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Real time imaging of intracellular hydrogen peroxide in pancreatic islets

Adam Neal[1], **Austin Rountree**[1], **Kelly Kernan**[1], **Brian Van Yserloo**[1], **Huiliang Zhang**[2], **Benjamin J. Reed**[1], **William Osborne**[1], **Wang Wang**[2], and **Ian R Sweet**[1,§]

[1]Department of Medicine, University of Washington, Seattle, WA, 98108, U.S.A.

[2]Department of Pain and Anesthesiology, University of Washington, Seattle, WA, 98195, U.S.A.

Abstract

A real time method to measure intracellular H_2O_2 would be very impactful in characterizing rapid changes that occur in physiologic and pathophysiologic states. Current methods do not provide the sensitivity, specificity and spatiotemporal resolution needed for such experiments on intact cells. We developed the use of HyPer, a genetic indicator for H_2O_2 that can be expressed in the cytosol (cyto-HyPer) or the mitochondria (mito-HyPer) of live cells. INS-1 cells or islets were permeabilized and the cytosolic HyPer signal was a linear function of extracellular H_2O_2, allowing fluorescent cyto-HyPer signals to be converted to H_2O_2 concentrations. Glucose increased cytosolic H_2O_2, an effect that was suppressed by overexpression of catalase. Large perturbations in pH can influence the HyPer signal, but inclusion of HEPES in the perfusate prevented pH changes, but did not affect glucose-induced cyto-HyPer signals suggesting this effect is largely pH-independent. Using the assay, two fundamental questions were addressed. Knockdown of SOD2, the mitochondrial form of SOD, completely suppressed glucose-induced H_2O_2. Further, glucose also induced mitochondrial superoxide and H_2O_2 production, which preceded the appearance of cytosolic H_2O_2. Therefore, glucose-induced H_2O_2 largely originated from mitochondria. Finally, glucose-induced HyPer signal was less than $1/20^{th}$ of that induced by toxic levels of H_2O_2. Overall, the use of HyPer for real time imaging allowed resolution of acute changes in intracellular levels of H_2O_2 and will have great utility for islet studies involving mechanisms of H_2O_2 mediating signaling and oxidative stress.

INTRODUCTION

Importance of H_2O_2 in islets

Failure of pancreatic β cells to compensate for increased insulin resistance is a major determinant of diabetes [1, 2]. Understanding the importance of specific control steps in the regulation of insulin secretion, such as glucokinase and ATP-sensitive potassium (K_{ATP})

§Corresponding Author: Ian Sweet, University of Washington, Diabetes Research Institute, Seattle, WA 98195-8055, U.S.A., Tel: 206 685-4775, Fax: 206 543-3567, isweet@u.washington.edu.

AUTHOR CONTRIBUTIONS

Real time H_2O_2 data was collected by A.N. and A.R., as well as the islet preparation and cell culture. Confocal imaging and generation of adviruses for HyPer and catalase were done by H.Z. and W.W. Oxygen consumption and insulin secretion data was collected by B.J.R. Molecular biology for SOD2 was carried out by K.K., B.V.Y. and W.O. Study design was conceived and tuned by W.W. and I.R.S. I.R.S. and W.W. also co-wrote the manuscript, with editing contributions from all authors.

channels has shed light on some causes of impaired insulin secretion [3, 4]. There is evidence that H_2O_2 can act as a signal mediating both basal [5] and glucose-induced insulin secretion [6, 7]. Indeed islets secrete insulin in response to low levels of H_2O_2 [8, 9]. It has been reported that intracellular H_2O_2 increases in response to glucose, and the effect of glucose on H_2O_2 and insulin secretion rate is blocked by scavengers of H_2O_2 [6, 7]. Sources of H_2O_2 in the β cell may reside in the cytosol from the action of NADPH oxidase [10] and the mitochondria through the action of the respiratory chain [11]. On the other end of the spectra, high levels of H_2O_2 may lead to oxidative stress, which is harmful to the islet [12]. This action of H_2O_2 may be mediating the loss of function and β cell death during episodes of excess glucose and/or fatty acids [13], as well as exposure to cytokines released in response to inflammation and autoimmune attack [14]. In these studies, intracellular H_2O_2 was determined by conventional indicators such as DCF (2',7'-dichlorofluorescein) [15], which are known to be non-specific and irreversible [16]. Thus, technical difficulties have precluded definitive testing of these highly significant findings. In addition, these indicators cannot distinguish between H_2O_2 residing in the cytosol from that in the mitochondria, critical knowledge needed for understanding the source and point of action of H_2O_2 signaling. What is needed is a highly specific and sensitive dye to H_2O_2 that can be directed at specific intracellular compartments.

Novel H_2O_2 indicator with high sensitivity

A recently developed genetic probe for H_2O_2, named HyPer, has high sensitivity, is specific to H_2O_2 relative to other ROS known to reside in the cells, and can be targeted to intracellular organelles [17, 18]. Previous studies have shown that the sensor expressed in islets was indeed sensitive to exogenous H_2O_2 and glucose [19]. However, these authors also pointed to the known pH-sensitivity of the dye that would be problematic when measuring glucose effects with the mitochondrial probe where the pH increases from 7.2 to 8 upon stimulation. Since HyPer is uniquely sensitive and specific (with respect to H_2O_2 and ROS) compared to other H_2O_2-sensitive dyes and sensors, we endeavored to use HyPer to measure H_2O_2 while correcting as needed for pH changes by concomitantly imaging a pH-sensitive dye with HyPer. Our data shows that for cytosolic H_2O_2, physiologic changes in pH (as that elicited by glucose) did not contribute to changes in HyPer signal. In addition, we found HyPer to be reproducible, dose dependent and chemically specific. The use of HyPer in islets will provide islet researchers with a powerful tool for the accurate monitoring of compartmentalized H_2O_2 signals to tackle key questions including the role of H_2O_2 in signaling and oxidative stress.

MATERIALS AND METHODS

Chemicals

Krebs-Ringer bicarbonate solution (KRB), used for all perifusion/imaging analysis, contained 98.5 mM NaCl, 4.9 mM KCl, 1.2 mM potassium phosphate, 1.2 mM magnesium sulfate, 25.9 mM sodium bicarbonate, 2.6 mM $CaCl_2$, varying amounts of glucose (all from Sigma Aldrich, St. Louis, MO), and 20 mM HEPES (Research Organics, Cleveland, OH). Potassium cyanide (KCN) and H_2O_2 were both purchased from Sigma-Aldrich.

Culture of INS-1 832/13 cells

INS-1 832/13 cells (henceforth referred to as INS-1 cells) were kindly provided by Dr. Christopher Newgard and were cultured as previously described [20]. Three days before the experiment, cells were harvested by exposure to Trypsin for 5 minutes, plated on to glass coverslips coated with Matrigel (Corning, Corning, NY) and the coverslips were placed in petri dishes containing the specified adviruses in RPMI Media 1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 2 mM L-Glutamine, 1 mM Pyruvate, 50 μM Beta-mercaptoethanol, 20 mM HEPES and 1% Pen/Strep.

Rat islet isolation and culture

Islets were harvested from Sprague-Dawley male rats (≈ 250 g, Charles River) anesthetized by intraperitoneal injection of sodium pentobarbital (35 mg/230 g rat). All procedures were approved by the University of Washington Institutional Animal Care and Use Committee. Islets were prepared and purified as previously described [21], and then cultured at 37°C in RPMI Media 1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) for specified times with HyPer dye or other adviruses.

