Vol. 114, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 29, 1983 Pages 835-842

AUTOFLUORESCENCE-ACTIVATED CELL SORTING OF PANCREATIC ISLET CELLS: PURIFICATION OF INSULIN-CONTAINING B-CELLS ACCORDING TO GLUCOSE-INDUCED CHANGES IN CELLULAR REDOX STATE

M. VAN DE WINKEL AND D. PIPELEERS

Dept. of Metabolism and Endocrinology,
Vrije Universiteit Brussel
Laarbeeklaan 103, B-1090 Brussels,
BELGIUM

Received June 9, 1983

Autofluorescence-activated cell sorting can be employed for the subfractionation of insulin-containing islet B-cells according to their responsiveness to their physiologic stimulus, glucose. The method utilizes a flow cytometric detection of the rapid variations in endogenous NAD(P)H - and FAD - fluorescence after exposure to 20 mM glucose. Under these conditions, a two-fold increase in NAD(P)H and a 40% decrease in FAD was observed in more than 75% of B-cells isolated from fed normal rats.

The technique makes it possible to study the metabolic behaviour of the B-cell population in (physio)pathological conditions of impaired glucose-induced insulin release; the availability of functionally homogenous B-cell preparations facilitates studies on stimulus-secretion coupling.

In view of the universal role of the cellular metabolic redox state in cell regulation, it is suggested that similar techniques can be developed for the metabolic analysis of other cell types and for their purification according to their responsiveness to specific stimuli.

A wide variety of techniques has been developed for the separation of cells according to differences in size, density or charge. While relying on relatively stable cell properties, these procedures often remained unsuccesfull in yielding highly pure preparations, mainly as a result of overlap of the respective cell distributions for the choosen parameter.

The recognition of cell-type specific markers has led to a new class of purification techniques with much higher discriminative power. Among these techniques, fluorescence-activated cell sorting (1) rapidly became a method of choice, as it allows the identification, quantification and purification of cells with

0006-291X/83 $1.50

fluorescently labelled surface antigens, and provides, in addition, the possibility to monitor these processes on visual display. Cell sorting has been mainly applicated in the field of immunology, where the availability of a whole series of surface antibodies made it possible to investigate various lymphocyte subpopulations in health and disease. So far, the technique has been less successful in identifying and isolating other cell types according to their functional characteristics, mainly as a result of the more limited knowledge in specific and physiologically relevant antigens.

In the present study, we demonstrate that fluorescence-activated cell sorting can be employed for analysing and purifying insulin-containing islet B-cells according to their responsiveness to their physiologic stimulus, namely glucose. The method is based upon cell-specific variations in redox state and the subsequent changes in the fluorescence of the endogenous dinucleotides FAD and NAD(P)H.

MATERIALS AND METHODS

Preparation of pancreatic islet cells.

Pancreatic islets were obtained from adult Sprague-Dawley rats using the collagenase digestion method of Lacy and Kostianovsky (2). Isolated islets were dissociated in calcium-free medium containing trypsin and desoxyribonuclease (3). The islet cell suspension was cleared from debris and dead cells via centrifugation through a Percoll layer of density 1.045 (3). After preincubating the cells for 20 min in CMRL-1066 (Gibco, Scotland), they were centrifuged at 800 g for 5 min and resuspended in Earle-Hepes. This medium is used throughout and is composed of Earle's salts (4) and supplemented with 10 mM Hepes, 0.5% (w/v) bovine serum albumin (Fraction V, Sigma, St. Louis, MO) and 50 mg% glucose.

Flow cytometry

The cells were analysed and separated in a fluorescence-activated cell sorter (FACS IV, Becton Dickinson, Sunnyvale, Ca) equipped with two argon lasers (Argon 164-06 and UV-argon 171-17, Spectra-Physics, Mountain View, Ca).

As 30 percent of the dissociated islet cells occured as aggregates of 2 to 20 cells, clogging of the circuit had to be prevented by inserting a 31 µm nylon filter at the beginning of the sample feed line. Reaggregation of the 70% single cells was minimized by keeping cell concentrations under 5.10^5 cells per ml and by gently stirring the sample solution. A 70 µm nozzle gave optimal results

in analysis and sorting. Cellular FAD and NAD(P)H fluorescence intensity was measured at 37°C, after 10 min incubation in the presence of 2.8 or 20 mM glucose.

