Studies of dispersed β cells have been used to infer their behavior in the intact pancreatic islet. When dispersed, β cells exhibit multiple metabolic glucose-response populations with different insulin secretion properties. This has led to a model for glucose-dependent insulin secretion from the islet based on a stepwise recruitment of individual β cells. However, previously reported synchronous and uniform Ca\(^{2+}\) activity and electrical responses indicate that β cell behavior within intact islets is more uniform. Therefore, uncertainty remains whether β cell metabolic heterogeneity is functionally important in intact islets. We have used two-photon excitation microscopy to measure and compare the glucose-induced NAD(P)H autofluorescence response in dispersed β cells and within intact islets. Over 90% of β cells in intact islets responded to glucose with significantly elevated NAD(P)H levels, compared with less than 70% of dispersed β cells. In addition, all responding β cells within intact islets exhibited a sigmoidal glucose dose response behavior with inflection points of ~8 mM glucose. These results suggest that β cell heterogeneity may be functionally less important in the intact islet than has been predicted from studies of dispersed β cells and support the role of glucokinase as the rate-limiting enzyme in the β cell glucose response.

Insulin secretion from pancreatic β cells is tightly coupled to glucose metabolism (1–4). When dispersed, marked variability in the metabolic responses of β cells to glucose has been observed (5, 6) using NAD(P)H autofluorescence as an index of the cellular redox state (7). Based on this, it has been suggested that metabolic heterogeneity plays a fundamental role in whole islet insulin secretion by a mechanism of variable activation thresholds of individual β cells (8). The observation that glucokinase, which has been postulated as the rate-limiting step in glucose transduction by β cells (3), exhibits heterogeneous immunoactivity among β cell also supports this model (9). However, other indicators of β cell function within intact islets, such as synchronous intracellular [Ca\(^{2+}\)] (10) and electrical (11) responses, indicate that intraislet β cells constitute a functionally more homogeneous population. Direct measurement of the metabolic behavior of cells within the intact islet, however, has not been performed. Here we report the first measurement of glucose-induced NAD(P)H autofluorescence changes within the intact islet at subcellular resolution using TPEM (12, 13). Using the same instrument, we also measured the glucose-induced metabolic behavior of isolated cells, thereby allowing a quantitative comparison of the metabolic response of β cells under both conditions.

A new alternative to confocal microscopy, TPEM allows collection of optical sections within thick samples, such as pancreatic islets, with greatly reduced photobleaching and photodamage. Previously, we have described a laser scanning microscope that is optimized for TPEM of UV excitable fluorophores, such as NAD(P)H (14). This instrument allows extended dynamic studies of many cells simultaneously, thereby permitting observation of the temporal and spatial organization of metabolic activity within the intact islet.

**EXPERIMENTAL PROCEDURES**

Islet Isolation and Culture—Rat islets were isolated by pancreatic distention followed by collagenase digestion (15). Only the splenic portion of the pancreas was digested in order to select an islet population enriched in α cells over ß and PP cells (16). Rats were treated under protocols approved by the Vanderbilt University Animal Care Committee. Before imaging, islets were cultured for 1–5 h in RPMI 1640 medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Life Technologies, Inc.), 100 IU/ml penicillin, and 100 μg/ml streptomycin. For imaging, islets were attached to a coverslip with Cell-Tak (Collaborative Biomedical Products, Bedford, MA), and the coverslip was placed in a temperature-controlled perfusion micro incu- bator (TLC-MI, Adam and List Associates, Westbury, NY). This micro incubator held the temperature of the sample at 37 °C (measured by thermocouple adjacent to the islet) by heating both the coverslip chamber and incoming perfusate. An air stream incubator (Nicholson Precision Instruments, Gaithersburg, MD) heated the objective to the perfusate temperature to eliminate heat transfer through the oil-glass objective interface. Individual islet cells were dispersed by the method of Pralong et al. (17) except that 15–25 islets were exposed to 50 μl of calcium-free 25 μg/ml trypsin solution for 5 min. Cells were triturated briefly and plated onto marked coverslips (Belco) coated with a monolayer of Celi-Tak.

