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Islet preconditioning via multimodal microfluidic modulation of intermittent hypoxia

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Abstract

Simultaneous stimulation of *ex vivo* pancreatic islets with dynamic oxygen and glucose is a critical technique for studying how hypoxia alters glucose-stimulated response, especially in transplant environments. Standard techniques using a hypoxic chamber cannot provide both oxygen and glucose modulations while monitoring stimulus-secretion coupling factors in real-time. Using novel microfluidic device with integrated glucose and oxygen modulations, we quantified hypoxic impairment of islet response by calcium influx, mitochondrial potentials, and insulin secretion. Glucose-induced calcium response magnitude and phase were suppressed by hypoxia, while mitochondrial hyperpolarization and insulin secretion decreased in coordination. More importantly, hypoxic response was improved by preconditioning islets to intermittent hypoxia (IH, 1min/1min 5%–21% cycling for 1 hour), translating to improved insulin secretion. Moreover, blocking mitochondrial K_{ATP} channels removed preconditioning benefits of IH, similar to mechanisms in preconditioned cardiomyocytes. Additionally, the multimodal device can be applied to a variety of dynamic oxygen-metabolic studies in other *ex vivo* tissues.

Introduction

Ex vivo study of islets of Langerhans, 50–400 μ m spheroidal aggregates of pancreatic endocrine cells, under controlled microenvironments is critical for understanding islet physiology. When islets are exposed to stimulatory glucose in the blood, uptake and glycolysis leads to ATP production, which opens up ATP-sensitive K⁺ (K_{ATP}) channels and results in calcium influx that triggers the exocytosis of insulin granules.^{1,2} Islets are capable of this glucose-insulin response because they are highly perfused *in vivo*, each within one cell length from a capillary vessel. Their insulin secretion is thus significantly influenced by the dynamics of blood flow and oxygen supply in addition to glucose gradients. Yet once the islets are isolated, both oxygen and nutrient supplies are constrained to a 100 μ m perimeter

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Author Contributions

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J.F.L., Y.W., J.O. and D.T.E. designed the experiments.

J.F.L. and Y.W. conducted the experiments.

A.B. and G.Y. designed the microinjectors.

H.J. and T.A.H. isolated the islets and ran ELISA.

Competing financial interests

The authors of the manuscript declare that they have no competing financial interests.

due to diffusion limitations.³ Recreating islet's physiological oxygen and glucose dynamics, key to *in vitro* and *ex vivo* studies, is difficult with hypoxic chambers that require elaborate flow and prevent continuous monitoring of islet functions. Additionally, transplant therapies for type I diabetes exposed isolated islets to hypoxia in the hepatic portal system,^{4,5} which has much lower PO₂ (<2%, 5–15 mmHg) compared to physiological pancreas (5.6%, 40 mmHg), for up to two weeks until revascularization.^{6,7} These hypoxic exposures are suggested to impair islet's glucose-insulin coupling mechanism.^{8,9} Among the coupling factors, calcium signaling, mitochondrial potential, and insulin kinetics can be easily monitored using microfluidics,^{10–13} but quantification of their hypoxic impairment is stymied by the lack of simultaneous stimulation and monitoring techniques. Therefore, combining microfluidic control of oxygen and islet monitoring can improve islet hypoxia studies.

Furthermore, tissues affected by ischemia and hypoxia could be trained or preconditioned for subsequent exposures. Preconditioning effects, first reported in Murry *et al.* 1986,¹⁴ demonstrated that pre-exposure to ischemia provided protection against subsequent cardiac infarctions. Preconditioning against cardiac infarction was also later demonstrated with intermittent hypoxia (IH).¹⁵ Similarly, ischemic preconditioning improves kidney graft function after transplantation¹⁶ and also protects against strokes in neural tissues,¹⁷ showing pervasive effects in numerous hypoxic tissues. In addition, evidences showed that K_{ATP} openers can benefit islets,^{18,19} suggesting preconditioning mechanism similar to cardiomyocytes may exist for islets. However, ischemic²⁰ and IH^{21,22} preconditioning have not been achieved in islets, despite the serious hypoxic exposures during pancreas procurement²³ and islet transplantation. Those dynamic hypoxia studies were complicated by the slow, inefficient hypoxic chambers that prevented real-time islet functional assessments. Thus, the simultaneous modulation of glucose and hypoxia, especially transient dynamics at microscale-level, represents a key technique to enable novel IH functional studies and achieve islet preconditioning.

