concentration per erythrocyte.

Measurement of Free Hemoglobin

To exclude the possibility that hemolysis contributed to the levels of ATP measured, after ATP determinations, samples were centrifuged at 500 x g at 4°C for 10 min and the presence of free hemoglobin in the supernatant was determined by light absorption at a wavelength of 405 nm. If any free hemoglobin was detected, the studies were not included to ensure that hemolysis was not influencing amounts of extracellular ATP measured.
**Data Analysis**

Statistical significance among groups was determined using an analysis of variance (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. Results are reported as means ± the standard error (SE). In all studies, ‘$n$’ refers to the number of different individuals from which erythrocyte samples were obtained. For each set of experiments, no sample from an individual was used twice. However, some individuals were studied in more than one experimental protocol.

**Institutional Approval**

The protocol used to obtain blood from humans was approved by the Institutional Review Board of Saint Louis University.
Results

Effect of Exposure of Human Erythrocytes to Low O₂ Tension on ATP Release

Exposure of erythrocytes to a gas composed of 15% O₂, 6% CO₂, balance N₂ for 30 min resulted in a pH, pCO₂, and pO₂ of 7.37±0.01, 35.4 ±0.7 mmHg, and 110.8 ±1.7 mmHg, respectively. Subsequent exposure of erythrocytes to a gas consisting of 4.5% O₂ for 10 min resulted in a pH, pCO₂, and pO₂ of 7.37±0.01, 36.0 ±0.7 mmHg, and 34.1 ± 0.6 mmHg, respectively. Finally, exposure of erythrocytes to 0% O₂ for 10 min resulted in a pH, pCO₂, and pO₂ of 7.38±0.01, 36.3 ±0.5 mmHg, and 12.3 ± 1.0 mmHg, respectively. The lower the O₂ tension to which erythrocytes were exposed, the greater the amount of ATP released (Figure 1).

Effect of Glybenclamide (10 μM) on Low O₂ Tension- and Iloprost-Induced ATP Release from Human Erythrocytes

Glybenclamide is an irreversible inhibitor of CFTR that prevents ATP release from erythrocytes in response to mechanical deformation (45). Here we used glybenclamide to determine if CFTR is a part of the low O₂ tension- or iloprost-induced ATP release pathways. Pretreatment of erythrocytes with glybenclamide (10μM) resulted in a decrease in ATP release in response to exposure to lowered O₂ tension (pO₂=17.7±0.8 mmHg) or iloprost (Figure 2). Importantly, glybenclamide (10 μM) was previously demonstrated to have no effect on intracellular ATP levels (45) so inhibition of ATP release cannot be attributed to decreased ATP synthesis. Because glybenclamide has been demonstrated to also inhibit ATP sensitive potassium channels, studies were performed to determine whether activation of ATP sensitive potassium channels would...
lead to ATP release. Cromakalim (10 µM, 30 min), an activator of ATP sensitive potassium channels, and saline (control) treated cells released 2.2 ± 1.12 and 1.3 ± 0.35 nmoles/4 x 10^8 RBCs, respectively (n=5), demonstrating that activation of ATP sensitive potassium channels in erythrocytes does not lead to significant increases in ATP release.

Effect of Carbenoxolone (100µM), Probenecid (100 µM) and ^10^panx1 peptide (200 µM) on Low O2 Tension-Induced ATP Release from Human Erythrocytes

To determine if pannexin 1 is involved in low O2 tension-induced ATP release pathways, erythrocytes were pretreated with carbenoxolone (100 µM), probenecid (100 µM), or ^10^panx1 peptide (200 µM) or their respective vehicles. ATP release was measured before and after the cells were exposed to lowered O2 tension in the presence of an inhibitor (pO2=10.8±1.0 mmHg) or its vehicle (pO2=10.9±1.2 mmHg). Carbenoxolone (n=10), probenecid (n=5) and ^10^panx1 peptide (n=5) inhibited ATP release in response to exposure of erythrocytes to reduced O2 tension (Figure 3). All three inhibitors had no effect on intracellular ATP levels (Table 1).

