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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

Measurement of DCF fluorescence as a measure of reactive oxygen species in murine islets of Langerhans

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

5 **DOI: 10.1039/b000000x**

In islets of Langerhans, oxidative stress induced by reactive oxygen species (ROS) is thought to be critically involved in β-cell dysfunction during the development of diabetes. However, ROS have also been hypothesized to play a role in cellular signalling. To aid in delineating the effects of ROS in living islets of Langerhans, the endocrine portion of the pancreas that contain β-cells, we sought to develop a 10 robust and reproducible protocol to measure these species using the fluorescent dye, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA). The protocol that was developed minimized photobleaching and leakage of H_2DCF from murine islets and utilized a normalization procedure to further reduce experimental variability. The method allowed for ~25 min of DCF measurement in living islets. We used the developed protocol to compare DCF fluorescence from batches of islets incubated in 15 varying glucose concentrations and observed ~1.5-fold higher fluorescence signals in 3 vs. 20 mM glucose. The effects of diazoxide, which clamps open K^+_{ATP} channels reducing intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) without affecting glucose metabolism, were also investigated. The presence of diazoxide increased DCF fluorescence at all glucose concentrations tested while addition of 30 mM K^+ to increase [Ca^{2+}]$_i$ reduced the fluorescence by ~15%. With the developed protocol, all experimental methods tested 20 to increase [Ca^{2+}]$_i$ resulted in a decrease in DCF fluorescence, potentially indicating involvement of ROS in intracellular signalling cascades.

Introduction

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, and include superoxide (O_2^-), 25 hydrogen peroxide (H_2O_2), and hydroxyl free radicals ($^{\cdot}OH$). A major source of O_2^- occurs at protein complexes I and III during oxidative phosphorylation.[1-4] O_2^- can then be converted to H_2O_2 by superoxide dismutase (SOD), which can then be broken down by catalase and glutathione peroxidases (GPxs).[5,6] ROS can also 30 be produced from other sources; one example being NADPH oxidase, which catalyzes the reduction of molecular oxygen to O_2^-.[7,8]

Pancreatic β-cells, located in islets of Langerhans, are the cells responsible for secretion of insulin in response to elevated 35 glucose levels. These cells show weak expression of antioxidant enzymes such as SOD and GPxs, indicating a lower antioxidant capacity compared with other tissues.[9,10] This lower antioxidant capacity may be a potential route for oxidative damage, and it may also be useful because ROS have been shown to potentiate 40 glucose-stimulated insulin secretion (GSIS).[11-15]

To help unravel the roles of ROS in β-cell physiology, robust methods to measure ROS in living islets are required. In general, the most suitable methods for live cell measurements are fluorescence-based where the fluorescence of the indicator is 45 influenced by the presence of ROS. One of the most popular indicators is 2',7'- dichlorodihydrofluorescein diacetate (H_2DCF-DA),[16] which is cell permeable until cleaved by intracellular esterases forming the anion, H_2DCF^-. This dye shows low fluorescence in its reduced state, but is highly fluorescent in its 50 oxidized state (DCF). Its drawbacks are that it is sensitive to photobleaching and can leak out of the cell because of its low charge state.

Due to the lack of a standard protocol for measuring ROS levels using H_2DCF-DA in living murine islets of Langerhans, we 55 set out to develop a robust and reproducible method. Once the method was developed, we evaluated the glucose-dependence of DCF fluorescence, the time course over which the fluorescence levels change in response to a glucose challenge, and the effects of pharmacological agents that affect glucose metabolism and 60 intracellular [Ca^{2+}] ([Ca^{2+}]$_i$). The results showed an inverse relationship between DCF intensity and [Ca^{2+}]$_i$ so that a decrease in DCF fluorescence was observed with increasing glucose concentration.