To enable functional studies of islet hypoxia and IH, we built a multilayered microfluidic device based on our previous islet perfusion (vs. physiological perfusion)^{24,25} and cell-culture oxygen gradient devices.²⁶ It combines proven islet perfusate control with direct oxygen control in a stimulation sandwich, as shown in Fig. 1a–d. Furthermore, as the gas is continuously delivered via computerized microinjectors, we can modulate the oxygen concentration from 21% to close to 0% digitally with transient time less than 60s. The dynamic controls of oxygen and glucose microenvironment at the microscope allow a real-time multimodal protocol that would not be possible or extraordinarily cumbersome using standard hypoxic chambers. Using this device, calcium signaling, mitochondrial potentials, and insulin kinetics were monitored, allowing us to investigate 1) quantitative hypoxia vs. islet glucose response, 2) IH preconditioning for hypoxia, and 3) multi-parametric dynamics of IH mechanism in islets.

Experimental

Microfluidics and microinjector system

The device is comprised of three microfluidic—glucose, gas, microwell—and one blank gasket polydimethylsiloxane (PDMS) layers, Figure 1a. The glucose channels were soft-lithographed on a 700 μm (two 350 μm) SU8-2150 master, while the gas and microwell features were made on 100 μm SU8-2100 masters. Then, degassed PDMS was spun onto the microwell master at 900 rpm twice to form a 200 μm gas-permeable membrane layer. Degassed PDMS was also molded over the glucose, gas, and blank masters at 80°C for 3 hours to form the remaining layers. Access ports for all layers were punched and an 8 mm diameter, 250 μL chamber was also punched in the gasket layer. The layers were bonded

with 30s of exposure from a plasma wand (ETP, Inc.) and then baked at 120°C for 3 hours. Lastly, the completed device was leak-tested with simultaneous loading of water and compressed air at 2 psi.

To control the oxygen, a microinjector system was built with two precision microdispensing nozzles acquired from The Lee Company and connected with their outputs in a T-shaped mixer tubed to the microfluidic device, Fig. 1c. Inputs of 0% and 21% oxygen with 5% CO₂ and balance nitrogen were connected to respective nozzles, with pressure adjusted to 2 psi equally. These microinjectors were controlled with a USB National Instrument TTL controller. A laptop ran the Labview script to control both nozzle openings, providing concentrations between 0–21% in 1% increments. The custom Labview program also provided up to five timing stages for modulating intermittent oxygen. In addition, the laptop was equipped with a USB FOXY Probe (Ocean Optics, Inc.) to measure gaseous and aqueous oxygen concentrations.

Islet isolation and preparation

C57BL/6 mice islet donors were euthanized with CO₂ and 3% isoflurane followed by cervical dislocation. Dissection was done with V-incision at the genital area and moving the bowel to clearly expose the bile duct. The ampulla on the surface of duodenum was clamped with a hemostat. The pancreas was then distended (inflated) through the bile duct with a 30-gauge needle syringe containing 2 ml of cold collagenase solution (0.375 mg/ml in HBSS) starting at the gall bladder. The pancreas was then removed and placed in 2 ml of collagenase solution in 37°C water bath for 12 minutes. At 80% the digestion was stopped and washed twice by adding cold HBSS and centrifuging at 284 g's (Beckman J6-MI) for 30 s at 4°C. The islets were purified using a discontinuous Ficoll gradient density of 1.096, 1.069, and 1.037 kg/m³ by centrifugation at 640 g and 4°C and picked up from the interface between 1.069 and 1.096 layers. The pellet was washed two more times with cold HBSS via the centrifuge. Then, the islet pellet was re-suspended in 10 ml RPMI-1640 containing 10% FBS, penicillin, streptomycin, and 20 mM HEPES and incubated in Petri-dishes (37 °C, 5% CO₂). All islets were cultured post-isolation for 1 day prior to use in experiments.

