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# Ultrastructure and membrane permeability of cultured pancreatic β-cells exposed to alloxan or 6-hydroxydopamine

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**Summary.** Stereological techniques on electron microscopy micrographs were used to evaluate the morphological changes of cultured islet β cells that had been exposed to alloxan or 6-hydroxydopamine.

Trypan Blue exclusion by cells cultured for 3 days indicated that the cells were 100% viable. Electron microscopy revealed that nearly all of the surviving cultured cells were β cells.

Exposure to 5 mmol/l alloxan or 1–5 mmol/l 6-hydroxydopamine for 10 or 30 min caused a general swelling of the cultured cells with a concomitant swelling of mitochondria and nuclei. The size of the secretory granules was not affected by the drugs. Only 3–10% of the cells excluded Trypan Blue after exposure to 5 mmol/l alloxan or 6-hydroxydopamine.

The data conform with the hypothesis that a primary action of alloxan and 6-hydroxydopamine is at the plasma membrane level of β cells.

**Key words:** Alloxan – Culture – Electron microscopy – 6-Hydroxydopamine – Pancreatic β-cells – Stereology

Alloxan has since long been used to induce diabetes in experimental animals (Rerup 1970). Plausible mechanisms of action were recently reviewed (Cooperstein and Watkins 1981). One line of research suggests that alloxan acts by generating free oxy-radicals which are toxic to the pancreatic islet β-cell (Heikkila et al. 1976; Grankvist et al. 1979a, 1981 and 1982; Fischer and Hamburger 1980a and b). The initial attack of these radicals could well

*Abbreviations and definitions.* $A_{cell}$ Cell profile area (μm²), surface area of one cell section surface; $V_n$ Nuclear volume density (%), number of points over the nucleus divided by the number of points over the total cell area × 100; $V_m$ Mitochondrial volume density (%), number of points over mitochondria divided by hits over the cytoplasm (points over the cell minus points over the nucleus) × 100; $V_g$ Granular volume density (%), number of points over granules divided by hits over the cytoplasm × 100

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be in the plasma membrane (Grankvist et al. 1979a). 6-Hydroxydopamine, another generator of free radicals (Heikkila and Cohen 1973) appears to affect isolated islets in a similar way (Grankvist et al. 1984). Others feel that alloxan primarily inhibits mitochondrial metabolism (Boquist 1977; Borg 1981). On the basis of electron microscopy, it has been proposed that the first morphological changes after alloxan in vivo occur in the plasma membrane, or the secretory granules, or the mitochondria (Williamsson and Lacy 1959; Wellman et al. 1967; Boquist 1977; Boquist and Lorentzon 1979; Falkmer and Olsson 1962; Orci et al. 1976).

The Trypan Blue dye exclusion test has been validated as an indicator of plasma membrane leakage and viability in alloxan-treated $\beta$ cells (Grankvist et al. 1979b). By a combination of electron microscopy and the Trypan Blue exclusion test, we examined the morphological changes and plasma membrane leakage of isolated cultured $\beta$-cells exposed to alloxan or 6-hydroxydopamine. By this approach of controlled alloxan treatment, an attempt was made to shed some light on the controversial problem of which subcellular structure is the primary target of alloxan.

## Materials and methods

*Animals and isolation of pancreatic islets.* Adult mice homozygous for the gene *ob* (Hellman 1965) were starved overnight. Islets were isolated by collagenase treatment of the pancreas and islet cells prepared essentially as described by Lernmark (1974). In brief, about 100 collagenase-isolated islets were pooled in 200 µl of $Ca^{2+}$-free Parker 199 medium which had been supplemented with 1 mM EGTA, 3 mM D-glucose, 5 µg/ml DNAase, 10 mg/ml bovine serum albumin, and 20 mM HEPES (pH 7.4). The cells were shaken for 10 s in polypropylene micro test tubes to obtain a cell suspension, and the cells were freed from cellular debris by centrifugation through dense albumin (40 mg/ml). The cells were resuspended in culture medium (see below) and 500 µl aliquots of this suspension were added to culture dishes.

*Culture of isolated islet cells.* Culture of isolated islet cells was performed in sterile plastic Petriperm® dishes with a diameter of 5 cm. Each dish contained 5.0 ml Parker 199 culture medium supplemented with 1 mM D-glucose, 10 mg/ml bovine serum albumin, 90 IE/ml benzylpenicillin and 55 µg/ml garamycin. The dishes were kept at 37° C for 3 days in a humidified athmosphere consisting of 5% $CO_2$ in air.

