VOLUME OF ISLET B CELL MITOCHONDRIA AND PHOSPHATE TRANSPORT IN LIVER MITOCHONDRIA OF MICE AFTER TREATMENT WITH ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE OR 3-ISOBUTYL-1-METHYLXANTHINE

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ABSTRACT

Stereologic technique was used for study of the B cell mitochondria of isolated mouse islets incubated in the presence of 3 mM glucose, 3 mM glucose and 1 mM 3-isobutyl-1-methylxanthine (IBMX), or 1 mM IBMX, and swelling technique was applied for investigation of inorganic phosphate (Pi) transport in isolated mouse liver mitochondria exposed to 1 and 10 mM IBMX and 0.01, 0.1 and 1 mM adenosine 3':5'-cyclic monophosphate (cAMP). No significant stereological difference was observed between the groups in volume or surface density, or in mean volume or mean surface area, whereas the numerical density was higher in the glucose group than in the glucose plus IBMX and IBMX groups. Comparison between the *in vitro* data of the present work and the *in vivo* data of a preceding work (15) disclosed higher values *in vitro* than *in vivo*, but a similar surface-to-volume ratio, suggesting absence of non-specific swelling *in vitro*. No effect of exposure to IBMX or cAMP could be disclosed on the Pi-induced swelling of isolated liver mitochondria. With the techniques and intervals used no evidence could be obtained for an effect of cAMP on mitochondrial volume and Pi transport in mouse B cells and liver.

KEY WORDS mitochondria / B cells / liver / phosphate transport / stereology / adenosine 3':5'-cyclic monophosphate

In preceding studies attention has been paid to compounds and factors which protect against the diabetogenic effect of alloxan (4, 5); the most well-known of these is glucose. Since we believe that the primary action of alloxan is inhibition of a sulfhydryl dependent mitochondrial carrier for inorganic phosphate (Pi) causing structural and functional changes both in the mitochondria and the whole B cells (5, 6), it is of interest that alterations in mitochondrial volume have been observed in mouse islet B cells subjected to

different concentrations of glucose *in vitro* (3, 9).

Glucose is the main stimulator of B cell function. Another factor of importance in B cell function is adenosine 3':5'-cyclic monophosphate (cAMP) which might affect mitochondrial Pi transport (1), and which has been reported to afford partial protection against the inhibitory effect of alloxan on glucose-induced insulin secretion (16), and to inhibit adenyl cyclase *in vitro* (11). However, Lacy *et al.* (14) found no evidence for a role of cAMP in the inhibition of insulin release caused by alloxan in isolated rat islets.

Against this background the present study was undertaken with the aim to see whether cAMP

and/or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) might affect the volume of mouse islet B cell mitochondria and/or the Pi transport of mouse liver mitochondria, studied by stereologic technique and swelling technique, respectively.

MATERIALS AND METHODS

Animals

The animals used were 3 to 6 months old non-diabetic C57BL-KsJ-+/+ mice from a local stock kept under standard laboratory conditions on a standard ration and at a constant temperature of 22°C. The animals were starved for 20 hr before experimentation.

Stereologic Study

The pancreatic islets for the stereological determinations were isolated with collagenase and treated as previously described (7). Incubation of isolated islets was carried out for 1 hr in the presence of 3 mM glucose alone (glucose group), 3 mM glucose and 1 mM IBMX (glucose+IBMX group), or 1 mM IBMX alone (IBMX group). Each group consisted of islets obtained from 4 to 5 mice.

After incubation the islets were fixed in 2.5% glutaraldehyde in 0.34 M Veronal acetate buffer, pH 7.4, followed by postfixation in 1% osmium tetroxide. Embedding was carried out in Epon 812 and the thin sections were stained with uranyl acetate and lead citrate.