Buffer, islet loading, and insulin ELISA

All perfusates were prepared in Krebs Ringer bicarbonate buffer: 129 mM NaCl, 5 mM NaHCO₃, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1 mM CaCl₂·2H₂O, 1.2 mM MgSO₄·7H₂O, 10 mM HEPES pH 7.35–7.40, 2 mM basal glucose, and 5% FBS. Stocks of Fura-2 AM in DMSO and Rh123 in 100% ethanol were added to Krebs buffer at final concentrations of 5 μM and 2.5 μM, respectively. The islets were incubated in 2 ml of Krebs buffer containing both Fura-2 and Rh123 for 30 minutes prior to loading via the aqueous microchannel. Approximately 10 islets were loaded for Fura-2 and Rh123 monitoring and 20 for insulin experiments. The islets were then perfused for 5 minutes to wash away excess dyes. Perfusate flow at 250 μl/min was provided by a Gilson Minipulse 2 peristaltic pump and heated on a 60°C hotplate upstream of the microfluidic device, which was mounted on an inverted Leica S6F with 37°C heated stage. There was no flow during the oxygen modulation to prevent convective disturbance of oxygen equilibrium at the islets. During glucose stimulation, effluent from the device was tubed to a Gilson 203 fraction collector and collected 4 minutes before stimulation and 3 minutes after wash at 1 sample/minute. Fractions were immediately frozen at –20°C and later thawed for 96-plate insulin ELISA measurements (Mouse Insulin, Mercodia, Sweden). The plates were read in a Biotek Synergy 2.

Data and statistical analysis

An inverted Leica S6F microscope provided simultaneous Fura-2 and Rh123 measurements. These dyes are sensitive to intracellular calcium and mitochondrial hyperpolarization, respectively. Fura-2 was excited at 340 and 380 nm and measured at 510 nm while Rh123 was excited at 495 nm and measured at 530 nm. When Fura-2 binds to available calcium, the dye's excitation maximum shifts from 380 to 340 nm, allowing a ratiometric measurement of 340 nm over 380 nm intensity to remove dye loading and photobleaching effects. Additionally, every experiment began with a standard pulse at 21% oxygen. Subsequent ratios were then normalized against the magnitude of this pulse and expressed as "Fura Ratio %". Rh123 fluorescence, which is quenched by mitochondrial hyperpolarization, was also analyzed with similar normalization. The insulin ELISA measurements were expressed as an index of the maximum value over the baseline average to account for the varying number of islets in the experiments. For transplants and islet physiology, it is common practice to express the stimulated insulin as an index over the basal levels.

In the hypoxia and IH calcium monitoring experiments, $n=3$ experiments were conducted for each condition. For the multi-parameter preconditioning experiments, $n=4$ experiments were conducted for calcium measurements while $n=3$ experiments were conducted for mitochondria and insulin measurements at each condition. 10–20 islets were loaded in each experiment as described in the islet loading section. For the multiparameter preconditioning experiments, two-tailed t -tests were performed to calculate p values for the differences between the means of 21% vs. 5%, 5% vs. preconditioning, and diazoxide vs. 5-hydroxydecanoate (5HD) conditions.

Results and Discussion

Multimodal stimulation of glucose, oxygen and flow

The multilayered microfluidic device provides controlled perfusate and oxygen microenvironments with aqueous flow in the top layer and gas modulation in the bottom. For perfusate control, stimulatory flow at 250 $\mu\text{l}/\text{min}$ can reach equilibrium within 3 minutes in the chamber, as shown by FITC dye mixing in Fig. 2a. With the islets loaded, stimulatory glucose concentrations—i.e. 7, 14, or 25 mM—can be introduced into the microwelled chamber. These concentrations correspond to physiological glucose levels for basal, post-meal, and diabetic conditions in mice, respectively. For oxygen control, microinjectors mixing of 0% and 21% O_2 inputs can deliver final oxygen concentrations of 0, 5, 10 or 21% at the microwelled membrane where the islets are resting. Similar to recent techniques^{27–29}, gas diffusion through this 200 μm PDMS membrane provides direct delivery of oxygen to the islets, in contrast with standard hypoxic chamber that requires circulating gas to cross millimeter thicknesses of bulk aqueous medium. Note that the diffusion time is inversely proportional to the square of the thickness, resulting in equilibration time in seconds for our device compared to hours with hypoxic chambers.³⁰

Additionally, the computerized gas control can modulate IH between 5% and 21%, achieving 2-minute cycles as shown in Fig. 2b. Other conditions can also be generated (see supplementary Fig. 1). Note that the oxygen sensor has a delay time of 15–20s in gas and 30–45s in aqueous media. Thus actual transient performance may be faster than measured. Gas fraction of 5% oxygen is a standard for hypoxia in cardiomyocytes and other tissue systems.^{31,32} The ability to vary with this range allows us to better quantify hypoxia and IH parameters at the ex vivo level with a precision previously not possible.