Viral packaging and expression of HyPer

Two forms of the H_2O_2 sensor were used: pHyPer-dMito vector and pHyPer-cyto vector (FP941 and FP942, Evrogen, Moscow, Russia) that localize to cytosol and mitochondria respectively [17]. Adenoviruses containing cyto-HyPer and mito-HyPer were generated by Vector Biolabs (Malvern, PA) as previous reported [22]. The H_2O_2 sensors were transduced in intact islets or cells during incubation in RPMI media supplemented with 10% heat-inactivated fetal bovine serum and the adenoviruses at a multiplicity of infection of 10–100 for three days at 37° C.

Permeabilization of INS-1 cells

To test the sensitivity of the HyPer sensor to known concentrations of H_2O_2, INS-1 cells expressing cyto-HyPer plated in 24-well dishes cells were permeabilized. Just prior to analysis, cells were washed with KRB followed by KG buffer (140 mM potassium glutamate, 5 mM ATP, 5 mM NaCl, 7 mM $MgSO_4$, 0.4 mM EGTA, 1% BSA, and 20 mM HEPES, pH 7.4). They were then incubated for 15 min with 0.5 ml of KG buffer containing varying concentrations of streptolysin-O (Sigma-Aldrich) to permeabilized the cells. Trypan blue exclusion test demonstrated that at 3.2 units/ml streptolysin-O more than 90% of the cells were permeabilized (Fig. S1). This concentration of streptolysin-O was used for permeabilizing cells while they were in the flow system for the time indicated.

Overexpression of cytosolic and mitochondrial catalase, and knockdown of superoxide dismutase

The mitochondrial targeted catalase construct was kindly provided by Dr. Peter Rabinovitch. The adenovirus construct and the adenovirus-packaged cytosolic catalase were both generated by Vector Biolabs. Freshly isolated islets from rats or cultured INS-1 cells were

infected with the above adenoviruses at a multiplicity of infection of 10–100 for 3 days. Appropriate gene expression was confirmed by Western blot or confocal imaging.

To make the SOD2 shRNA, the most efficient knockdown construct for SOD2 was screened and identified (see Supplemental Methods for details), which was then placed into the pLenti6/BLOCK-iT0222-DEST expression vector. The lentiviral vector was inserted in HEK-293T cells by calcium phosphate-mediated co-transfection with plasmids containing viral genome components. Vector preparations were concentrated 100 fold. INS-1 cells were plated at a density of 100,000 cells per 35 mm dish and, after 24 h of proliferation, were transduced with 50 μl of concentrated virus in 1 ml of INS-1 media containing 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO) overnight. This amount was found to result in an 80% decrement in SOD2 mRNA as reflected by Quantitative PCR performed on an Mx3005P® Multiplex QPCR System (Stratagene, La Jolla, CA) with samples loaded in triplicate using ~60 ng cDNA.

Western Analysis for catalase

Protein samples were prepared from islets samples using a lysis buffer (Cell Signaling) containing protease inhibitors (Sigma). After SDS-PAGE, proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories), and blocked in 5% nonfat milk before incubation with primary antibodies: anti-catalase (1:1000, Athens Research and Technology) and anti-Actin (1:5000, Sigma). The secondary antibodies (1:5000, Invitrogen) were labeled with Alexa Fluor detection reagent, and images taken by Odyssey Infrared Technology (LI-COR).

Confocal imaging of HyPer indicators

To determine the intracellular localization of cyto-HyPer and mitochondrial localization of mito-HyPer, we loaded the mito-HyPer expressing islets with MitoTracker Red FM (500 nM for 20 min, M22425, Thermo-Fisher Scientific). Confocal imaging was carried out using a Leica TCP SP8 confocal microscope with a 40x, 1.3 NA oil immersion objective. Cyto-HyPer and mito-HyPer were sequentially excited with 405 and 488 nm lasers and the emissions were collected at 505–550 nm. For MitoTracker Red, 552 nm excitation was used and emission collected at >560 nm.

Single and Dual channel perifusion system for real time imaging

Real time imaging experiments were carried out while islets or INS-1 cells were perifused using a commercially available temperature controlled perifusion dish (as previously described [23]). In order to accommodate side-by-side comparison of islets with and without overexpression of catalase, the standard single port perifusion dish (Bioptechs, Butler, PA), was custom-modified so that it had two inlet and outlet ports (Fig. 1). Two rectangular sections were cut from a gasket made out of two stacked circles of Parafilm so that the voids created when the gasket was placed into the perifusion dish acted as individual perifusion chambers, each fed and drained by one of the two ports.

Real time epi-fluorescent imaging of intracellular H_2O_2, superoxide and pH

After the islets or cells were loaded into the perifusion chambers, the chambers were sealed and mounted on to the stage of a Nikon Eclipse TE-200 inverted microscope. KRB was pumped through the two chambers at flow rates of 130 μL/min for each using a Masterflex L/S peristaltic pump (Cole-Parmer, Vernon Hills, IL). The HyPer signals were generated by dual fluorescence excitation via a xenon arc lamp (Lambda LS-1620, Sutter Instrument Company, Novato, CA) through either a 405/30nm or a 480/40nm bandpass filter and detected at 510nm through a longpass dichroic mirror with a cutoff of lower 500nm. The images were taken using a digital camera (Photometrics Cool Snap HQ2 CCD camera, Tucson, AZ) through a 40x Super Fluor Nikon objective (DIC H/N2). At the end of each experiment, an inhibitor of the electron transport chain (KCN) was added to the media to stop the generation of H_2O_2 from the mitochondria, and the steady state values in the presence of KCN used for background correction. Note that KCN may also have other effects in addition to blocking cytochrome c oxidase that might prevent the signal from going all the way to baseline, but it did induce a level of signal that was lower than all other conditions that were investigated. Data was expressed ratiometrically, where the excitation intensities at 480nm excitation were divided by those obtained during excitation at 405nm. MitoSOX Red (M36008, Thermo-Fisher Scientific, Waltham, MA), a dye sensitive to superoxide that localizes to the mitochondria, and SNARF-1 (5-(and-6)-Carboxy SNARF® −1, Acetoxymethyl Ester, Acetate, C1271, Thermo-Fisher Scientific), a pH-sensitive dye that distributes in the cytosol, were visualized by excitation at 546 nm and detection at 605 nm.

Continuous measurement of oxygen consumption rate and insulin secretion rate

A flow culture system was used that concomitantly measured oxygen consumption rate while collecting outflow fractions for subsequent measurement of insulin (described previously [21, 24, 25]). OCR was calculated as the flow rate (approximately 80 μL/min) times the difference between inflow and outflow oxygen tension and measured by detecting signal from an oxygen-sensitive dye painted on the inside of the perifusion chamber [21]. Insulin in the outflow fractions was measured using an RIA kit (Linco Research Inc., St. Charles MO).

Static assessment of insulin secretion

Insulin secretion rate was determined statically with multiple conditions as previously described [26]. Briefly, islets were handpicked in parallel into a Petri dish containing KRB, 0.1% BSA and 3 mM glucose and incubated at 37 °C/5% CO_2 for 60 min. Subsequently, islets were placed into wells of 96-well plates (10 islets/well) containing indicated amounts of glucose, and incubated for 60 more min. At the end of this period, supernatant was assayed for insulin using a Linco RIA kit.

Viability staining with Acridine Orange and Propidium Iodide

Islets were imaged after staining of islets with acridine orange (10 mmol/L) and propidium iodide (15 mmol/L) (AO/PI) [27].