Quantitative NAD(P)H Imaging in Living Islets and Isolated β Cells—Two-photon excitation microscopy was performed using a previously described instrument (14). Briefly, a 76-MHz train of 100 femtosecond pulses of 710-nm light from a mode-locked Ti:Sapphire laser (Coherent Mira, Mountain View, CA) was focused on to the sample by the objective, separated from the excitation light by a dichroic mirror (550 DCLP Ext R, Chroma Technology, Brattleboro, VT), filtered to remove scattered red light (550 SP with blocking from 700 to 750 nm, Chroma Technology), and directed to a photomultiplier tube detector (Hamamatsu R268). Output from the detector was integrated, stored,
and displayed by computer.

For the dynamic NAD(P)H images, the perfusion was turned off, image acquisition was started, and 8 s later a small volume of test agent was added at the far edge of the sample chamber, which initially contained 3 ml of basal buffer (DME base (Sigma) with 2 mM glutamine and 1 mM glucose). In these experiments, it was more convenient to use a glass-bottomed 35-mm dish (PG35G-0-14-gm, Matek Corporation, Ashland, MA) than the coverslip chamber, but temperature was still maintained by heating both the stage and the objective lens. Pre-heated test solutions were added as follows: 100 ml of 1 mM glucose, 60 ml of 1 mM mannoheptulose, or 100 ll of 100 mM NaCN to give the approximate final concentrations of 30, 20, and 3 mM, respectively. TPEM images were acquired continuously for 136 s at 8 s/scan.

For the steady-state glucose response measurements, islets were perfused at 1.2 ml/min and 37 °C with Krebs-Ringer bicarbonate buffer (120 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 5 mM NaHCO3, and 10 mM HEPES, pH 7.4) containing 3, 5, 7.5, 12, or 23 mM glucose, and NAD(P)H autofluorescence was measured after 10 min of equilibration with each different concentration of glucose. In a parallel experiment using 12 mM glucose, images acquired at 5-min intervals after the addition of glucose, showed that 10 min was sufficient to reach the response plateau.

NAD(P)H measurements of dispersed cells were performed by the same techniques as the glucose response, except that perfusion rate was lowered to 0.5 ml/min and glucose levels were varied between 1 mM and 30 mM to compare directly with the dynamic NAD(P)H affords unprecedented clarity in optical sections throughout the islet. The scale bar is 10 μm.

Digital Image Analysis—Digital image processing of time series was performed on a Macintosh Power PC 6100AV computer running Alice (Perceptive Systems, Inc., Boulder, CO) using the “Particle Series” custom add-on. Means were taken in 25 pixel circular regions of interest in the cytoplasm of single islet cells to generate a time course or glucose response for NAD(P)H autofluorescence. The traces shown are each representative of at least 30 cells analyzed in this manner.

RESULTS

We have measured NAD(P)H autofluorescence at the subcellular level throughout intact pancreatic islets (Fig. 1) by combining TPEM (14) with established quantitative laser scanning microscopy methods (18). Subcellular structures, such as the nuclei (which appear dark in the NAD(P)H image) are clearly visible. The spatial discrimination and collection efficiency are sufficient to quantitatively assess NAD(P)H levels in individual β cells, with temporal resolutions of 4–8 s/image. A series of control experiments were performed to demonstrate that the fluorescence changes observed were due to changes in [NAD(P)H] and to establish the limits of islet viability during TPEM (Fig. 2). Inhibition of respiration using cyanide produced a 2-fold elevation in autofluorescence, whereas inhibition of glucose phosphorylation with mannoheptulose led to a ~50% reduction. The effect of these metabolic perturbations on NAD(P)H levels agrees with similar measurements using UV excitation (7, 19). Laser irradiation of ~3 mW (average power at the sample) generated signals sufficient for imaging without

rabbit IgG-FITC (Jackson Immunoresearch) and incubated for 8 h. Following washing, islets were mounted in Aqua-Polymount (Polysciences, Inc.) and imaged sequentially for insulin and glucagon using the 543- and 488-nm lines of a HeNe and Ar-Kr laser, respectively, with a Zeiss LSM 410 confocal microscope. Dispersed β cells were identified by insulin and glucagon immunostaining, respectively. After measuring the autofluorescence responses by TPEM, islets were

Fig. 1. NAD(P)H autofluorescence in an optical section of an isolated rat islet. Individual β cells and their nuclei are visible 40 μm into an islet mounted on a coverslip. The reduced light scattering and absorption associated with TPEM (red light instead of UV light is used to excite NAD(P)H) affords unprecedented clarity in optical sections throughout the islet. The scale bar is 10 μm.