Islet glucose-calcium response is depressed by hypoxia

During islet's glucose-insulin response, a stimulatory pulse of glucose results in a pulse in intracellular calcium, which triggers insulin secretion. This calcium pulse has a bi-phasic profile with an initial dip (phase 0) corresponding to calcium sequestration in the endoplasmic reticulum, followed by an overshoot (phase 1) and oscillatory plateau (phase 2) that are both associated with transient membrane K_{ATP} channels.^{33,34} To quantify this calcium response to hypoxia, Fura-2 labeled islets were loaded into the multimodal device and exposed to 21%, 10%, and 5% oxygen while monitoring their calcium signaling. Under 21% oxygen flow, normoxic for *ex vivo* islets, the calcium influx showed a standard bi-phasic profile with magnitude 1.3 times above baseline in response to 14 mM glucose. Subsequent hypoxic pulses were normalized against this normoxic pulse. When the islets were exposed to 5% hypoxia for 30 minutes (zero flow to prevent convective oxygenation), their baseline calcium increases, as shown in Fig. 3a. After this exposure, the calcium pulse lost both magnitude and phase information. Additional incubation of 10 minutes hypoxia followed by a second pulse of glucose did not alter the response. Quantitatively, the pulse height was depressed below 50% of the normoxic pulse, for both 10% and 5% hypoxia. Qualitatively, 5% hypoxia showed softened phase 0 dip, rounded phase 1 overshoot, and suppressed phase 2 oscillations compared to both 10% and normoxic profiles, as shown in Fig. 3b.

We further investigated the time and glucose-dependence exposure of 5% hypoxia. At 14 mM glucose, 0 minute exposure, i.e. no prior exposure, reduced phase 2 magnitude to 75% of the original pulse. After 10 minutes of exposure, this dropped to below 50%. Beyond 20 and 30 minutes, the hypoxic magnitude settled around 30% as shown in Fig 3c. On the other hand, with 20 minutes of exposure at 5%, all three physiological glucose concentrations—7, 14, and 25 mM—resulted in depressed pulse magnitudes compared to 21%. In summary, prominent hypoxic response occurred at 5% oxygen concentration and the effects could not be compensated with increased glucose concentrations.

In vitro, intracellular calcium increases resulted from exposure to hypoxia have been reported and attributed to cell membrane channel deficiencies or calcium release from endoplasmic reticulum and mitochondria storages.³⁵ Our data also showed a baseline calcium increase during exposures to 5% hypoxia, Fig. 3a. Furthermore, hypoxia has been previously demonstrated to suppress insulin secretion^{8,9}, with 5% hypoxia inducing stronger suppression than 10%.^{31,32} The calcium monitoring results in this study corroborate this finding. One distinction that needs to be made is that physiological oxygenation reduces PO_2 to 40 mmHg (5.6%) in native pancreas, while isolated islets are exposed to 150 mmHg (21%) as their normoxic level. However, both reduction of oxygen to 5% *ex vivo* and corresponding transplant to hepatic portal vein at 5–15 mmHg or <2% have strong hypoxic effects.^{21,31} Furthermore, our dynamic device demonstrated more than just lower calcium response amplitudes, but also reduced phase 0–2 features as evident in Fig. 3b. This reduction or dampening of the K_{ATP} -dependent overshoot and oscillation in hypoxia could possibly be due to the decrease of ATP at the mitochondria.³⁶ In contrast, other studies have only reported end point changes without dynamics, compared to our *in situ* hypoxic measurements.

IH improves islet glucose-calcium response under hypoxia

To improve islet's response under hypoxia, islets were pre-exposed to IH at 1 minute 5% and 1 minute 21% cycling totaling 60 minutes as a preconditioning to hypoxia, as shown in Fig. 4a. Additional incubation of 10 minutes hypoxia did not reverse the response back into hypoxia, suggesting the protection cannot be removed by further exposure to continuous

hypoxia. This IH protection improved islet response magnitude to 80% of the normoxic pulse, even operating under hypoxia, as shown in Fig. 4b.