RESULTS

Sensitivity of expressed H_2O_2 sensor: Response of cyto-HyPer to extracellular H_2O_2

To test the sensitivity and response time of the cytosolic H_2O_2 sensor, INS-1 cells were permeabilized with streptolysin O prior to loading them into the perifusion system. Various concentrations of H_2O_2 ranging from 30 nM up to 1 μM were added to the inflow at 20-minute intervals (Fig. 2A). Starting at 65 nM, the fluorescence increased in proportion to the H_2O_2 concentration until 600 nM was reached (Fig. 2B). At 1 μM, the signal began to saturate. The response time of the sensor was slightly delayed at low extracellular concentrations, but was within 1 minute at 600 nM. Since the concentration of H_2O_2 was certainly reduced by anti-oxidant machinery in the cell, this estimate of response time was an upper limit. Taken together, the relation between cyto-HyPer signal and cytosolic concentrations of H_2O_2 is linear between 65 and 600 nM, and has a kinetic resolution that is at most on the order of a few minutes.

Test of H_2O_2 specificity by cyto-HyPer

Having characterized the sensitivity of cyto-HyPer sensor to H_2O_2, we next addressed whether it was specific for H_2O_2. To do this, we over-expressed cytosolic catalase, an enzyme that converts H_2O_2 to water with very high specificity, in islets and INS-1 cells. Catalase was overexpressed after 2 days of incubation with an adenovirus containing DNA for either cytosolic or mitochondrial catalase, as reflected by Western blot (Fig. 3A). Using the two-channel perifusion system, we conducted side-by-side comparisons between real time responses of cyto-HyPer to glucose in islets or INS-1 cells with and without high levels of catalase. In the absence of catalase over-expression, glucose led to an increased fluorescence ratio in both islets (Fig. 3B) and INS-1 cells (Fig. 3C). Over-expression of cytosolic catalase in islets, and mitochondrial catalase in INS-1 cells, completely inhibited the glucose-induced increases in signal, indicating that the change in fluorescence in response to glucose was largely determined by H_2O_2. There was, however, a slight decrease in cyto-HyPer fluorescence in response to glucose in the INS-1 cells, when mitochondrial catalase was being overexpressed, an effect that we did not investigate.

During glucose stimulation of the islet, changes in pH do not effect H_2O_2 measurements

A previous study described the sensitivity of HyPer fluorescence to elevated pH when islets were exposed to high levels of base [19]. Accordingly we endeavored to test whether stabilization of changes in intracellular pH could be accomplished by use of the pH buffer HEPES, and whether pH changes in the physiologic range affect HyPer fluorescence. When perifused in standard KRB buffer (in the absence of HEPES), islet pH (as reflected by the pH sensor SNARF-1) decreased in response to 20 mM glucose, an effect which was rapidly reversed upon returning to 3 mM glucose (Fig. 4A). In contrast, when 20 mM HEPES was added to the KRB buffer, pH was no longer sensitive to changes in glucose (Fig. 4A). Therefore, at least for changes in glucose, the use of HEPES maintains intracellular pH so it could not contribute to glucose changes in cyto-HyPer. We then measured the effect of glucose-induced changes in pH on cytosolic H_2O_2 by comparing the response of H_2O_2 in the presence and absence of HEPES in the buffer. Somewhat unexpectedly, the H_2O_2 responses to glucose were identical whether the pH changes were occurring or were prevented by

HEPES (Fig. 4B). This data is actually in concordance with the original methods paper introducing HyPer where it was shown that near physiologic pH, the cyto-HyPer signal is insensitive to pH, and it is only at high pH when the effects on HyPer are significant ([17], Supplementary Fig. 1). Thus it appears that for glucose stimulation, pH sensitivity of HyPer is not an issue, and the use of HEPES provides a method to stabilize changes in pH to test this assumption.

Identification of source of cytosolic H_2O_2 in islet cells

Previous studies have suggested that sources of H_2O_2 in islets are located in the cytosol, including NADPH oxidase [28], autooxidation [29], and the hexosamine pathway [30], as well as mitochondrial oxidation [31] (Fig. 5). Since in many cell types mitochondria are the major source of ROS [32], we tested whether mitochondrial superoxide is the major source of cyto-HyPer-detected H_2O_2 by knocking down mitochondrial superoxide dismutase (SOD2) in INS-1 cells. The SOD2 shRNA was titrated to achieve an 80% reduction in SOD2 mRNA in INS-1 cells ((0.56 vs. 2.75 ng SOD2/ng RNA in the transfected vs. control INS-1 cells)). Similar to the over-expression of catalase, knock down of SOD2 prevented the glucose-induced increase in cytosolic H_2O_2 (Fig. 6). This suggests that the major source of the intracellular H_2O_2 is from the conversion of mitochondrial superoxide to H_2O_2 by the action of SOD2. Attempts made to knockdown islet SOD2 were not successful for reasons that were not clear.

Glucose responses of intracellular generation of superoxide and mitochondrial and cytosolic H_2O_2

To further test the source of H_2O_2 and temporal resolution for the sensors, the kinetics of the response of superoxide, mitochondrial H_2O_2 and cytosolic H_2O_2 were measured by the dyes MitoSOX Red, mito-HyPer and cyto-Hyper, respectively. To confirm that the mito-HyPer was specifically distributed in the mitochondria, confocal images of islets expressing mito-Hyper and cyto-Hyper were compared to islet fluorescence after loading with MitoTracker Red. The fluorescence pattern of MitoTracker Red was localized to forms consistent with mitochondrial distribution. The spatial distribution of the fluorescence for mito-HyPer and MitoTracker Red were well correlated (Fig. 7A), and in contrast to cyto-Hyper, showed a lack of signal from nuclei and cytosol. Without expression of HyPer, islets did not have significant non-specific fluorescent signal.

From the previous data, intracellular H_2O_2 is largely being produced from mitochondrially-generated superoxide, which then traverses the mitochondrial membrane to the cytosol. Consistent with this scenario, superoxide responded to glucose prior to the increase in mitochondrial H_2O_2, and there was a lag in cyto-HyPer of about 5 minutes relative to mito-HyPer (Fig. 7B). As H_2O_2 is generally thought to be freely diffusible across membranes, we sought to confirm this finding by an alternate method. To do this, we used permeabilized INS-1 cells expressing mito-HyPer, and exposed them to increasing concentrations of H_2O_2, in a similar fashion to the experiment shown in Fig. 2A performed with cyto-HyPer. In contrast to cytosolic H_2O_2, which increased in response to 65 nM extracellular H_2O_2, increases in mitochondrial H_2O_2 was seen at 600 nM but not 200 nM extracellular H_2O_2. Thus, in two different experimental conditions, and when H_2O_2 transport was in the

direction of cytosol to mitochondria, or vice versa, there was a significant barrier to equilibrium between the two compartments.

Comparison of physiologic and toxic levels of H_2O_2

It is a hallmark of type 2 diabetes that beta cell mass is decreased [33]. High levels of ROS generated by increased glucose metabolism have been proposed to result in damage to beta cells, providing a mechanism for how hyperglycemia could lead to decreased beta cell mass [34]. An accurate, and calibrated assay for H_2O_2 allows comparison of the H_2O_2 levels achieved by increased glucose and the amount of H_2O_2 needed to result in damage to the beta cell. To implement this test, we first determined the amount of exogenous H_2O_2 that is needed to bring about significant islet cell death. Islet membrane integrity was evaluated for periods of up to 24 hours following a 15-minute exposure to varying amounts of H_2O_2 (Fig. 8A). At low concentrations (below 50 μM) no dead cells were detected, whereas exposure to 100 μM resulted in some cells on the periphery of the islet to stain for propidium iodide entry (reflecting breakdown of the plasma membrane). At 500 μM H_2O_2, much of the islet stained red for propidium iodide within 6 hours (although the core was spared). Thus, under these conditions, it takes more than 100 μM H_2O_2 to damage the islets. We also performed experiments where islets were chronically exposed to H_2O_2 for 24 hours, and statically assessed for changes in insulin secretion rate. No changes were observed by islets exposed to 10, 30 or 100 μM H_2O_2, but the rate was reduced by more than 90% after exposure to 500 μM H_2O_2 (data not shown), a dose response similar to that obtained and using boluses and membrane integrity as an endpoint and in a previous study [28].