Materials and methods

65 Chemicals and reagents

Sodium chloride, sodium phosphate dibasic, potassium chloride, potassium phosphate monobasic, tricine, magnesium chloride, calcium chloride, bovine serum albumin (BSA), and penicillin-streptomycin were purchased from Sigma–Aldrich (Saint Louis,

Analytical Methods Accepted Manuscript

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MO). H_2DCF-DA was obtained from Life Technologies (Carlsbad, CA). Dextrose and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were from Fisher Scientific (Pittsburgh, PA). Diazoxide was from Spectrum Chemical (Gardena, CA). Mn(III) tetrakis (4-benzoic acid) porphyrin chloride (Mn-TBaP) was obtained from Cayman Chemical (Ann Arbor, Michigan). Cosmic Calf Serum (CCS) was from HyClone Laboratories (South Logan, Utah). Sodium hydroxide was purchased from EMD Chemicals (San Diego, CA). Collagenase P was purchased from Roche Diagnostics (Indianapolis, IN). RPMI 1640 was from Mediatech (Manassas, VA). H_2O_2 was from Avantor Performance Materials (Center Valley, PA). Ethanol was from Koptec (King of Prussia, PA). Gentamicin was from Lonza (Walkersville, MD). All solutions were made with ultrapure deionized water (NANOpure® Diamond system, Barnstead International, Dubuque, IA).

Isolation of islets of Langerhans

All experiments were performed under guidelines approved by the Florida State University Animal Care and Use Committee, protocol #1235. Islets were isolated by collagenase digestion of the pancreas from male mice (20-40 g) as previously described.[17] Briefly, mice were sacrificed by cervical dislocation and a 0.86-mg/mL solution of collagenase P was injected into the pancreas through the pancreatic duct. The pancreas was collected and incubated in 5 mL of the collagenase solution at 37 °C for 8 min with shaking every 2 min. A centrifuge and a 100 µm nylon filter (BD Biosciences, San Jose, CA) were used to filter the islets from the exocrine tissue. After washing with Hanks' Balanced Salt Solution, the islets were hand-picked under a microscope to ensure high purity of the preparation and cultured in RPMI 1640 medium containing 11 mM glucose, 10% CCS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 10 µg/mL gentamicin at 37 °C in the presence of 5% CO_2. Islets were used within 5 days after isolation.

Experimental protocol

Before incubation, H_2DCF-DA stock solution was made by dissolving 100 mg of the powder into anhydrous DMSO, which was then diluted to 0.5 mM and stored as 100 µL aliquots in a -20 °C freezer. The stock solution and aliquots were made in the dark and with nitrogen flushing to avoid light and air that may cause degradation of the dye. For each experiment, a new aliquot was used and any remaining was discarded. Each batch of aliquots was used within 2 months.

All of the dye incubation steps described below were performed in the dark. Islets were first transferred from the culture media to the experimental buffer, which consisted of balanced salt solution (125 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, and 25 mM Tricine) with either 3, 11, or 20 mM glucose and placed back in the incubator at 37 °C in the presence of 5% CO_2 for 30 min. After that time, H_2DCF-DA was added into the dish to a final concentration of 20 µM and the dish was placed back in the incubator for another 30 min. Finally, islets were quickly rinsed with dye-free experimental buffer containing the same concentration of glucose in which they were previously incubated. After this time, the islets were transferred into a 2 mL chamber (P35G-1.5-14-C, MatTek, Ashland, MA) that was mounted on a heating element (TC-E35x15, Bioscience Tools, San Diego, CA). The imaging buffer was the same as the experimental buffer and was pre-warmed to 37 °C and maintained at this temperature while imaging. In some experiments, 250 µM diazoxide and/or 30 mM K^+ was added to the experimental buffer, and images were acquired as described below.

To perform the time course measurements, one batch of islets, consisting of 5-9 islets, was transferred from the culture media to the experimental buffer (BSS) containing 3 mM glucose. They were then incubated in 3 mM glucose for 30 min followed by dye loading for an additional 30 min. After that time, islets were rinsed with fresh BSS and transferred into experimental buffer that contained 20 mM glucose and a fluorescence image was recorded every 5 min. Another set of experiments was performed in an opposite manner, incubating islets in 20 mM glucose and imaging in 3 mM glucose.

Positive controls were performed by adding H_2O_2 to a final concentration of 100 µM to the islet chamber that contained the experimental buffer and measuring the resulting fluorescence. A negative control was conducted by adding Mn-TBaP to a final concentration of 10 µM during all incubation steps. To test the effect of $[Ca^{2+}]$ on DCF fluorescence, 20 µM H_2DCF-DA and 100 µM H_2O_2 were added into the imaging chamber containing the experimental buffer but without islets and the fluorescence measured. The $[Ca^{2+}]$ was then increased to 500 nM and the fluorescence measured again.