Cellular FAD was excited at 488 nm by a 200 mW laserbeam (Argon 164-06) (5) and the emitted fluorescence was selected between 510 and 550 nm by using a suitable band pass interference filter (Schott, Mainz, Fed. Rep. Germany). Cellular NAD(P)H was excited by 250 mW UV-light at 363.8 nm (single line) or 500 mW UV-light in the multi line mode (UV-argon 171-17). Laser blue stray light was rejected by an excitation filter, selecting light between 275 and 375 nm (Coloured glass filter, UG 11, Schott). Emission was measured between 400 and 470 nm, as selected by the corresponding band pass interference filter (Schott).

Scatter was measured as near forward scattered light intensity at 488 nm; in order to eliminate other scattered light, in particular UV-light, a narrow band interference filter with transmission at 488 nm was placed in front of the photodetector (488 laser line filter, MA 3-0.5, Schott).

RESULTS

1. FAD/scatter analysis of islet cells.

When dissociated islet cells were analysed in an Earle-Hepes buffer at 37°C and 50 mg% glucose, two distinct populations were easily discerned in FAD/scatter dot plots (Fig. 1); comparable results have been previously obtained in Krebs-Hepes buffer at room temperature (5); population A was more than 95% pure in islet non-B-cells and population B more than 95% pure in islet B-cells.

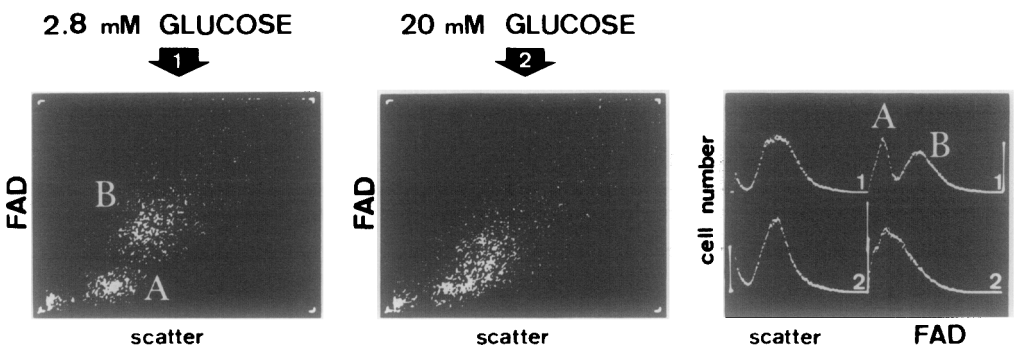

Figure 1 : FACS-analysis of unpurified islet cells examined for their FAD fluorescence and scattering intensity. Dot plots and histograms were obtained after 10 min incubation in 2.8 mM glucose (1) or in 20 mM glucose (2). At 2.8 mM glucose (1), single B-cells (B) are clearly distinguished from single islet non-B-cells (A) (2); this was not the case at 20 mM glucose, which markedly decreased FAD fluorescence of B-cells(2).

After 10 min incubation with 20 mM glucose, the two populations were less well defined, as glucose decreased the FAD-fluorescence intensity of B-cells but not of the other islet cells (Fig. 1). In view of these results, further metabolic studies were exclusively conducted on B-cells purified by FAD/scatter based sorting at 50 mg% glucose.

2. Effect of 20 mM glucose upon FAD- and NAD(P)H-autofluorescence of single B-cells.

Single purified B-cells were incubated for 10 min at 37°C in the presence of 2.8 mM or 20 mM glucose, before they were analysed in FAD/scatter or in NAD(P)H/scatter.

Raising the glucose concentration to 20 mM reduced the FAD-related endogenous fluorescence by 40 percent, while increasing the cellular NAD(P)H-fluorescence at least 100 percent (Table 1, Figure 2).

3. Sorting of single, glucose-responsive B-cells.

The observation that 20 mM glucose markedly and significantly altered both FAD- and NAD(P)H-fluorescence of single B-cells created the possibility to sort glucose-responsive B-cells by dual laser excitation with simultaneous measurement of their FAD and NAD(P)H-autofluorescence.

TABLE 1 : Effect of glucose upon the relative FAD- and NAD(P)H-fluorescence intensities of pancreatic B-cells.