In order to evaluate the potential for glucose metabolism to produce enough H_2O_2 to have toxic effects, we compared the amount of glucose-generated intracellular H_2O_2 to that which was present when islets were exposed to toxic amounts of exogenous H_2O_2. To do this, fluorescent data was converted to actual concentrations of H_2O_2 by permeabilizing the cells at the end of the experiment and carrying out a stepwise concentration dependency as was done to generate Fig. 2A. An increase in glucose (to 20 mM) also increased cytosolic H_2O_2 to about 50 nM (Fig. 8B). When the exogenous H_2O_2 was raised to 100 μM, the minimal amount needed to induce a loss of viability, cytosolic H_2O_2 was increased to more than 20 times that reached at high glucose. Thus, increases in endogenously generated H_2O_2 by high glucose were many times lower than that needed to induce toxic effects in vitro.

To further confirm that high glucose levels do not impair metabolic or secretory function, oxygen consumption and insulin secretion by islets were measured continuously for 24 hours using a flow culture system while exposed to various concentrations of glucose in islet culture media. Oxygen consumption rates were proportional to the concentration of glucose, and did not decrease for any of the glucose levels (Fig. 8C). Insulin secretory rates were also proportional to glucose concentration, but as observed in previous studies [35], waned over time after about 10 hours following the stimulation by glucose. At the beginning and end of the experiment, the ability of the flow-cultured islets to respond acutely to an increase in glucose from 3 to 20 mM was assessed. All islet oxygen consumption and insulin secretion responses were the same, except for islets exposed to 3 mM glucose overnight whose responses were much diminished. Thus, with respect to 24 hour exposures, exposure to high

glucose resulted in more metabolically and functionally robust islets, consistent with the scenario that glucose cannot alone induce toxic levels of H_2O_2.

DISCUSSION

H_2O_2 has been hypothesized to have a major role in islet function and pathology, both as a signaling molecule [6, 7] and as a mediator of beta-cell damage during oxidative stress [11, 36]. However, a major barrier to progress in testing and further investigating these biochemical concepts is the lack of a real time assay for measuring the time course and absolute concentrations of H_2O_2 in intact islets. A previous study demonstrated the ability of HyPer to express in islets and track H_2O_2 levels [19], however, pH-sensitivity was thought to be an obstacle to its general use. Accordingly, we built upon this previous study, addressed the pH-sensitivity and validated the H_2O_2 assay for use in islets. We then applied the assay to address two fundamental questions about the source and concentration range of intracellular H_2O_2.

Validation of a real time H_2O_2-specific assay

The validation of cyto-HyPer involved two basic tests: sensitivity and specificity. The assay response was linear between 65 and 600 nM, occurred with less than minute-to-minute resolution, and catalase, an enzyme with high specificity for H_2O_2, completely suppressed glucose-induced increase in H_2O_2. In general, the responses in INS-1 cells and whole islets were similar, but INS-1 cells were used when the adviruses would not result in desired changes in protein levels, for instance for knocking down SOD2. In addition, in the presence of HEPES, pH didn't change in response to glucose, and the H_2O_2 response to glucose was the same in both the presence and absence of HEPES. Therefore, while we present a method to circumvent the potential pH-sensitivity of the H_2O_2 signal by buffering intracellular pH, it proved unnecessary since the small change in pH induced by glucose did not alter the HyPer signal. This was likely due to the fact that the HyPer protein sensitivity to pH below about 7.6 is greatly reduced ([17], Supplementary Fig. 1). With respect to mito-HyPer, we did not have a method for measuring the mitochondrial pH, nor do we have an approach that necessarily stabilizes it. However, with the exception of a delay in the cyto-HyPer signal relative to the mito-HyPer, the signals were in most cases qualitatively similar, making it likely that the mito-HyPer is also a valid kinetic measure of H_2O_2. Thus, cyto-HyPer can quantify physiologic changes of H_2O_2 in real time in intact whole islets, and the glucose response was specific for H_2O_2 and not affected by pH.

Physiologic vs. harmful levels of H_2O_2 in islets

As islets in the transition to diabetes are exposed to lengthy durations of hyperglycemia, it has been proposed that high metabolic flux through the electron transport chain generates harmful levels of ROS [12], which in turn could lead to loss of beta cell mass. In our study, consistent with other reports [37, 38], glucose caused an increase in both cytosolic and mitochondrial islet H_2O_2. However, this is the first study that quantified the intracellular concentrations of H_2O_2, which increased from 20 to 50 nM when islets were exposed to 3 and 20 mM glucose respectively. In contrast, the amount of H_2O_2 observed in the presence of toxic levels of H_2O_2 was more than 20 times that seen in the presence of 20 mM glucose.

This is consistent with the stability of oxygen consumption observed during exposure to high glucose, as well as another study where viability and secretory function of isolated islets cultured in vitro was maintained for 7 days or more in the presence of very high levels of glucose [39]. To be sure, conditions in vivo may result in different/higher levels of intracellular H_2O_2, and furthermore the effects of H_2O_2 could be more potent in in vivo conditions. But nonetheless, it seems that the vastly different concentration dependency of toxicity vs. H_2O_2 levels would suggest that a mere 2.5-fold increase of H_2O_2 levels would be unlikely to result in H_2O_2-mediated damage.

Generation of H_2O_2 in islets mainly by conversion of mitochondrial superoxide

Due to the potential for endogenously generated H_2O_2 to induce damage and/or act as a physiologic signal, there has been much interest in the intracellular mechanisms mediating H_2O_2. Past publications have considered several biochemical pathways operating in the cytosol of the islet that lead to production of H_2O_2 including the actions of NADPH oxidase [28], autooxidation [29], and the hexosamine biosynthesis pathway [30]. In contrast, in regard to many cell types, the mitochondria are thought to be the main source of H_2O_2 [32]. The results of our studies, including the loss of glucose-stimulated H_2O_2 response after mitochondrial SOD knockdown, and the delay in appearance of H_2O_2 in the cytosolic relative mitochondrial H_2O_2, strongly suggest that mitochondrial superoxide is the major source of H_2O_2 in the islet. During this experimental investigation it was interesting to observe an unexpected asymmetry in the transfer of H_2O_2 between the cytosol and the mitochondria. Although it is commonly held that H_2O_2 is very permeable to lipid membranes and that the mitochondrial membrane would not represent a kinetic barrier, we observed a delay in response of cytosolic H_2O_2 in response to mitochondrially-generated H_2O_2, and a complete lack of increase in mitochondrial signal at low levels of cytosolic H_2O_2 induced by extracellular H_2O_2. Whether this was due to impermeability of H_2O_2 by the mitochondrial membrane, or the action of antioxidants around the cytosolic side of the mitochondrial membrane is not established by this study. However, the time course of H_2O_2 distribution into different compartments has implications for the potential for H_2O_2 to act as a signal.