Fluorescence detection

The fluorescence of DCF was measured on the stage of a Nikon Eclipse Ti microscope. Light from a Xenon arc lamp was first passed through a 0.8 neutral density filter and an excitation filter (482 ± 35 nm). This light was then reflected by a 510 nm dichroic mirror, and into the back of a 10X, 0.5 NA objective. Emission light passed through the same objective and was filtered by a bandpass filter (536 ± 40 nm) prior to collection by a charge-coupled device (Photometrics Cascade TC253, Roper Scientific, Tucson, AZ). All of the filters were combined onto a single filter set (FITC-3540B-NTE), which was purchased from Nikon Instruments Inc. (Melville, NY). Three fluorescent images were taken, once every 10 s, for each group of islets at each condition and were acquired directly after bringing the islets to the imaging chamber. Up to 9 islets were imaged at one time and 10-30 were tested for each condition.

Data analysis

All DCF fluorescence intensities were background subtracted and normalized to the background-subtracted 3 mM glucose value. Results are shown as mean ± S.E. for the indicated number of islets. A two-tailed Student's t-test was performed between groups and results deemed significant at $p < 0.05$.

Results and discussion

The conventional pathway of GSIS involves glycolysis and oxidative phosphorylation increasing the ATP/ADP ratio. This increased ratio then closes K^+_{ATP} channels, resulting in depolarization of the plasma membrane. In response, voltage-dependent Ca^{2+} channels open, increasing $[Ca^{2+}]_i$, resulting in insulin secreted from beta cell granules. Using H_2DCF-DA, the

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amounts of ROS measured from islets incubated in 3 mM glucose has been observed to be both lower[14,18] and higher[19-21] compared to islets incubated at elevated glucose levels. Due to these discrepancies, we set out to develop a robust analytical procedure for the use of H_2DCF-DA in living murine islets.

Fluorescence normalization and protocol

In murine islets, the fluorescence intensity of DCF decreased rapidly, especially during the first 10 min of fluorescence imaging. These results were observed regardless whether the images were acquired continuously or in intervals ranging from 1-10 min using minimal light intensity as measured out of the microscope objective (2 mW). This decrease in fluorescence was attributed

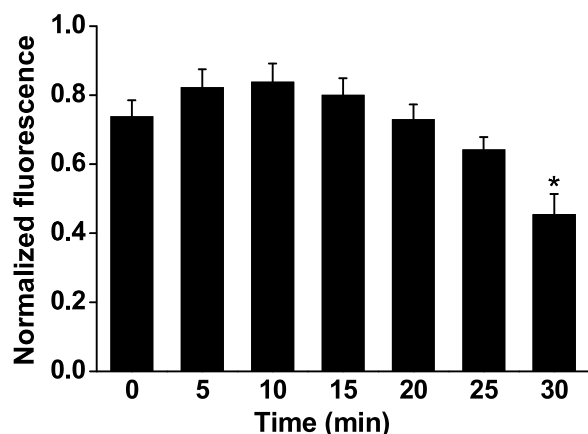

Fig. 1 Stability of DCF fluorescence. The average normalized DCF fluorescence is shown when 9 islets were incubated in 20 mM glucose. Fluorescence images were acquired every 5 min. *: $p < 0.05$ compared to 0 min