Effect of Carbenoxolone (100µM), Probenecid (100 µM) and ^10^panx1 peptide (200 µM) on Iloprost-Induced ATP Release from Human Erythrocytes

In contrast to the results with cells stimulated with lowered O2 tension, cells incubated with the prostacyclin receptor agonist, iloprost, showed no decrease in ATP release in the presence of any of the three inhibitors of pannexin 1 (Figure 4).
Regulated release of ATP occurs in many tissues and contributes to complex signaling pathways within organ systems (18, 38, 39). Erythrocytes have been demonstrated to release ATP in response to physiological and pharmacological stimuli (5, 31, 33, 44). ATP that is released from the erythrocyte binds to purinergic receptors on the vascular endothelium which results in the local generation of vasodilators including nitric oxide and prostaglandins (20, 46).

The signaling pathways by which erythrocytes release ATP are under active investigation. However, the identity of the final conduit(s) for ATP release has been elusive. The recognized mechanisms by which cells can release ATP are through channels or transporters, via vesicular release, or through lysis of the cell. Since lysis of erythrocytes does not occur through a regulated signaling pathway and erythrocytes do not possess the cellular machinery required for the formation of vesicles (52), erythrocytes must release ATP through channels or transporters. Our laboratory has demonstrated that erythrocytes release ATP in response to physiological stimuli such as mechanical deformation (45) and exposure of erythrocytes to lowered O₂ tension (5, 48). In addition, erythrocytes possess prostacyclin receptors and release ATP in response to incubation with prostacyclin analogs, including iloprost (33, 44). It has previously been demonstrated that release of ATP in response to low O₂ tension or prostacyclin analogs requires the activation of two distinct heterotrimeric G proteins (30-33). Exposing erythrocytes to lowered O₂ tension activates the G protein, Gi (31, 32), whereas, exposing erythrocytes to iloprost stimulates the prostacyclin receptor which is
coupled to the G protein, Gs (33, 44). Activation of either G protein leads to the stimulation of adenylyl cyclase activity and the accumulation of cAMP (43, 44, 47).

It has also been demonstrated that CFTR is involved in mechanical deformation-induced ATP release from rabbit erythrocytes. Treating erythrocytes with glybenclamide or niflumic acid, known inhibitors of CFTR, decreases ATP release in response to this stimulus (45). In addition, erythrocytes from patients with cystic fibrosis do not release ATP in response to mechanical deformation (25). However, the role of CFTR in the pathways for ATP release in response to lowered O₂ tension or prostacyclin analogs has not been previously determined. In the present study, we demonstrate that glybenclamide also inhibits iloprost- and low O₂ tension-induced ATP release, demonstrating that CFTR is a part of the associated ATP release signal transduction pathways. The inhibitory effect of glybenclamide on ATP release was demonstrated to be through its action on CFTR and not due to an effect on ATP sensitive potassium channels (45). Although CFTR is clearly involved in these signaling pathways, studies have demonstrated that CFTR is not an ATP conduit itself but rather that it regulates the release of ATP via another channel (1, 7, 19).

Other candidates for ATP conduits include other ATP binding cassette proteins (MDR1)(2), maxi anion channels (27), connexins (10, 11), and more recently, pannexins (4, 22, 36). Pannexin 1 is classified as a ‘gap junction protein’ due to the sequence homology it shares with innexins (23, 26). Pannexins form channels with properties that make them attractive candidates for ATP conduits. These channels are voltage sensitive and can be opened by depolarization, mechanical perturbation, or increased concentrations of intracellular calcium (22, 36). In addition, unlike connexin
hemichannels, pannexin channels can be activated at normal physiological extracellular calcium concentrations (12).