*Exposure to cytotoxic agents, and staining with trypan blue.* 555 µl of concentrated alloxan or 6-hydroxydopamine in acidified (HCl, pH 2.5) culture medium was added to the culture dishes to yield a final concentration of the drugs of 1–5 mM. After 0.5–30 min, glutaraldehyde dissolved in Krebs-Ringer-HEPES-buffered medium (pH 7.4) was added to a final concentration of 2.5%. To half of the dishes, whether untreated or treated with alloxan or 6-hydroxydopamine, 2% Trypan Blue had been added approximately 2 min before the glutaraldehyde. 50 µl of the dyed and fixed cell suspension was placed under a microscope and the frequency of cells with Trypan Blue-stained cells estimated by counting about 100 randomly selected cells.

*Electron microscopy and stereological analysis.* The glutaraldehyde-fixed cells were postfixed in 1% $OsO_4$, dehydrated in graded ethanol solutions and embedded in Epon® 812 plastic. 70 nm sections were cut on an LKB Ultratome, collected on naked copper grids, post-stained with uranyl acetate and lead citrate, and examined in a Philips EM 300 or a JEOL JEM-100 CX electron microscope.

For stereological measurements, $\beta$-cells with displayed nuclei were chosen at random and

micrographs taken. From each test group, 31 to 56 electron micrographs were printed at a final magnification of 10,000×. The magnification was calibrated against a reference grid. Random sampling of the micrographs was used as the β-cells were not oriented to the plane of section (Weibel 1979). A multipurpose grid with a line length of 10 mm was used when making point-counting measurements. Measurements were made by the same person without prior knowledge of the specific treatment of each cells. The method error of the point-counting procedure was estimated from repeated analysis of 21 cells and found to represent ± 1.8% of the mean cell profile area (Eränkö 1955).

The distribution of individual hits over the β-cells were analyzed by a sign test (Geigy Scientific Tabels 1982). None of the distributions in the groups deviated significantly from symmetry. Student's *t*-test for non-paired observations was used to evaluate treatment effects.

*Chemicals.* Collagenase and 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) were from Boehringer Mannheim GmbH, Mannheim, West Germany. Bovine serum albumin was from Miles Laboratories Inc., Kankakee, IL, USA. 6-Hydroxydopamine was from Sigma Chemical Co., St. Louis, MO, USA. Alloxan monohydrate was from United States Biochemical Corp., Cleveland, OH, USA. Trypan Blue was from BDH Chemicals, Poole, Dorset, U.K. Tissue Culture Medium 199 was from SBL, Stockholm, Sweden. Benzyl penicillin was from

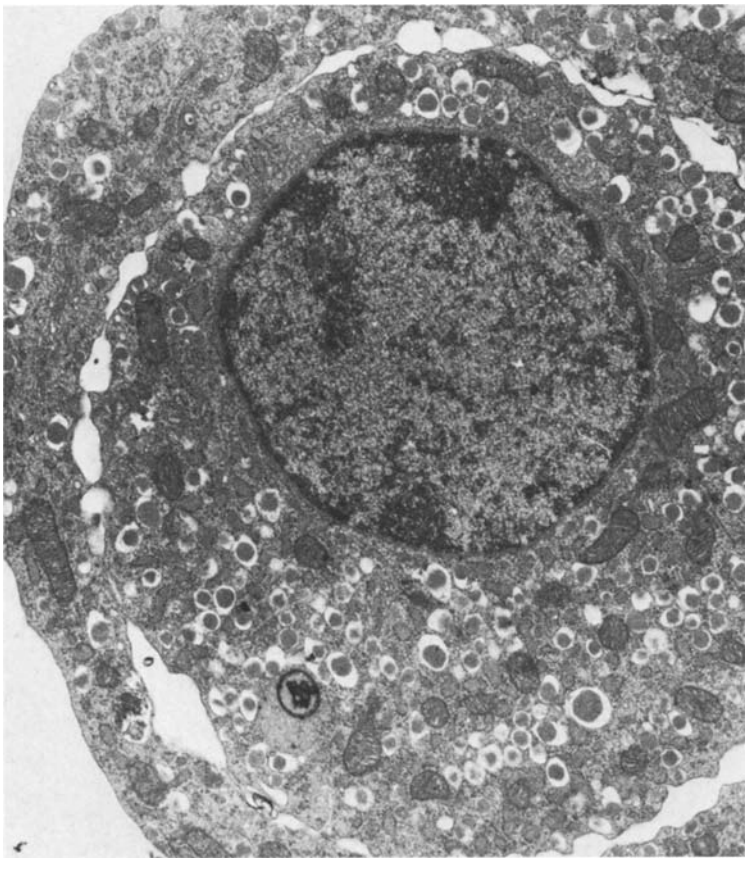

**Fig. 1.** β-Cells cultured for three days, showing well preserved morphology. A β-cell with visible nucleus is seen to be surrounded by extensions of another. (×9,760)