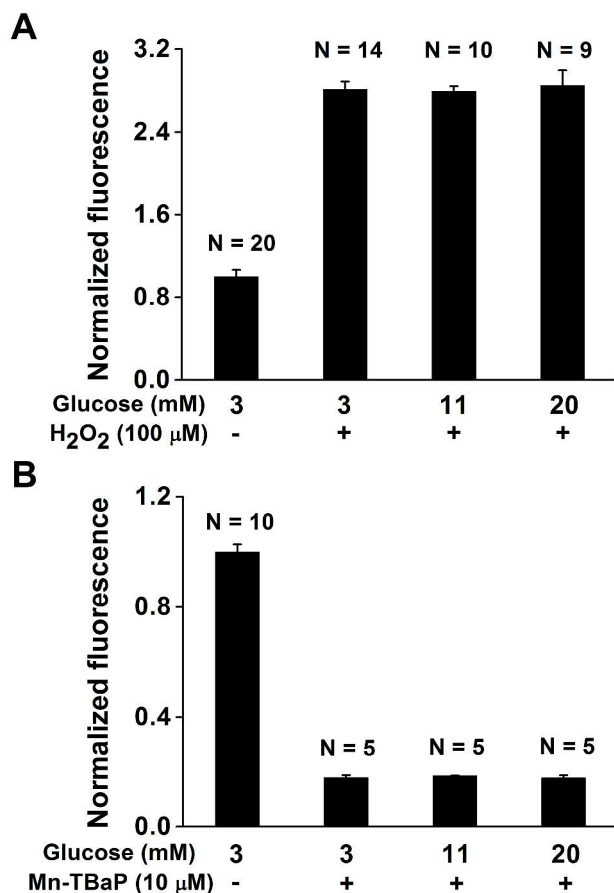

Fig. 2 Control experiments for DCF. For all plots, the average and normalized fluorescence intensities are shown where N represents the number of islets tested for each condition. **(A)** A positive control was performed by adding 100 μM H_2O_2 to islets incubated in different glucose concentrations as shown under the graph. The average normalized fluorescence intensity of islets incubated in 3 mM glucose without H_2O_2 is shown as a reference. **(B)** The negative control was performed by incubating islets at different glucose concentrations with Mn-TBaP during all incubation and imaging steps. The resulting fluorescence intensities were similar to background levels (not shown). The average normalized fluorescence intensity of islets incubated in 3 mM glucose without Mn-TBaP is shown as a reference.

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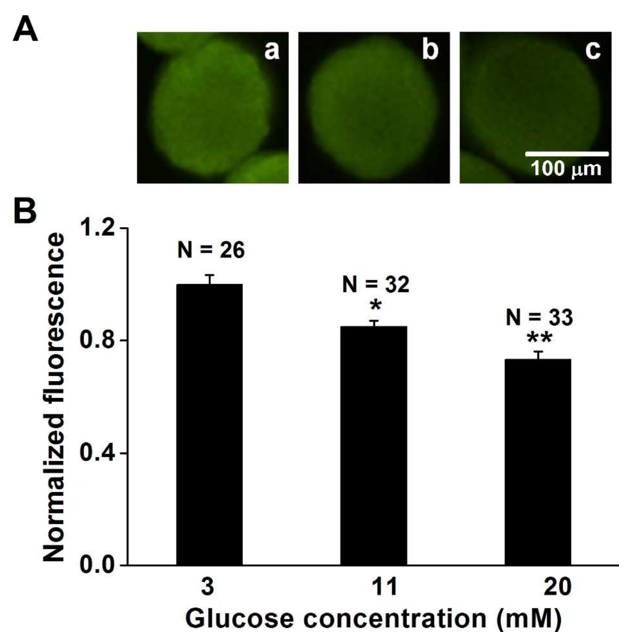

Fig. 3 Effects of glucose concentration on DCF fluorescence in islets. (A) Images of islets at (a) 3 mM glucose, (b) 11 mM glucose, (c) 20 mM glucose. (B) An average of normalized fluorescence intensities from islets incubated at different glucose concentrations tested is shown. N represents the number of islets used for each condition tested. *: $p < 0.05$ compared with 3 mM glucose, **: $p < 0.02$ compared with 11 mM glucose.

to both photobleaching and dye leakage. To minimize these effects, the optimized protocol consisted of performing all experimental steps, consisting of both incubation and measurement steps, in the dark, and limiting the time from rinsing to measurement of DCF fluorescence to 1 min. Another source of error in the fluorescence measurements was found to be due to day-to-day differences in experimental conditions such as lamp intensity and dye concentration. To account for these variations, the average background-subtracted DCF fluorescence intensity from a batch of islets incubated in 3 mM glucose was measured at the start of each day. Experiments were then performed throughout the day to measure DCF fluorescence under different experimental conditions. The results of these experiments were normalized by dividing the background-subtracted intensities of each islet to the average background-subtracted intensity of islets incubated in 3 mM glucose. This normalization gave the 3 mM glucose a normalized intensity of 1.0 for all experiments.

Under these conditions, the fluorescence stability of DCF was tested by measuring fluorescence at 5 min intervals in a batch of 9 islets at a constant glucose concentration over time (Figure 1). It was observed that the fluorescence was stable over the first 25 min, and after that time, a significant decrease was observed in the measured fluorescence intensity.