It was demonstrated that carbenoxolone, a glycyrrhizic acid derivative shown to inhibit pannexin 1, decreased ATP release from erythrocytes in response to hypotonic stress (28). In that same study, the authors demonstrated that when erythrocytes were exposed to lowered O₂ tension, increased uptake of the dye carboxyfluorescein occurred in the erythrocyte, suggesting that a channel large enough for ATP to pass had opened. Although no data were presented, it was noted that treating erythrocytes with carbenoxolone decreased the amount of carboxyfluorscein taken up by these cells in response to lowered O₂ tension. Thus, these studies demonstrated that, in erythrocytes, pannexin 1 serves as a conduit for ATP release in response to hypotonic stress and suggested that pannexin 1 could be involved in ATP release in response to the more physiologically relevant stimulus of exposure of these cells to lowered O₂ tension. In the present study, we measured ATP release from human erythrocytes in response to lowered O₂ tension and demonstrated that treating erythrocytes with three chemically dissimilar agents known to inhibit pannexin 1 inhibits ATP release in response to this stimulus (Fig 3). In addition, we measured ATP release from human erythrocytes in response to the prostacyclin receptor agonist, iloprost. We demonstrate that pannexin 1 inhibitors do not inhibit ATP release in response to this stimulus (Fig 4).

Carbenoxolone, probenecid, and ¹⁰panx1 peptide have all been demonstrated to inhibit pannexin 1 in other cell types (28, 34, 42). In addition to inhibiting pannexin 1, carbenoxolone has also been demonstrated to inhibit other channels, including connexins and volume rectifying anion channels (VRAC), thought to serve as ATP
conduits in non-erythroid cells (6, 8, 54). In the study by Locovei et al., the presence of connexin 43 was not detected in erythrocytes (28). In addition, the channel properties for the opening of connexins (ie. low extracellular calcium concentration (12, 35)) makes it highly unlikely that the inhibitory effects of carbenoxolone on ATP release from the erythrocytes is due to inhibition of connexins. Because carbenoxolone can also inhibit VRAC (54), we examined the effects of 2 other inhibitors of pannexin 1. Probenecid was first shown to be capable of inhibiting pannexin 1 in a study using frog erythrocytes (42). In addition, probenecid does not inhibit currents formed by connexin 46 or the chimeria connexin 32E143 (42). To date, no studies have demonstrated that probenecid is capable of inhibiting VRAC. The last inhibitor used was $^{10}\text{panx1}$ peptide, a mimetic peptide that was shown to inhibit pannexin 1 through a steric block of the channel (13, 53). Like carbenoxolone, the $^{10}\text{panx1}$ peptide has been demonstrated to also block connexins. However, no studies have demonstrated that this peptide is capable of inhibiting VRACs. The ability of all three chemically dissimilar pharmacological agents known to inhibit pannexin 1 to decrease ATP release in response to lowered O$_2$ tension supports the role of pannexin 1 as an ATP conduit in the erythrocyte activated by this stimulus.

In addition to its role in the erythrocyte, pannexin 1 has been implicated as an ATP conduit in many cell types including astrocytes (23), airway epithelial cells (36), and taste buds (22). In many of these cell types the stimulus for ATP release was exposure of the cell to hypo-osmotic stress (mechanical perturbation) or depolarization, both stimuli that are known to be capable of directly activating pannexin currents. The
mechanism by which pannexin 1 is activated in the erythrocyte in response to the stimulus of lowered O$_2$ tension is not known.

Previous studies, coupled with the work presented here, establish that CFTR is a required component of diverse signaling pathways for ATP release from erythrocytes. Based on previous studies demonstrating the importance of anion counter-transport in anoxia induced ATP release from erythrocytes, the role of CFTR in low O$_2$ tension-induced ATP release could be as a regulator for chloride currents (19). It was demonstrated by Bergfeld et al. that substitution of permeant anions in the buffer surrounding erythrocytes with methanesulphonate, an impermeant anion, resulted in a significant reduction in ATP release in response to the combination of hypoxia and hypercapnia (5). These results suggest that permeant extracellular anions are necessary for ATP efflux in response to hypoxic conditions. Based on their findings, the authors theorized that the extracellular anions are required in order to maintain a charge balance across the membrane. Although no studies have been performed to determine the importance of permeant extracellular anions in iloprost-induced ATP release, the presence of CFTR in that signaling pathway would suggest that entry of extracellular anions is also required for ATP release in response to this stimulus.