	FAD-fluorescence	NAD(P)H-fluorescence
2.8 mM glucose	100 (21.7)	100 (27.8)
20 mM glucose	58 (34.6)	225 (17.3)
20 mM fructose	96 (22.0)	109 (22.3)

Mean fluorescence intensities expressed as a percent of the intensities in "basal" glucose conditions (2.8 mM glucose). Coefficients of variation are indicated between parenthesis.

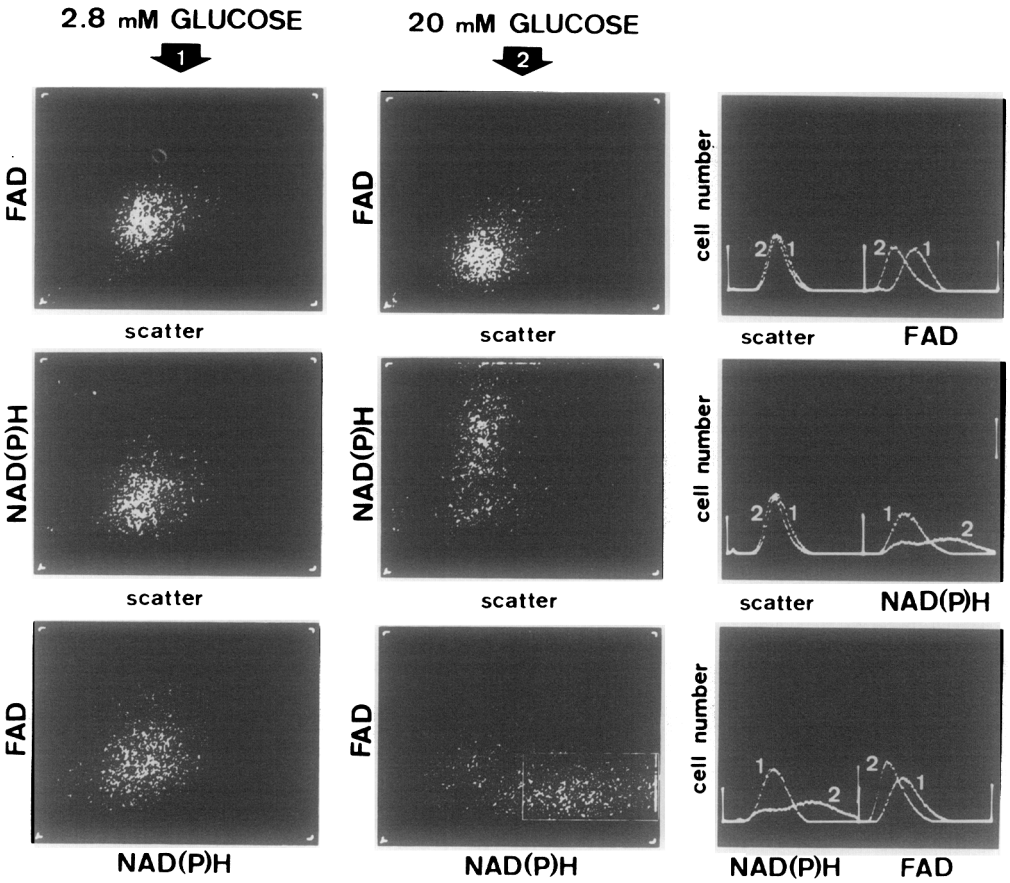

Figure 2 : FACS-analysis of single purified B-cells, incubated in 2.8 mM (1) or 20 mM (2) glucose. The cells are examined in FAD/scatter (upper series of three illustrations), in NAD(P)H/scatter (middle series) and FAD/NAD(P)H (lower series). Raising the glucose concentration to 20 mM was followed by a reduction in cellular FAD-fluorescence (upper series) and an increase in cellular NAD(P)H-fluorescence (middle series). The lower series illustrates this glucose effect in single B-cells, examined simultaneously for their FAD- and NAD(P)H fluorescence intensities; a distinct population of glucose-responsive cells can be distinguished on the basis of a decreased FAD and increased NAD(P)H fluorescence intensity (square); unresponsive cells are recovered in "basal" position, as defined by analysis in 2.8 mM glucose.

Control cells were incubated at 2.8 mM glucose and 37°C and analysed to locate single B-cells in "basal" redox state; this location served as a reference in the analysis of B-cells, that were incubated for 10 minutes at 20 mM glucose and 37°C (Fig. 2).