A potential problem in comparing DCF fluorescence from islets incubated under different glucose conditions is glucose-dependent dye uptake. The positive control was used to test this effect by comparing the H_2O_2-induced fluorescence from islets that were exposed to different glucose concentrations. In this way, the fluorescence recorded is expected to be proportional to the intracellular [DCF]. As shown in Figure 2A, islets incubated in different glucose concentrations showed similar fluorescence intensities ($p > 0.05$), which indicated that the dye concentrations were equal and dye loading was independent of glucose concentration. In a different set of experiments, Mn-TBaP was added during all incubation steps to scavenge free radicals[22] for a negative control. The normalized fluorescence from islets in the presence of Mn-TBaP (Figure 2B) showed a

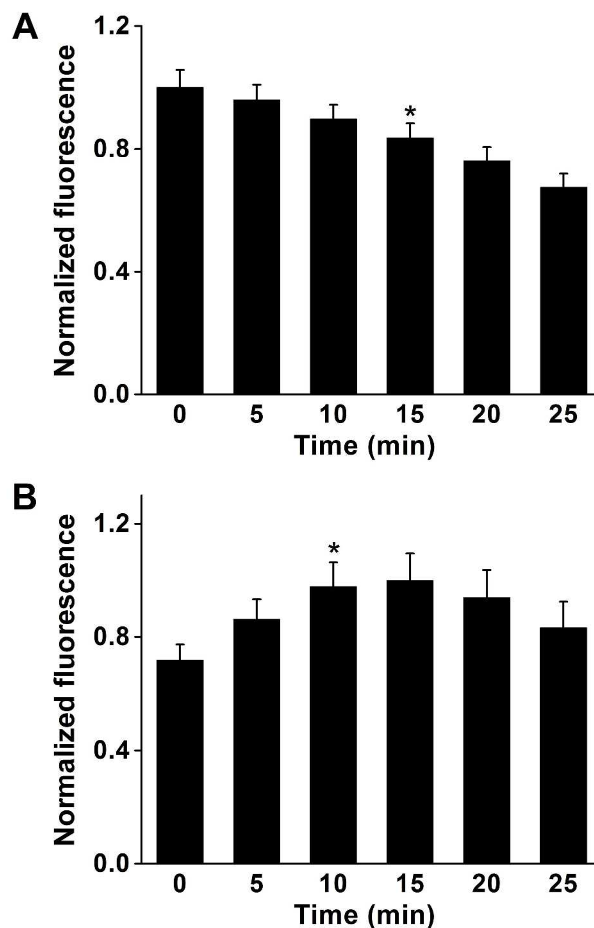

Fig. 4 Time course measurements. The average normalized fluorescence from 10 islets is shown. **(A)** Islets were incubated in 3 mM glucose for 60 min and imaged in 20 mM glucose at the times indicated at the bottom. *: $p < 0.05$ compared with 0 min. **(B)** A separate group of islets were incubated in 20 mM glucose for 60 min and imaged in 3 mM glucose at the times indicated below the plot. *: $p < 0.05$ compared with 0 min.

signal similar to the background at all glucose concentrations tested indicating that DCF fluorescence was sensitive to ROS.

Effects of constant glucose concentrations on ROS generation

With the developed protocol, the glucose-dependence of ROS formation in murine islets of Langerhans was investigated. Groups of islets were incubated for a total of 60 min in one of three glucose concentrations: 3, 11, or 20 mM (Figure 3A). An inverse relationship between the average normalized fluorescence intensity of DCF and glucose concentration was observed (Figure 3B). The signal at 20 mM glucose was ~75% of the signal at 3 mM glucose, and 11 mM glucose produced a value between the two other concentrations, significantly different than both ($p < 0.05$ compared to 3 mM, $p < 0.02$ compared to 20 mM). These results are representative of islets taken from ~10 mice and across

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a time span of 2 months. It was noticed that as the dye became older, the fluorescence intensity was reduced compared to when the dye was new. However, the daily normalization procedure improved the intra-day reproducibility to < 5% RSD and inter-day reproducibility to < 10% RSD.

Time course measurement

Because the developed protocol allowed stable measurements to

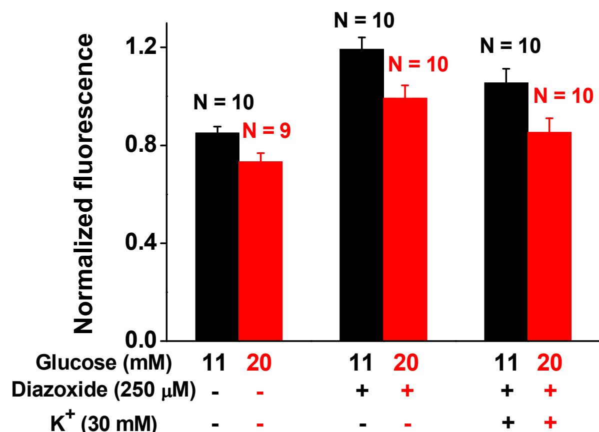

Fig. 5 Effects of diazoxide on DCF fluorescence. Fluorescence images were acquired from islets under different conditions as indicated below the plot. A "+" indicates the presence of either 250 μM diazoxide or 30 mM K^+.