In summary, the studies presented here provide a mechanism by which ATP exits erythrocytes exposed to low O$_2$ tension. We demonstrate that both CFTR and pannexin 1 are integral components of the low O$_2$ tension-induced ATP release pathway in human erythrocytes. Although CFTR activity is required for iloprost-induced ATP release, the ATP conduit in this pathway remains unknown.
Figure Legends

Figure 1: Effect of exposing human erythrocytes to lowered O₂ tension on ATP release

Erythrocytes were exposed to gas with a composition of 15% O₂, 6 % CO₂, balance N₂ in a tonometer. The cells were then exposed sequentially to gases with compositions of 4.5 % O₂, 6% CO₂, balance N₂ and 0% O₂, 6% CO₂, balance N₂. ATP was measured 30 min after exposure to 15% O₂ and 10 min after exposure to 4.5% O₂ and 0% O₂ (n=20). Values are the means ± SE. † p< 0.01, different from 15/6 group; *p<0.05, different from 4.5/6 group, n = the number of different individuals studied.

Figure 2: Effect of glybenclamide on low O₂ tension-(A) and iloprost (ILO)-(B) induced ATP release from human erythrocytes

(A) Erythrocytes were incubated with glybenclamide (10 µM) or its vehicle (DMF) for 30 min while exposed to gas with a composition of 15% O₂, 6 % CO₂, balance N₂ in a tonometer. The cells were then exposed to a gas with a composition of 0% O₂, 6% CO₂, balance N₂. ATP was measured 30 min after exposure to 15% O₂ and 10 min after exposure to 0% O₂ (n=7). Values are the means ± SE. * p< 0.05, different from all other values, n = the number of different individuals studied.

(B) ATP release was measured 5, 10, and 15 min after the addition of ILO (1 µM, n=5) to erythrocytes that were pre-incubated with glybenclamide (10 µM) or its vehicle (DMF) for 30 min. Peak ATP values are reported. The average time for the maximal increase in ATP release was 12.0 ±1.3 min after the addition of
iloprost. Values are the means ± SE. † p< 0.01, different from all other values, n= the number of different individuals studied.

Figure 3: Effect of carbenoxolone, probenecid, and $^{10}$panx1 peptide on low O$_2$ tension-induced ATP release from human erythrocytes

Erythrocytes were incubated with carbenoxolone (A, n=10, 100 µM), probenecid (B, n=5, 100 µM), $^{10}$panx1 peptide (C, n=5, 200 µM) or their respective vehicle (PBS) for 30 min (20 min for experiments with $^{10}$panx1 peptide) while exposed to gas with a composition of 15% O$_2$, 6% CO$_2$, balance N$_2$ in a tonometer. The cells were then exposed to a gas with a composition of 0% O$_2$, 6% CO$_2$, balance N$_2$. ATP was measured 30 min after exposure to 15% O$_2$ and 10 min after exposure to 0% O$_2$. Values are the means ± SE. † p< 0.01, different from all other values, n = the number of different individuals studied.

Figure 4: Effect of carbenoxolone, probenecid, and $^{10}$panx1 peptide on iloprost (ILO)-induced ATP release from human erythrocytes

ATP release was measured 5, 10, and 15 min after the addition of ILO (1 µM) to erythrocytes that were pre-incubated with (A) carbenoxolone (100 µM, n=6), (B) probenecid (100 µm, n=6), (C)$^{10}$panx1 peptide (200µM, n=6) or their respective vehicles for 30 min (20 min for experiments with $^{10}$panx1 peptide). Peak ATP values are reported. The average time for the maximal increase in ATP release for cells treated with carbenoxolone, probenecid, or $^{10}$panx1 peptide was 8.6 ±1.1, 12.1 ±1.1, or 12.9 ±1.1 min after the addition of iloprost, respectively. Values are the means ± SE.
*different from respective baselines, p<0.05; † different from respective baselines, p<0.01, n = the number of different individuals studied.
Acknowledgments

The authors thank J.L. Sprague and V. Sridharan for inspiration.

This work was supported by National Institutes of Health grants HL-64180 and HL-89094.
References


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Figure 2

A

![Bar graph showing ATP levels for Vehicle (DMF) and 10 μM Glybenclamide at 15/6 and 0/6 time points. The graph displays standard error bars.](image)

B

![Bar graph showing ATP levels at Baseline and ILO for Vehicle (DMF) and 10 μM Glybenclamide. The graph includes standard error bars.](image)