**Table 1.** Frequency (%) of β-cells excluding Trypan Blue after having or not having been exposed to 5 mM alloxan or 6-hydroxydopamine for 10 or 30 min. $n=4$ for each group tested.

| | |
|---|---|
| Controls | 100±0 |
| 5 mM alloxan for 10 min | 8±3 |
| 5 mM alloxan for 30 min | 3±1 |
| 5 mM 6-hydroxydopamine for 10 min | 10±4 |
| 5 mM 6-hydroxydopamine for 30 min | 8±3 |

Kabi AB, Stockholm, Sweden. Garamycin was from Scheringer Co., Kemilwork, NJ, USA. Epon 812® was from Electron Microscope Sciences, Fort Washington, PA, USA.

Commercially available reagents of analytical grade and twice distilled water were used throughout.

## Results

### *General morphology of cultured cells*

Isolated islet cells that had been cultured for 3 days showed well preserved ultrastructure (Fig. 1). β-cells were easily identified by their typical secretory granules. More than 99% of the cultured cells had morphological characteristics of β cells; only 6 out of 750 studied cells showed morphological characteristics of other islet cell types. Virtually no fibroblasts were seen.

### *Trypan Blue uptake of cells exposed to alloxan or 6-hydroxydopamine*

Cells which had only been treated with acidified medium, i.e. the vehicle of alloxan or 6-hydroxydopamine, did not accumulate Trypan Blue. Cells that had been incubated with 5 mM alloxan or 5 mM 6-hydroxydopamine for 10 or 30 min, readily accumulated the dye (Table 1).

### *Electron microscopy of cultured β cells exposed to alloxan*

After 10 min of incubation with 1 mM alloxan no significant change in cell profile area ($A_{cell}$), nuclear volume density ($V_n$), mitochondrial volume density ($V_m$), or granular volume density ($V_g$) was seen (Table 2). After 10 min incubation in 5 mM alloxan, $A_{cell}$ significantly increased while $V_g$ significantly decreased as compared to control cells. Incubation in 5 mM alloxan for 30 min also caused an increase in the cell profile area ($A_{cell}$), although the effect was less pronounced than after 10 min. $V_g$ remained low after 30 min with values comparable to those after 10 min of incubation (Table 2). Neither the nuclear volume density nor the mitochondrial volume density changed significantly in response to alloxan.

**Table 2.** Effects of alloxan on $\beta$-cell ultrastructure. Group I: control; group II: 1 mM alloxan, 10 min incubation; group III: 5 mM alloxan, 10 min incubation; and group IV: 5 mM alloxan, 30 min incubation. The values represent mean values ± SEM for 32–56 cells. [a] $P<0.002$, [b] $P<0.02$, [c] $P<0.003$, and [d] $P<0.006$ for difference from group I

| Parameter | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|
| $A_{cell}$ | 59.8 ± 2.3 | 65.9 ± 3.6 | 75.8 ± 5.2[a] | 68.9 ± 3.0[b] |
| $V_n$ | 26.2 ± 1.1 | 27.2 ± 1.6 | 26.8 ± 1.5 | 26.6 ± 1.8 |
| $V_m$ | 13.0 ± 0.7 | 12.9 ± 1.0 | 11.0 ± 0.8 | 11.7 ± 0.8 |
| $V_g$ | 23.3 ± 1.3 | 20.6 ± 1.5 | 17.2 ± 1.3[c] | 18.2 ± 1.1[d] |

**Table 3.** Effects of 6-hydroxydopamine on $\beta$-cell ultrastructure. Group I: control; group II: 1 mM 6-hydroxydopamine, 10 min incubation; group III: 5 mM 6-hydroxydopamine, 10 min incubation; and group IV: 5 mM 6-hydroxydopamine, 30 min incubation. The values represent mean values ± SEM for 31–56 cells. [a] $P<0.02$, [b] $P<0.05$, [c] $P<0.002$, and [d] $P<0.01$ for difference from group I

| Parameter | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|
| $A_{cell}$ | 59.8 ± 2.3 | 71.0 ± 4.6[a] | 67.8 ± 3.6[b] | 73.2 ± 3.9[c] |
| $V_n$ | 26.2 ± 1.1 | 25.2 ± 1.6 | 29.7 ± 2.1 | 26.1 ± 1.6 |
| $V_m$ | 13.0 ± 0.7 | 12.4 ± 1.0 | 11.3 ± 1.0 | 12.4 ± 0.8 |
| $V_g$ | 23.3 ± 1.3 | 20.1 ± 1.3 | 17.8 ± 1.8[d] | 20.3 ± 1.3 |

### *Electron microscopy of cultured cells exposed to 6-hydroxydopamine*

The effects of 6-hydroxydopamine on $\beta$-cell structure is summarized in Table 3. $A_{cell}$ increased in incubations for 10 min with 1 or 5 mM, or 30 min with 5 mM 6-hydroxydopamine. A significant decrease in $V_g$ was seen in cells incubated for 10 min with 5 mM 6-hydroxydopamine.