be made during the first 25 min of the experiment (Figure 1), the dynamics of DCF fluorescence when islets were exposed to a changing glucose concentration were investigated. The average fluorescence of DCF decreased by 6% within 5 min when the glucose concentration was increased from 3 to 20 mM (n = 5, Figure 4A). After 25 min, the signal had decreased to 33% of the original signal. When the DCF fluorescence was measured as the glucose concentration decreased from 20 to 3 mM, a ~39% increase in the fluorescence signal was observed within the first 15 min (Figure 4B). The net results of these experiments were similar to those obtained by incubating different batches of islets at constant 3, 11 or 20 mM glucose concentrations (Figure 3). As the glucose concentration increases, the measured DCF fluorescence decreases. These results are similar to those observed elsewhere, which used H_2DCF-DA [19,20] or other techniques[21] to measure ROS levels.

The effect of intracellular Ca^{2+} on ROS formation

Among the various factors that can contribute to ROS formation, $[Ca^{2+}]_i$ plays an important role and its potential effects are complicated. Increases in $[Ca^{2+}]_i$ are essential for GSIS, but increases in this cation will also affect mitochondrial membrane potential, and therefore, ROS levels. As $[Ca^{2+}]_i$ rises, the flux of Ca^{2+} into the mitochondria also rises where it can lead to hyperpolarization of the mitochondrial membrane potential via activation of enzymes involved in the TCA cycle, and it can also lead to mitochondrial membrane depolarization because of the net increase of Ca^{2+} ions.[23-25] These results would have differing effects on the amount of ROS generated.

To help delineate the mechanism of how GSIS reduces DCF fluorescence, diazoxide was used. Diazoxide clamps K^+_{ATP} channels open ensuring the plasma membrane does not become depolarized during GSIS and maintains $[Ca^{2+}]_i$ at basal levels.[26] As a result, by measuring DCF fluorescence in the presence of diazoxide, the effect of $[Ca^{2+}]_i$ on ROS production during oxidative phosphorylation may be indirectly tested.

As shown in Figure 5, the fluorescence of DCF increased in the presence of diazoxide at all glucose concentrations tested ($p < 0.001$ vs. 11 and 20 mM glucose alone). This indicates that reduction of $[Ca^{2+}]_i$, which will be observed in the presence of diazoxide, results in increased DCF levels. A control experiment indicated that in the absence of islets, the fluorescence of DCF itself was insensitive to high Ca^{2+} levels, although this experiment was performed on the uncleaved dye. In some experiments, 30 mM K^+ was added to islets exposed to diazoxide. High levels of K^+ depolarize the plasma membrane independent of metabolic factors causing $[Ca^{2+}]_i$ levels to rise. In islets incubated in either 11 or 20 mM glucose, the fluorescence of DCF decreased ~13% when K^+ was added ($p < 0.05$ compared to diazoxide alone) but not to the levels observed in the absence of diazoxide.

These results could be interpreted as the consumption of ROS, in part, via Ca^{2+}-dependent processes that occur during GSIS. Because Ca^{2+}-dependent processes would be inactive at low $[Ca^{2+}]_i$, the ROS levels would not be consumed and therefore increase as indicated by higher DCF fluorescence. Application of K^+ would increase $[Ca^{2+}]_i$ and re-activate the Ca^{2+}-dependent processes to an extent leading to a depletion of ROS. More experimental evidence, such as simultaneous measurement of NADH, $[Ca^{2+}]_i$, mitochondrial membrane potential, and DCF would need to be acquired to confirm this hypothesis.

Conclusions

The method described here provides a stable and reproducible protocol for measuring ROS in murine islets of Langerhans using H_2DCF-DA at both constant and dynamic glucose levels. The results indicated that low glucose levels induced a higher DCF fluorescence and that these results were inversely proportional to the expected $[Ca^{2+}]_i$. The results observed in the presence of glucose and diazoxide both may indicate that ROS are potentially used in intracellular signaling cascades.

Acknowledgements

This work was supported in part by a grant from the National Institutes of Health (R01 DK080714).

Notes and references

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