Conclusions

1. We have developed an assay that measures H_2O_2 levels in real time, where only mild changes in pH are occurring;
2. Data supports predominant source of H_2O_2 under glucose stimulation is mitochondrial superoxide;
3. H_2O_2 is not freely permeable to either the plasma membrane or the mitochondrial membrane;
4. The concentrations of glucose-stimulated H_2O_2 in isolated islets are far below those that are acutely toxic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED:

AO	acridine orange
cyto-HyPer	pHyPer-cyto
DCF	2', 7'-dichlorofluorescein
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2	hydrogen peroxide
INS-1 cells	INS-1 813/32 cells
KCN	potassium cyanide
KRB	Krebs-Ringer bicarbonate
mito-HyPer	pHyPer-dMito
PI	propidium iodide
ROS	reactive oxygen species
SOD2	superoxide dismutase 2

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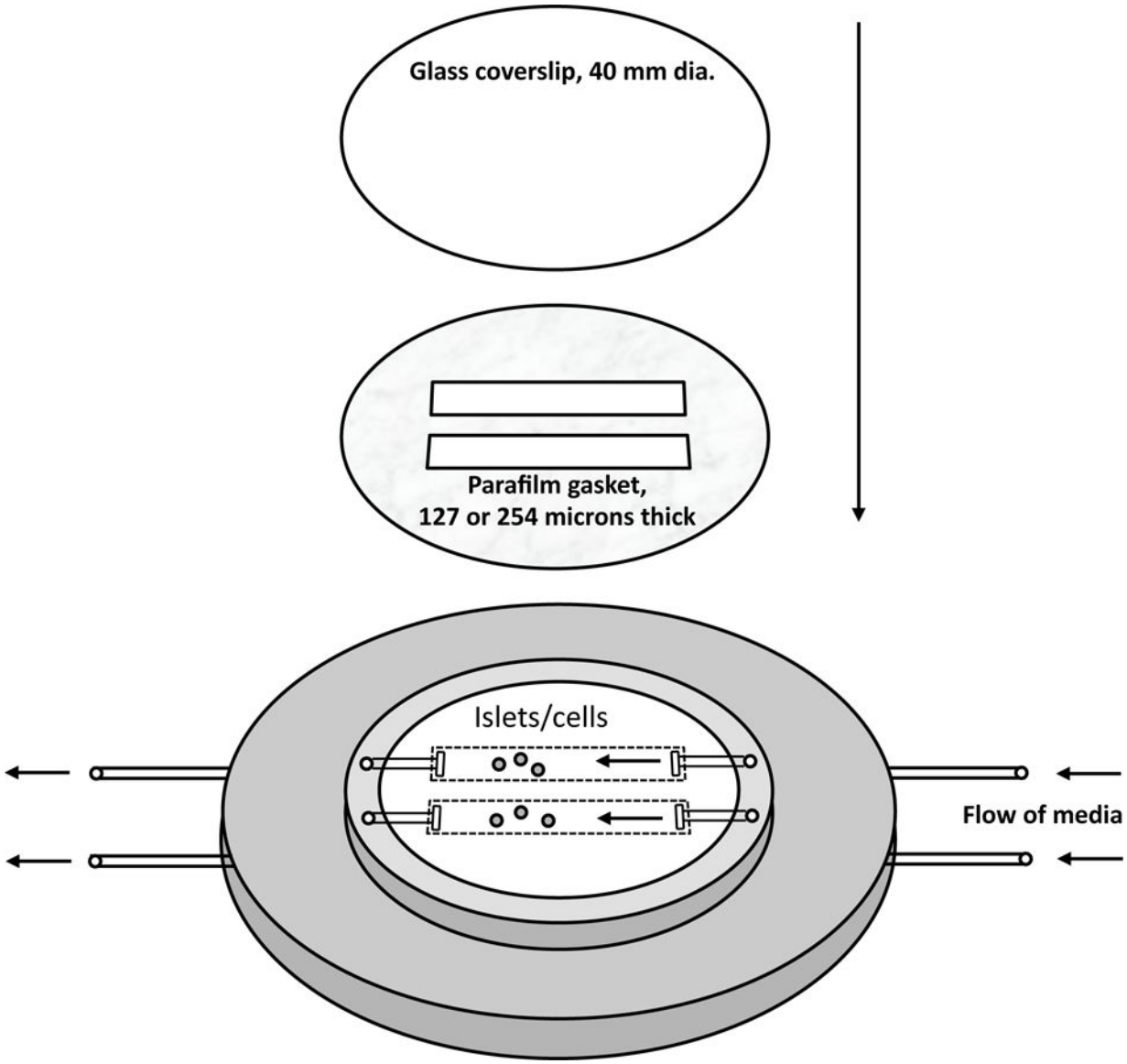

Figure. 1. Schematic of the dual channel fluidics/imaging chamber.

A Bioptechs FCS2, a closed system, parallel plate flow cell, was modified in order to accommodate two inflow and outflow ports. The gaskets were then cut so that the ports supplied and drained flow to and from two separated cell channels. For cells one Parafilm gasket was used, and for islets, two were stacked prior to cutting the rectangular holes. This system allowed for two perifusions to be carried out in parallel such as when comparing genetically altered islets or cells to control cells.

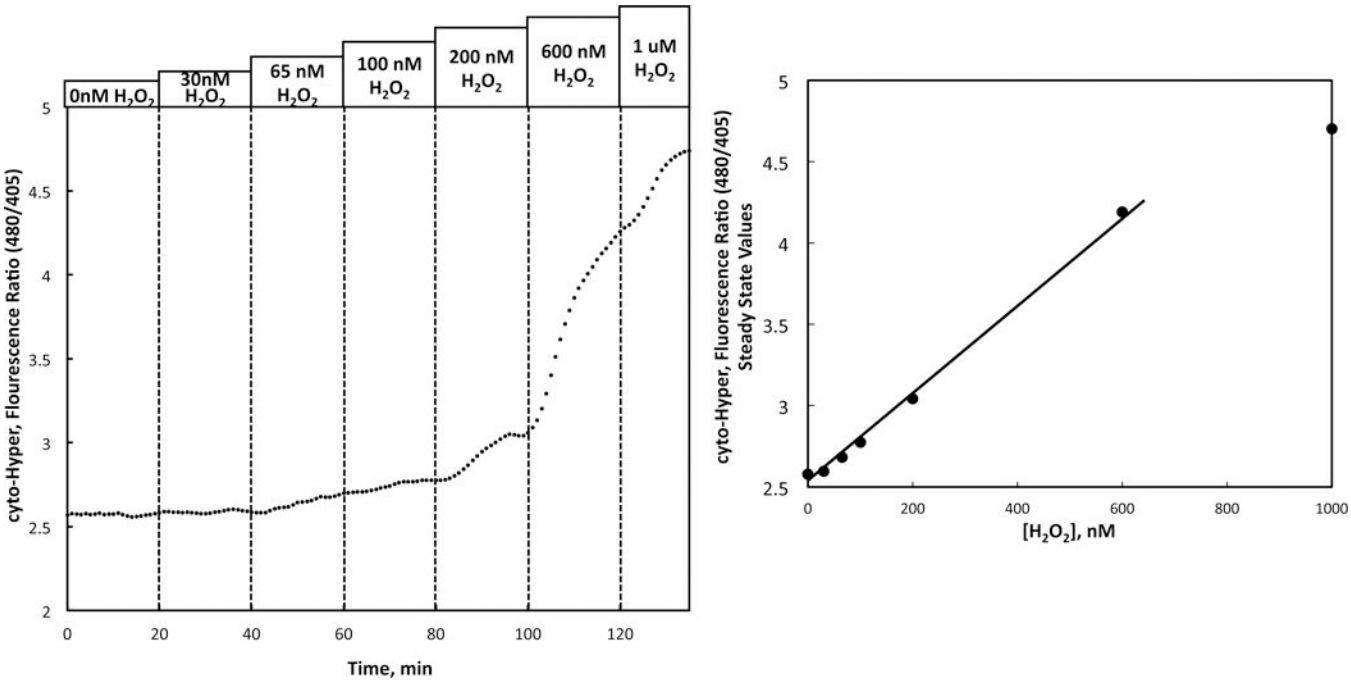

Figure 2. Real time (A) and steady state (B) responses of cyto-HyPer in permeabilized INS-1 cells.