We further characterized the timing and cycling parameters of IH in order to optimize the preconditioning of islets. Shortening the hypoxic cycling to 1min/1min, as shown in Fig. 4c, provided the best pulse magnitude. When the period was fixed at 1min/1min and the total number of IH cycles was increased from 4 to 30, there was an improvement in magnitude as well. However, when the cycling was increased to 60, the preconditioning benefit was removed. This cut-off in preconditioning duration (<2 hours) could be a result of the limited volume (250 μ L) of Krebs Ringer buffer in the chamber compared to a continuous flow of culture medium.

In comparison to other preconditioned tissues, IH applied to rat myocardium at five 12 min cycles (6%/21% oxygen) showed protection against ischemia-reperfusion injury up to 24 hours after treatment.⁵ Furthermore, myocardium IH protocols (1 minute cycles) showed preference for longer treatment durations, with 4 hours IH having smaller infarction size than 30 minutes IH protocols.³¹ Consistent with myocardium, islet preconditioning demonstrated here is also optimal with shorter minute-scaled cycling for hour-long durations.

Insulin secretion is in turn improved by IH preconditioning via mitochondrial K_{ATP} channels

The effects of hypoxia and IH preconditioning on calcium dynamics were paralleled by changes in insulin secretion and mitochondrial potential, as measured by off-chip insulin ELISA and Rhodamine 123 (Rh123) fluorescence, respectively, Fig. 5. Insulin fractions were collected at 1 minute intervals and expressed as an index of stimulation over baseline. The hypoxic pulse rendered insulin response down to basal levels (in some cases below baseline) while preconditioning improved insulin to 85% of normoxia. Mitochondria hyperpolarization, monitored with Rh123 uptake and self-quenching, was improved with IH preconditioning compared to hypoxia exposure alone. Preconditioning also showed sensitization of hyperpolarization with faster response than normoxic pulses (see supplementary Fig. 2).

The observed benefit in hyperpolarization, suggestive of mitochondrial K_{ATP} channel involvement in preconditioning, was investigated using channel opener diazoxide and blocker 5-hydroxydecanoate (5HD). These channel modifiers are extensively used to demonstrate cardiomyocyte preconditioning.^{37,38} Islets were incubated in 100 μ M diazoxide for 10 minutes and washed in Krebs Ringer buffer for 10 minutes prior to hypoxia and glucose stimulation. Diazoxide incubation provided preconditioning benefits to islets under 5% hypoxia (see supplementary Fig. 3 for Fura-2 profile). Conversely, islets treated with IH (1min/1min cycle, 60 min total) and 100 μ M 5HD simultaneously failed to be preconditioned and showed typical hypoxic response (see supplementary Fig. 4 for Fura-2 profile). Both mitochondrial hyperpolarization and insulin secretion were improved with diazoxide compared to IH+5HD, as shown in Fig. 5. Though not statistically significant, diazoxide had higher average calcium and insulin responses while its mitochondrial hyperpolarization was less than that of IH preconditioning alone. This suggests IH preconditioning is better at enhancing mitochondrial response rather than solely altering calcium-dependent insulin secretion.

By applying rapid, minute-scaled IH, we were able to demonstrate the first *ex vivo* preconditioning for pancreatic islets with improvements in insulin secretion and dynamics. By optimizing cycling to 2 minute periods totaling 60 minute durations, hypoxic islets were preconditioned to achieve calcium, mitochondria, and insulin responses that are over 80% of

the normoxic response. Although islet preconditioning is not well-understood, our islet preconditioning results are consistent with the mechanistic view of mitochondrial K_{ATP} channel preconditioning.^{37–39} Specifically, our results showed IH-preconditioned mitochondria hyperpolarizes more than hypoxic conditions, but also does so faster than normoxia (see supplementary Fig. 2). Furthermore, mitochondrial specific blocker 5HD⁴⁰ unlike diazoxide, does not affect glucose-related K_{ATP} responses and insulin secretions. Taken together, our first demonstration of IH preconditioning in pancreatic islets suggests that mitochondria and its associated K_{ATP} channels play a critical role in the preconditioning process while glucose-related K_{ATP} channels are unmodified. We achieved this because our device can modulate mitochondrial preconditioning both pharmacologically and metabolically (i.e. diazoxide/5HD and IH) while simultaneously probing glucose-insulin functions.