Fig. 2. Cellular autofluorescence detected in islets by TPEM is due to NAD(P)H. Digital image analysis was used to measure autofluorescence in single β cells after image acquisition. NaCN (3 mM), which inhibits cellular respiration, caused a ~2.5-fold rise in cellular autofluorescence (7) (●, n = 5 islets). Mannoheptulose (20 mM), which inhibits glucose utilization by competitive inhibition of glucokinase, resulted in a ~50% decrease in autofluorescence (●, typical β cell, n = 3 islets). Glucose stimulation (30 mM) increased autofluorescence ~2-fold (■, typical β cell, n = 8 islets). An average laser power of 3 mW at the sample produced NAD(P)H autofluorescence signals adequate for imaging without detectable photobleaching (x). Continuous exposure to excessive doses of two-photon irradiation (~5 mW average power) caused a 1.5-fold increase in autofluorescence after 136 s (▲). This cellular photodamage was caused by two-photon interactions because islets pre-exposed to 15 mW of defocused red light showed no change in NAD(P)H levels (data not shown). The scale bar is 10 μm.
evidence of cellular damage (e.g. autofluorescence increase, detectable photobleaching, or degradation of glucose response). However, extended laser irradiation >5 mW resulted in increased autofluorescence (Fig. 2) and the loss of any glucose-induced autofluorescence response (data not shown). To determine if the laser-induced autofluorescence increase was caused by two-photon excitation or the incident red light, islets were exposed to intense laser illumination focused into the coverslip. This exposed the entire islet to unfocused red light without generating any two photon excitation within cells. In this situation, even 15 mW of unfocused red light did not affect autofluorescence, and the subsequent glucose-induced NAD(P)H response was unaffected. Thus, islet cells are sensitive only to the amount of two-photon excitation and not single-photon interactions from the red light.

To allow quantitative comparison of cells in the intact islet and after dispersion, we measured NAD(P)H responses of cells in both situations. In the case of intact islets, dynamic behavior was also observed over several successive images. The temporal metabolic response to glucose of a single optical section of a cellular autofluorescence is shown in A–H. After measuring dynamic changes, β and α cells (β, green for insulin and red for glucagon) were identified by immunofluorescent staining and confocal microscopy. The NAD(P)H images shown (time course generated as in Fig. 2) begin one scan before any increase was detected and continue at 8-s intervals thereafter. NAD(P)H autofluorescence in central β cells lagged that of the periphery by about 40 s, consistent with glucose diffusion into the islet.

To validate the results obtained from intact islets, we also examined the NAD(P)H response of isolated β cells. Fig. 5

![Fig. 3. Serial images showing the temporal metabolic responses of cells visible in an optical section of an intact islet. Two-photon excitation of cellular autofluorescence is shown in A–H. After measuring the dynamic changes, β and α cells (β, green for insulin and red for glucagon) were identified by immunofluorescent staining and confocal microscopy. The NAD(P)H images shown (time course generated as in Fig. 2) begin one scan before any increase was detected and continue at 8-s intervals thereafter. NAD(P)H autofluorescence in central β cells lagged that of the periphery by about 40 s, consistent with glucose diffusion into the islet.](http://160.129.157.26)
The glucose-induced metabolic responses from the β cells in intact islets were strikingly uniform and contrast significantly with the heterogeneous behavior of isolated β cells (Figs. 3 and 5). In our experiments, greater than 90% of β cells in intact islets were metabolically active, whereas less than 70% of dispersed β cells responded to the same glucose concentrations. These isolated cell measurements are in excellent agreement with previous reports where the metabolically responsive population of isolated β cells in the presence of 20 mM glucose was determined to be 70% (5, 8). In addition, as shown in Fig. 5, the amplitude of response varied more greatly in the dispersed cells.