More than 75 percent of glucose-exposed B-cells were recovered at lower FAD- and higher NAD(P)H-fluorescence intensities, and

constituted a clearly distinguishable population, which could be easily separated from the cells, that had remained in the initial "basal" state (Fig. 2).

No significant variations in FAD- and NAD(P)H-fluorescence were measured after addition of 20 mM fructose - which is not metabolized by the pancreatic B-cell.

DISCUSSION

We have previously shown that single insulin-containing B-cells can be purified from other pancreatic islet cells by fluorescence-activated cell sorting (5). The separation is based upon differences in FAD-fluorescence and light-scattering properties. Although the sorted B-cells were more than 95% pure, they might still represent a functionally non-homogenous population, either as a characteristic of a physiological or pathophysiological condition, or as a result of the preparation procedure. It might therefore be useful - both for analytical purposes as for methodological reasons - to further purify the pancreatic B-cells according to their functional characteristics.

The insulin-containing B-cells play a central role in glucose-homeostasis, as they provide a tight hormonal regulation of plasma glucose levels. Their rapid and appropriate release of insulin requires a sensitive glucose recognition unit, capable of eliciting a dose-dependent hormonal discharge. Evidence has accumulated that glucose-stimulated insulin release is mediated via the generation of reducing equivalents, leading to an increased NAD(P)H to NAD(P) ratio and a decreased FAD to $FADH_2$ ratio (6); these alterations in B-cell redox occur within 3 minutes after the rise in glucose and persist during the time of exposure (6,7). The simultaneous measurement of the cellular NAD(P)H- and FAD-levels thus represents a valid parameter for the glucose sensitivity of pancreatic B-cells, and can consequently be

used for the isolation of B-cells according to their metabolic response to their main physiologic regulator, glucose.

More than 75% of the single B-cells that were isolated from fed normal rats, responded to 20 mM glucose by a marked increase in their NAD(P)H and a less pronounced decrease in their FAD-levels; the mean amplitude of these responses was certainly not lower than that measured in intact islets (6,7). The smaller fraction of B-cells, which maintained an unaltered redox state after addition of glucose may correspond to metabolically inactive or glucose-unresponsive B-cells.

The present technique appears useful in studying the B-cell population from conditions with impaired glucose-induced insulin release, such as fasting or certain forms of diabetes mellitus. It also provides functionally homogenous B-cell preparations which are a requisite for the analysis of the main regulatory mechanism in glucose homeostasis, namely glucose-induced insulin release.

Although no other cell systems have been tested, it seems attractive to develop similar procedures for the isolation of other cell types according to their biological activity. Earlier studies have indicated the possible usefulness of NAD(P)H and FAD in the characterization of cells under basal conditions (5,8); variations in cytoplasmic redox state have also been shown to regulate a variety of cell functions in different cell types. Physiologic conditions, known to exert a cell-specific effect via an alteration in cellular redox, might thus expand the applications of fluorescence-activated cell sorting to the biochemical characterization and subfractionation of various cell types.

ACKNOWLEDGEMENTS

We thank S. Duys for her careful technical assistance and A. Kiggen for her continuous secretarial help. This work was supported by grants from the Belgian Fonds Geneeskundig Wetenschap-

pelijk Onderzoek (grant 3.0021.80) and from the Belgian Ministerie van Wetenschapsbeleid (80-85/9).

REFERENCES

1. Herzenberg, L.A., Sweet, R.G., and Herzenberg, L.A. (1976) Scient. Am. 234, 108-117.
2. Lacy, P.E. and Kostianovsky, M. (1967) Diabetes 16, 35-39.
3. Pipeleers, D.G. and Pipeleers-Marichal M.A. (1981) Diabetologia 20, 654-663.
4. Earle, W.R. et al. (1943) J. Natl. Cancer Inst. 4, 165.
5. Van De Winkel, M., Maes, E., and Pipeleers, D. (1982) Bioch. Biophys. Res. Comm. 107, 525-532.
6. Malaisse, W.J., Hutton, J.C., Kawazu, S., Herchuelz, A., Valverde, I., Sener, A. (1979) Diabetologia 16, 331-341.
7. Panten, U., Ishida, H., Schauder, P., Frerichs, H., Hasselblatt, A. (1977) Anal. Bioch. 82, 317-326.
8. Thorell, B. (1980) Blood cells 6, 745-751.