The thin sections were viewed in a Siemens Elmiskop 1A, and electron micrographs from 2 islets from each animal were taken at a primary magnification of 12,530. A grating replica of 2,160 lines per mm was used for calibration purposes. A specified corner of the copper grid was used as a reference system in order to ensure random sampling on the microscopic field (18). The electron micrographs were enlarged $\times 2.7$ and printed at a final magnification of 33,830. The total number of micrographs taken was 52, 49 and 43 for the glucose, glucose+IBMX and IBMX groups, respectively.

Standard stereological methods were applied for estimation of the mitochondrial volume, surface and numerical densities, the mean mitochondrial volume, and the mean mitochondrial surface area (13, 17, 18, 19). A multipurpose grid containing 50 lines of constant lengths was used. Volume estimates were made by point counting. The volumetric density of mitochondria (V_{mit}) was estimated by placing the

grid with $P_T=100$ test points on a micrograph and determining the fraction ($P_{Pi}=P_i/P_T$) of these points enclosed within the profiles of mitochondria: $V_{mit}=P_{Pi}$. The envelope surface density (S_{mit}) was determined by counting the intersection points I_i of the surface contour of profiles with test lines of known length L_T : $S_{mit}=2I_i/L_T$.

The numerical density of mitochondria (N_{mit}) was estimated by counting the number of mitochondrial profiles in a test area of known size using the formula:

$$N_{mit} = \frac{1}{\beta} \times \frac{N_{Ai}^{3/2}}{V_{mit}^{1/2}} \times K$$

N_{Ai} is the number of profiles per unit test area. The shape coefficient β , which is dependent on the axial ratio of the mitochondria, was estimated to 1.8 for all three groups and the size distribution coefficient K was arbitrarily set to 1.05 (19).

Student's *t*-test for non-paired observations was used for statistical treatment of data.

Stereological Abbreviations and Definitions

V_{mit}	cm^3/cm^3	volume density; volume of mitochondria per unit volume of tissue
S_{mit}	m^2/cm^3	envelope surface density; surface area of mitochondria per unit volume of tissue
N_{mit}	cm^{-3}	numerical density; number of mitochondria per unit volume of tissue
V_{mit}/N_{mit}	μ^3	mean mitochondrial volume; volume of one mitochondrion
S_{mit}/N_{mit}	μ^2	mean mitochondrial surface area; the surface area of one mitochondrion

Study of Mitochondrial Phosphate Transport

The livers were removed under ether anaesthesia and the mitochondria were isolated by homogenization and differential centrifugation in a medium containing 0.25 M sucrose, 5 mM Tris and 1 mM EDTA, pH 7.4. Centrifugation was carried out for 10 min at 1,000 g_{av} , and then for 10 min at 10,000 g_{av} , after which the pellet was resuspended and centrifugated for another 10 min at 10,000 g_{av} . All steps were carried out at 0 to 5°C. The protein concentration was measured spectrophotometrically and the concentration used in the experiments was 0.3

Table 1 *Stereological Parameters*

Parameter	Glucose (I)	Glucose + IBMX (II)	IBMX (III)	Statistical significance
V_{mit} (cm ³ /cm ³)	0.091 ± 0.007	0.080 ± 0.008	0.078 ± 0.009	I-II N.S. I-III N.S. II-III N.S.
S_{mit} (m ² /cm ³)	0.931 ± 0.071	0.776 ± 0.072	0.762 ± 0.077	I-II N.S. I-III N.S. II-III N.S.
N_{mit} (cm ⁻³)	534 × 10 ⁹ ± 12.6 × 10 ⁹	367 × 10 ⁹ ± 11.3 × 10 ⁹	405 × 10 ⁹ ± 11.3 × 10 ⁹	I-II <i>P</i> < 0.001 I-III <i>P</i> < 0.01 II-III N.S.
V_{mit}/N_{mit} (μ ³)	0.171 ± 0.012	0.219 ± 0.022	0.192 ± 0.021	I-II N.S. I-III N.S. II-III N.S.
S_{mit}/N_{mit} (μ ²)	1.743 ± 0.133	2.114 ± 0.196	1.881 ± 0.190	I-II N.S. I-III N.S. II-III N.S.