## Discussion

After three days of culture the dispersed islet cells showed an excellent ultrastructural morphology, and were 100% viable as judged by the Trypan Blue dye exclusion test. Freshly isolated islet cell suspensions are contaminated by a significant proportion of nonviable cells (Lernmark 1974; Grankvist et al. 1979b). Clearly the culture procedure was advantageous in increasing the percentage viable cells in the preparation. Damaged cells probably lysed and desintegrated into the culture medium. The surviving cells were almost exclusively $\beta$-cells as judged from their electron-microscopical characteristics. Although whole islets from *ob/ob*-mice contain more than 90% $\beta$-cells there is a significant proportion of A and D cells (Hellman 1965). It therefore seems as if the culture procedure also increased the relative amount of $\beta$-cells in the cell preparation. Our culture of dispersed $\beta$-cells appeared to result in better preserved ultrastructure than the culture of

whole isolated islets from NMRI mice, in which a marked swelling of organelles has been reported (Borg et al. 1975).

Several papers have been written on morphological changes of β-cells after alloxan exposure. Most authors report the first morphological changes to occur in the plasma membrane, mitochondria or secretory granules (Cooperstein and Watkins 1981). By the technique of freeze-fracture of islets treated in vitro with alloxan Orci et al. (1976) found a decrease in the number of intramembranous particles of islet cell plasma membranes. Nakamura (1978), too, found the first morphological changes of β-cells in alloxan-treated rats in paramembranous cytoplasmic areas. However, Boquist and Lorentzon (1979) and Lorentzon and Boquist (1979), presented morphological data to suggest that the mitochondria are the primary site of alloxan action.

Some biochemical studies point to the plasma membrane as a first site of alloxan action. Watkins et al. (1970) showed that alloxan markedly increased the penetration of D-(1-$^{14}$C)mannitol and $^{14}$C-insulin into toadfish islet slices, signifying penetration of these extracellular markers through damaged plasma membranes. According to Tomita and Watanabe (1976) alloxan increased the permeation of horseradish peroxidase into rat islet cells. We have previously reported that alloxan rapidly induces permeation of Trypan Blue into islet cells and inhibits their capacity to accumulate $^{86}Rb^+$ (Grankvist et al. 1977; 1979a; Idahl et al. 1977) and that extracellularly located scavengers of free radicals can protect islet cells against the toxic actions of alloxan in vitro and in vivo (Grankvist et al. 1979a, 1981). Alloxan also inhibits mitochondrial enzymes (Borg et al. 1979), but it seems unclear whether these effects are subsequent or secondary to effects occurring in the plasma membrane.

The present study shows that cultured β-cells undergo a rapid swelling together with a concomitant swelling of mitochondria and nuclei on exposure to alloxan. The secretory granules seem, however, not to be initially affected. That the insulin secretory granules are strikingly resistant to cytolytic influences has previously been observed in islets damaged by complement (Idahl et al. 1980). The general swelling of the β-cell after alloxan exposure does not seem to have been noticed in other studies where a marked mitochondrial swelling after alloxan was interpreted as signifying a primary mitochondrial action of the drug (Boquist and Lorentzon 1979; Lorentzon and Boquist 1979). The present data are compatible with the view that the plasma membrane is rapidly damaged by alloxan. They also indicate that the mere swelling of mitochondria cannot be taken as evidence for an initial effect of alloxan on this organelle.

Like alloxan, 6-hydroxydopamine generates free radicals and destroys isolated nerve terminals (Heikkila and Cohen 1971; Kirpekar et al. 1983). 6-Hydroxydopamine also inhibits $^{86}Rb^+$ accumulation in isolated mouse islets (Grankvist et al. 1984). The present study shows that 6-hydroxydopamine induces alloxan-like morphological changes and Trypan Blue uptake of cultured β-cells.

Our results on isolated cultured β-cells increase the evidence for a prima-

ry action of alloxan at the plasma membrane level. Thus they emphasize the importance of taking effects on the plasma membrane into account when the actions of alloxan or 6-hydroxydopamine on intracellular organelles are evaluated.

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