Two explanations for the differences observed between isolated cells and those in the intact islet seem possible, and each has different implications. The first explanation is that proteolytic damage during islet and single-cell isolation procedures may degrade the NAD(P)H response of certain cells without compromising their viability. In intact islets, all nonresponsive cells were at the periphery, and cells on the islet exterior would obviously be more prone to this kind of damage. A similar damage mechanism may also be responsible for the increased fraction of nonresponsive dispersed β cells, because islet dispersion requires even more aggressive proteolytic treatment. If this is the case, then models developed from studies of isolated β cells are unlikely to accurately describe islet function.

A second explanation for the differences between isolated β cells and those in intact islets could be that intercellular communication might act to overcome the heterogeneities of individual β cells. Cell-cell contact may allow less responsive cells to be entrained by the more responsive cells, but this was not
observed in the dissociated cells (Fig. 5) where cells in contact
were not found to respond more uniformly than completely
isolated ones. In intact islets, individual β cells never displayed
earlier responses than neighboring cells (Fig. 3), so any en-
trainment mechanism would need to act faster than a few
seconds. Such coupling cannot be electrical because electrophysiologi-
ical and \( [Ca^{2+}] \), measurements show a time lag be-
 tween the NAD(P)H increase and membrane depolarization of
>15 s (23). Such a mechanism would likely involve a small,
easily diffusible messenger. This messenger could also diffuse
rapidly toward the islet core and generate an activation signal
ahead of the primary glucose-induced autofluorescence wave,
but no such signal was observed (Fig. 3). Although it may be
possible to construct a model with a messenger that can equil-
ibrate between adjacent cells within a few seconds and not
trigger cells toward the islet center sooner than the measured
40 s delay, we conclude that such an entrainment mechanism is
unlikely.

In the absence of a defined entrainment mechanism, the
uniform metabolic response results in the whole islet appear to
be inconsistent with significant metabolic heterogeneity among
β cells, but the images do show autofluorescence heterogeneity
at the initial glucose concentration of 1 mM (Fig. 3A), as do the
isolated β cells (Fig. 5A). This basal autofluorescence heteroge-
nity suggests that a metabolic thresholding based on the ab-
solute ATP/ADP ratio (related to the absolute NAD(P)H/
NAD(P)\(^+\) ratio) could generate heterogeneous β cell function
even in the presence of a uniform NAD(P)H response. However,
such basal heterogeneities in whole islets are difficult to inter-
pret, because they could possibly arise due to variable optical
density or the subcellular distribution of NAD(P)H. First, cells
in the middle of the islet generally have more tissue between
them and the objective lens than do cells on the periphery, and
more fluorescence signal is indeed detected in the peripheral
cells (Fig. 3). Secondly, because the 1-μm-thick optical section
passes through each cell differently, the amount of NAD(P)H
detected in each cell may differ as well.

The metabolic heterogeneity found in dispersed β cells has
been shown to correlate with heterogeneity in insulin secretion
and has thus led to a model for islet function based on variable
activation thresholds (8). However, our measurements of
NAD(P)H changes as a function of glucose concentration indi-
cated a sigmoidal dose response with an inflection point at 8
mM glucose (Fig. 4). This coincides with the \( K_m \) of glucokinase,
the β cell glucose sensor (3, 21), and contradicts a model where
the sigmoidal insulin secretory response from the whole islet is
generated by a stepwise recruitment of individual β cells with
variable activation thresholds (8). None of the β cells examined
here exhibited different inflection points, as would be predicted
by the stepwise recruitment model. We conclude that a step-
wise recruitment of β cells does not describe insulin secretion
from whole islets. However, in the absence of direct insulin
secretion measurements from individual cells within the islet,
models of functional heterogeneity cannot be ruled out. The
results may also be inconsistent with previous descriptions of
pronounced β cell glucokinase heterogeneity as detected by
immunohistochemical methods (9). Further studies of the dif-
fferences between intact islet and isolated β cells using TPEM
should lead to a resolution of these controversies and a greater
understanding of intraislet metabolic response dynamics.

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