(A) INS-1 cells were imaged during perifusion with stepwise increasing amounts of H_2O_2 in the buffer as indicated (typical calibration curve). (B) Steady state values were calculated as the average of the final five minutes of each H_2O_2 concentration. A trendline was drawn through the linear region from 65 to 600 nM, where 30 nM was indistinguishable from 0 nM fluorescence

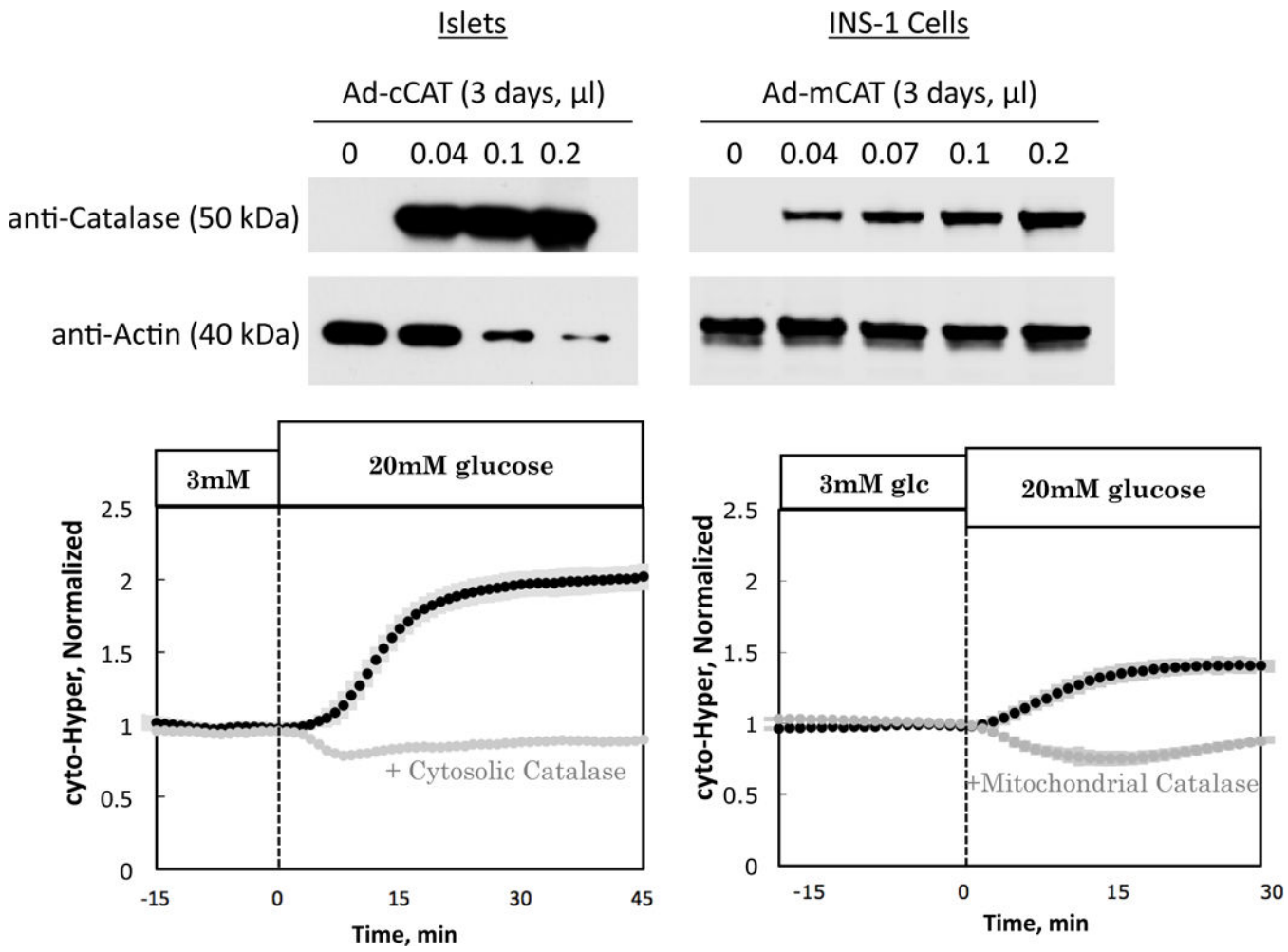

Figure 3. Effect of catalase overexpression on cyto-HyPer.

(A) Western analysis of cytosolic catalase in islets and mitochondrial catalase in INS-1 cells Glucose-induced increase in cyto-HyPer signal is suppressed in response to **(B)** overexpression of cytosolic catalase in rat islets (n = 4) and **(C)** overexpression of mitochondrial catalase in INS-1 cells (n = 4), demonstrating specificity of HyPer for H_2O_2.