Conclusion

We have demonstrated a novel microfluidic device capable of simultaneous modulation of oxygen and perfusate deliveries at islet's microscale-level. It enabled detailed quantification of the hypoxia impairments of glucose-insulin coupling factors. More importantly, the use of the device provided the first report of IH preconditioned islets with improved insulin secretion under hypoxia, a feat previously unachievable using either hypoxic chambers or competing microfluidic devices. Additionally, we demonstrated that IH preconditioning of islets is consistent with reported mitochondrial K_{ATP} preconditioning mechanism. In the future, additional gas-controlled perfusate channels could be added to enable stable oxygen concentrations while maintaining rapid membrane-diffused oxygen to the islets without disruption.

A direct application of islet IH preconditioning is improving islet function prior to transplants. The ability to improve islet function not only addresses hypoxia injury while awaiting revascularization but can also optimize the number of isolated islets required for each transplant. Beyond islet preconditioning, the multimodal device described here can be applied to studying general IH and hypoxia conditioning for other microtissues or tissue preparations such as brain slices or embryoid bodies. For example, detailed studies of K_{ATP} channel function could explain why preconditioning works so well in cardiomyocytes, and be used to benefit other tissues. Additionally, mechanisms in lipotoxicity and glucosetoxicity are suspected to be dependent on reactive oxygen species and high oxygen metabolism.⁴¹ Both aspects can be investigated by modulating gas phase oxygen in addition to aqueous oxygen scavengers using this multimodal device. Lastly, many developmental and regenerative mechanisms in stem-cell differentiation and cell-cell interactions depend on oxygen gradients. The ground work laid out by this multimodal oxygen device can be adapted to many of these challenging unanswered questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Multimodal islet hypoxia device

(a) Cross-section view of the microfluidic stimulation sandwich. (b) Multiparametric staining: Rhodamine 123 in red, Fura-2 340 nm in green and 380 nm in blue, and composite live-dead assay (calcein and ethidium bromide in green and red, respectively). (c) Schematic of microscope-based multimodal device. (d) Protocol for simultaneous modulation of oxygen (blue oxygenated, red hypoxic), flow, and glucose stimulations at the islets.

Figure 2. Simultaneous oxygen and glucose stimulations

(a) Static controls. Top: chamber introduction of FITC molecule at 250 $\mu\text{l}/\text{min}$ stabilizes within 3 minutes of mixing time. Bottom: gas control provides stable delivery of 5, 10, and 21% oxygen at the membrane. (b) Temporal control. Cycling of oxygen between 5–21% is possible with 1 minute period as measured both at the gas delivery (diffused) and surface of microwells (dissolved).

Figure 3. Response of islets to hypoxia

(a) Representative overview of islet calcium response showing depressed hypoxic pulse after 30 minutes of 5% exposure, compared to normoxic pulse. Subsequent hypoxia exposure continues to depress response. (b) Overlaid comparison of mean normoxic, 10%, and 5% hypoxic pulses under 14 mM glucose stimulation, showing impairments in magnitude and phase information at 5%. (c) Islet hypoxia parameters. Top: Longer exposure to 5% hypoxia induces stronger suppressions pulse magnitude. Bottom: Various concentrations of glucose at 7, 14, and 25 mM cannot resuscitate hypoxic response. Error bars represent standard deviations. 100% represents normoxic magnitudes as shown in (b).

Figure 4. IH preconditioned islets have improved response to hypoxia

(a) Representative overview of preconditioning using IH, totaling 30 minutes exposure at 5%, showing enhanced hypoxic response compared to normoxic pulse (same islet batch in a previous experiment). 10 more minutes of subsequent hypoxia still retains preconditioning. (b) Overlaid comparison of mean normoxic, hypoxic, and preconditioned hypoxic responses. Inset shows representative traces with recovery of oscillation behavior. (c) Islet preconditioning optimizations. Top: Decreasing the period of cycling improves both the response magnitude as well as consistency. Bottom: Increasing the number of IH cycle improves the response. However, beyond 60c or 120 minutes this protocol is not optimal. Error bars represents standard deviations.

Figure 5. Insulin secretion, along with calcium and mitochondrial hyperpolarization, is also improved by preconditioning via mitochondrial K_{ATP} channels

Combined Fura-2, insulin, and Rh123 responses for different oxygen and chemical conditions: 21% oxygen, 5% oxygen, preconditioning (P+5%), diazoxide (D+5%), and 5HD (5HD+P+5%). Diazoxide is consistent with preconditioning while 5HD negates the benefits of preconditioning by opening and blocking K_{ATP} channels, respectively. Two-tailed t-tests: 5% vs. 21%, 5% vs. P+5%, D+5% vs. 5HD+P+5%; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.