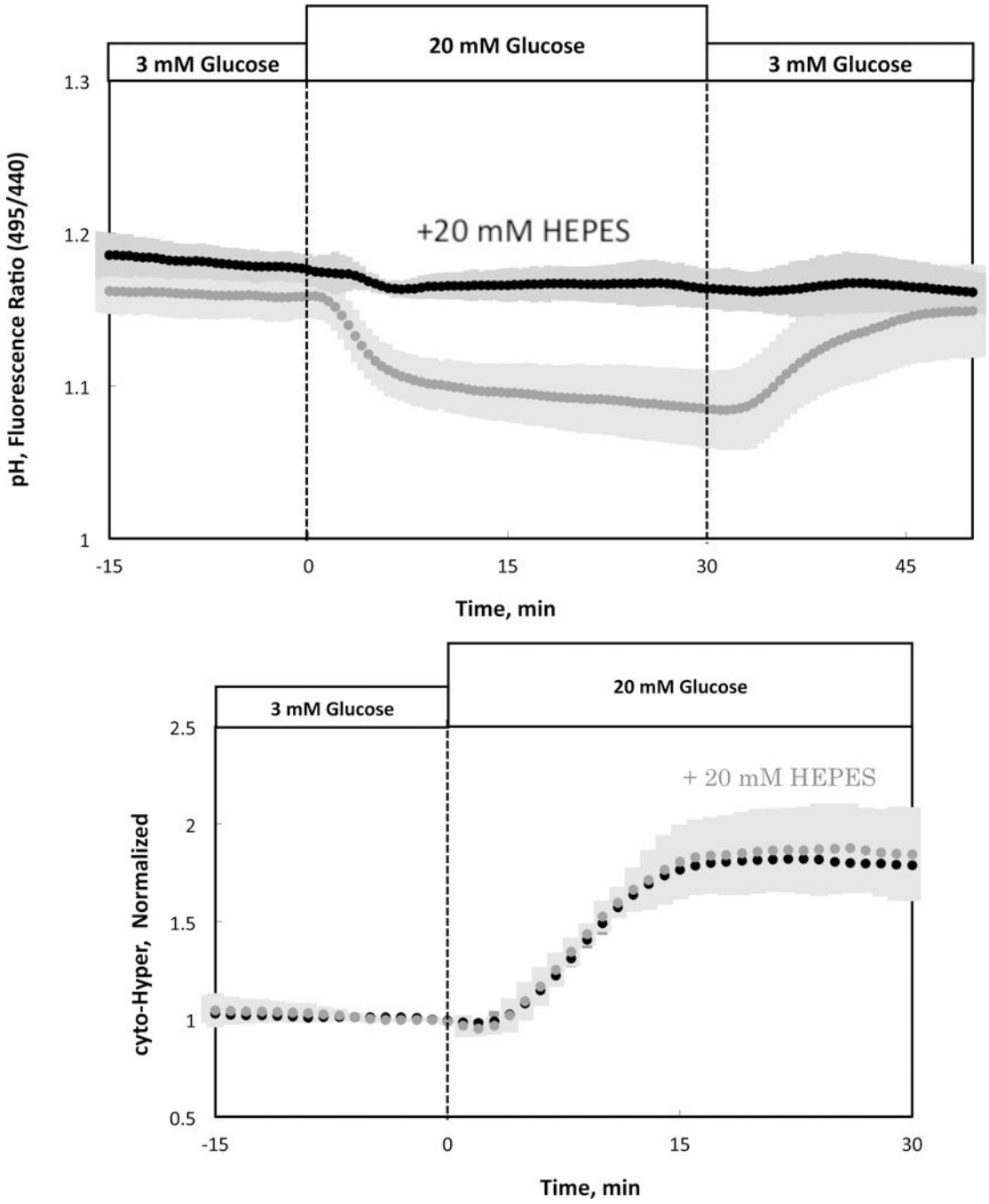

Figure 4. Lack of effect of glucose-induced changes in pH on cytosolic H_2O_2.

(A) Glucose-stimulated pH in the presence and absence of 20 mM HEPES in the buffer (n = 3). (**B**) Glucose-induced changes in cytosolic H_2O_2 by islets in the presence or absence of 20 mM HEPES (n = 3).

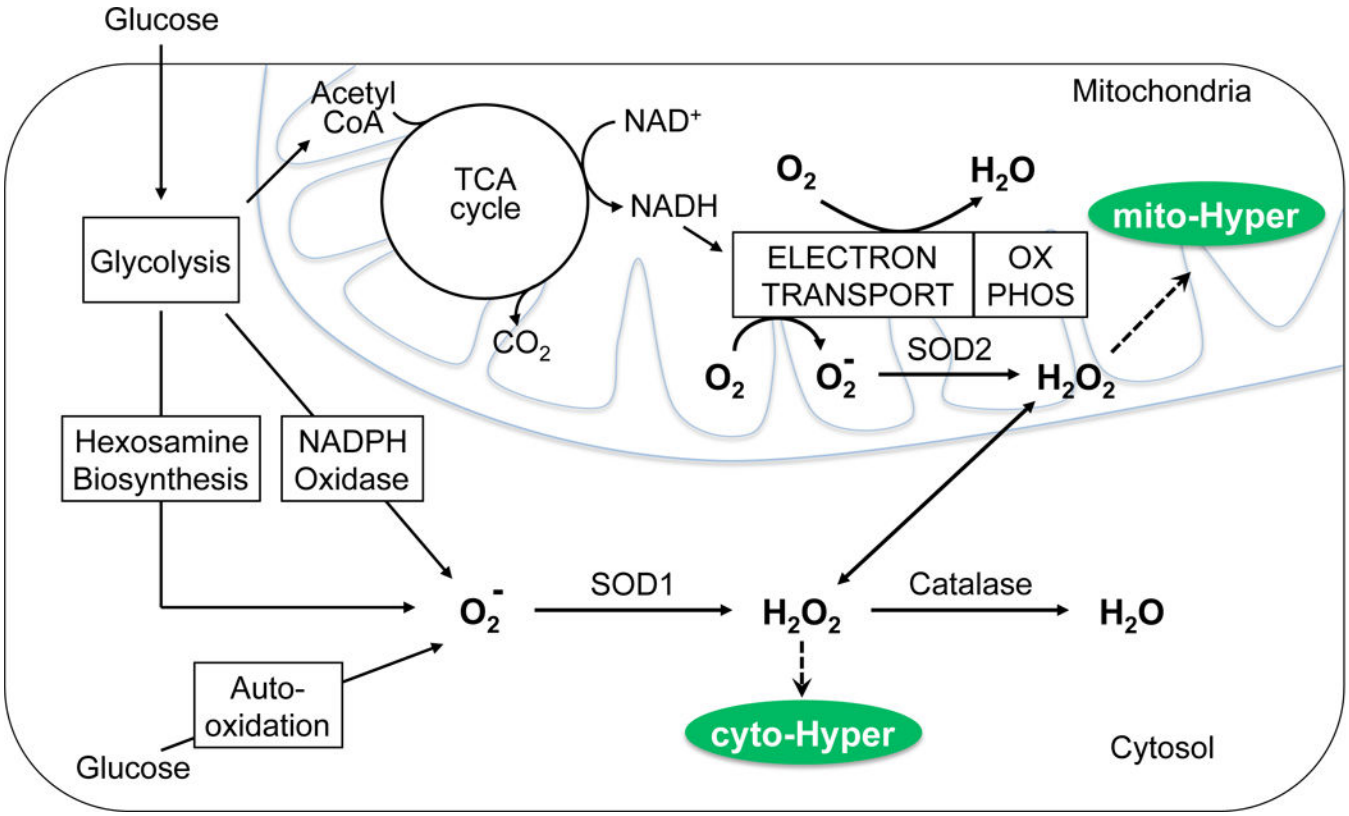

Figure. 5. Production and conversion of H_2O_2.

H_2O_2 is produced in the cytosol and mitochondria by conversion of superoxide and the action of SOD. H_2O_2 in the cytosol and mitochondria are detected by cyto-HyPer and mito-HyPer respectively.

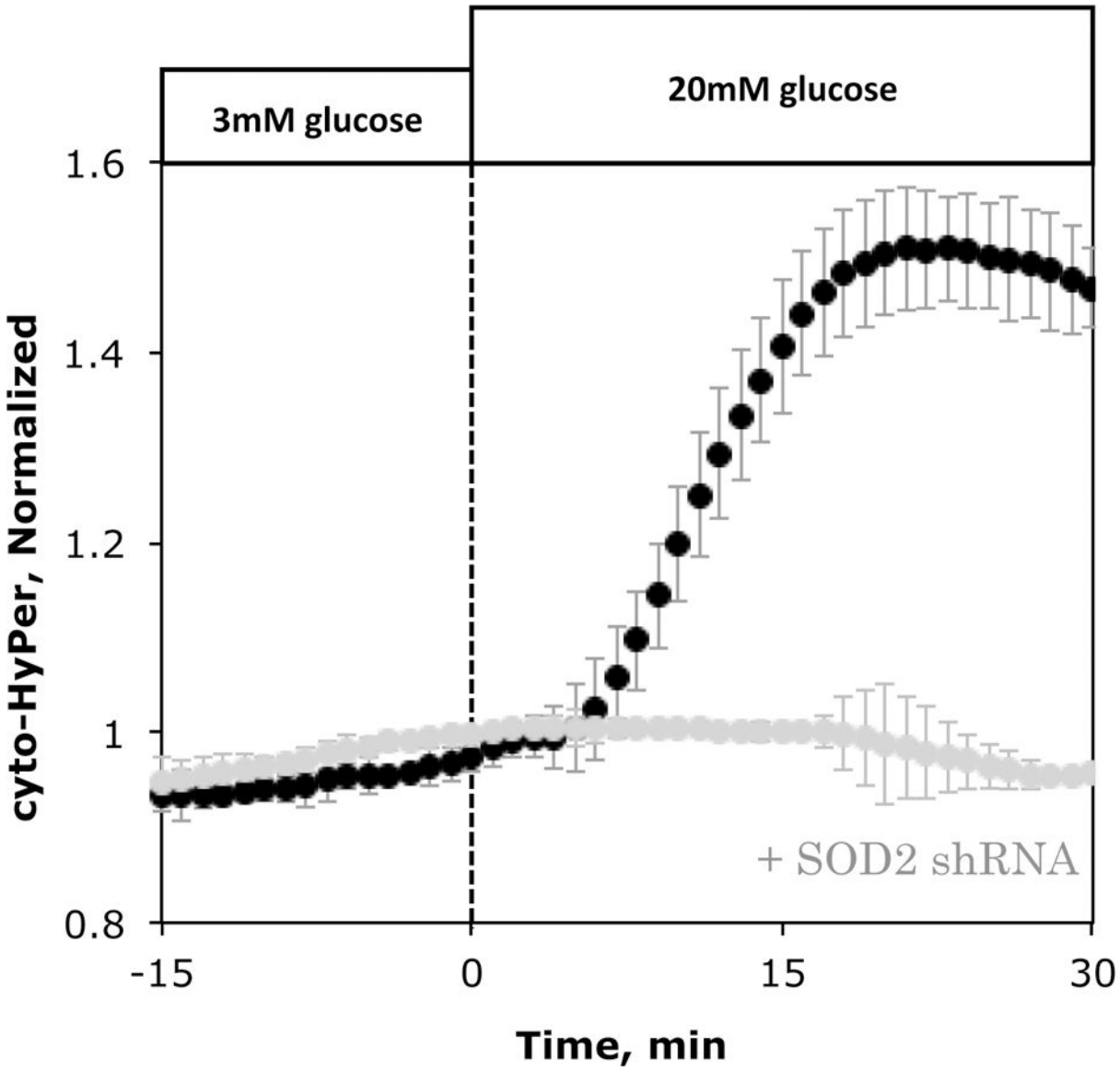

Figure 6. Effect of SOD2 (mitochondrial SOD) knockdown on glucose-induced increase in H_2O_2.
Response in H_2O_2 to glucose by INS-1 cells cultured for 3 days in the presence (n= 2) or absence of shRNA (n = 4) for SOD2.

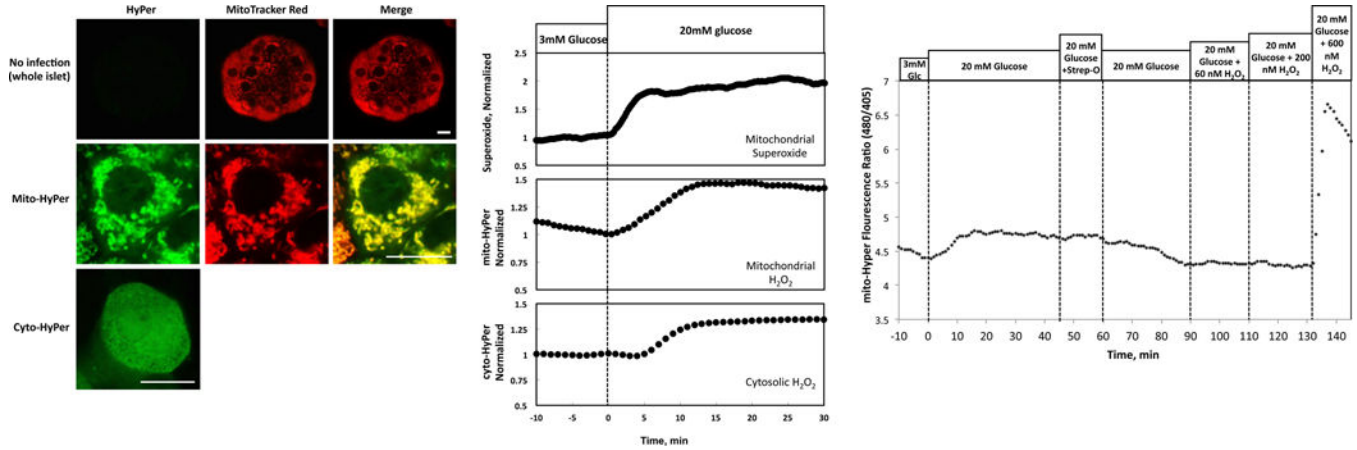

Figure 7. Kinetics of superoxide, mitochondrial and cytosolic H_2O_2 in response to glucose.

(A) Confocal imaging of islets and islet cells infected with or without mito-HyPer and cyto-HyPer. Top row of panels: images of whole islets loaded with or without MitoTracker Red. Middle row of panels: single islet cell expressing mito-HyPer and loaded with MitoTracker Red. Bottom panel: image of single islet cell expressing cyto-HyPer. White scale bar is 10 microns in length. **(B)** Kinetic responses of islets loaded with MitoSOX Red (a sensor for mitochondrial superoxide), or expressing mito-HyPer or cyto-HyPer (n = 4). **(C)** Lack of response of mito-HyPer to low levels of extracellular H_2O_2 in permeabilized islets (typical response).

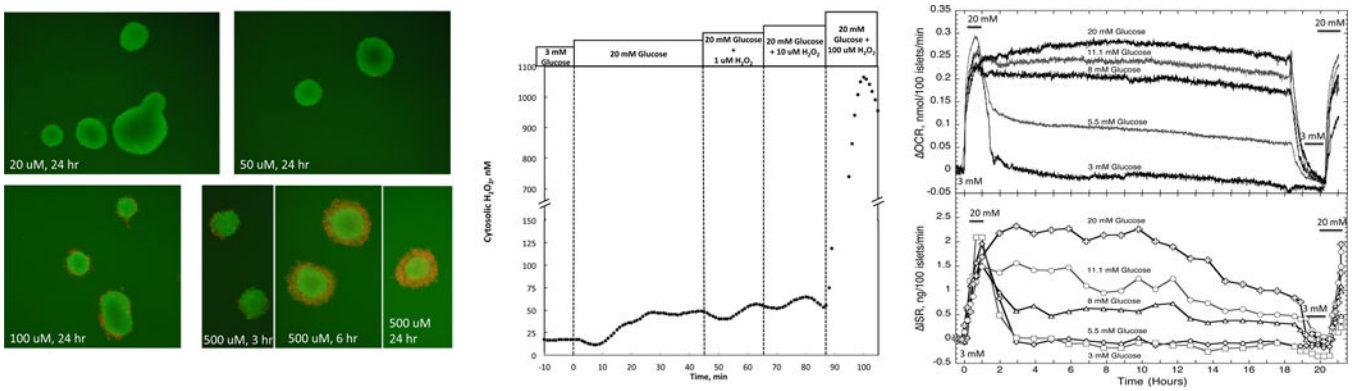

Figure 8. Comparison of glucose-induced H_2O_2 levels vs. H_2O_2 observed in the presence of toxic concentrations of extracellular H_2O_2.

(A) Islets were incubated for 15 minutes in the presence of the indicated concentrations of H_2O_2 and then assessed for viability as reflected by membrane patency at the times shown. (Red cells no longer have membrane integrity.) **(B)** Cytosolic H_2O_2 in response to glucose and extracellular H_2O_2 (typical response). Toxic concentrations of extracellular H_2O_2 were accompanied by supraphysiologic cytosolic levels of H_2O_2. (**C)** Oxygen consumption rate and insulin secretion rate as a function of exposure to various concentrations of glucose in the culture media (n = 3). Prior and subsequent to the culture period, acute islet response to an increase in glucose concentration from 3 to 20 mM was assessed.