Stereological parameters for the B cell mitochondria of isolated islets incubated for 1 hr in the presence of 3 mM glucose, 3 mM glucose and 1 mM 3-isobutyl-1-methylxanthine (IBMX), or 1 mM IBMX. The figures represent mean values ± SEM. N.S. = no significance

Table 2 *Comparison of Stereological Parameters*

	<i>In vitro</i>	<i>In vivo</i>	Statistical significance
V_{mit} (cm ³ /cm ³)	0.091 ± 0.007	0.046 ± 0.003	<i>P</i> < 0.001
S_{mit} (m ² /cm ³)	0.931 ± 0.071	0.476 ± 0.035	<i>P</i> < 0.001
N_{mit} (cm ⁻³)	534 × 10 ⁹ ± 12.6 × 10 ⁹	356 × 10 ⁹ ± 6.1 × 10 ⁹	<i>P</i> < 0.001
V_{mit}/N_{mit} (μ ³)	0.171 ± 0.012	0.130 ± 0.009	<i>P</i> < 0.01
S_{mit}/N_{mit} (μ ²)	1.743 ± 0.133	1.341 ± 0.098	<i>P</i> < 0.05

Comparison of stereological parameters for the B cell mitochondria from mouse islets exposed for 1 hr to low (3 mM) glucose concentration *in vitro* (data from the present work) with those from islets of mice injected with saline (data from Lorentzon and Boquist, 1979). The figures represent mean values ± SEM.

mg/ml.

The effect of cAMP and IBMX on mitochondrial phosphate transport was studied with swelling technique at room temperature using a spectrophotometer at 520 nm and a basal medium containing 5 mM TES, 0.1 M ammonium-orthophosphate (Pi), and 1 μg rotenone, pH 7.4. cAMP was dissolved in isolation medium and IBMX was dissolved in dimethyl sulfoxide (DMSO). Mitochondrial preparations were preincubated for 2 min with 0.01, 0.1 and 1 mM cAMP, and with 1 and 10 mM IBMX. In addition, preincubation for 15 sec was performed of mitochondria and 1 mM cAMP. Control experiments were carried out with plain isolation medium and with plain DMSO. Control experiments were also performed with 1 mM

mersalyl which is a well-known inhibitor of Pi transport in liver mitochondria.

IBMX was from Aldrich-Europe, Janssen Pharmaceutical, Beerse, Belgium; cAMP, DMSO, mersalyl, TES and Tris were from Sigma Chemical Co., St. Louis, Mo., U.S.A.; rotenone and sucrose were from BDH Chemicals Ltd., Poole, England; and EDTA was from E. Merck, Darmstadt, West Germany. All other chemicals were of highest quality available commercially.

RESULTS

Stereology of B Cell Mitochondria

The numerical density (N_{mit}) was significantly higher in the glucose group than in the glucose

plus IBMX and IBMX groups, whereas the numerical density in the glucose plus IBMX group was not significantly different from that in the IBMX group (Table 1). No significant difference was observed between the groups of mitochondria in any of the other stereological parameters investigated. Thus, the volume density (V_{mit}), surface density (S_{mit}), mean volume (V_{mit}/N_{mit}) and mean surface area (S_{mit}/N_{mit}) were similar in the three groups of mitochondria.

Since the presently applied *in vitro* technique might affect the B cell mitochondria, a comparison was made between the stereological data for the glucose group of the present work with those for the saline (control) group of a preceding *in vivo* stereological study of B cell mitochondria (15). It should be noted that the glucose (3 mM) used in the present work represents a low glucose concentration which is generally considered not to cause functional stimulation of the B cells.

This comparison disclosed that the values for all investigated stereological parameters (V_{mit} , S_{mit} , N_{mit} , V_{mit}/N_{mit} and S_{mit}/N_{mit}) were significantly higher *in vitro* than *in vivo* (Table 2). The surface-to-volume ratio (S_{mit}/V_{mit}) on the other hand, was approximately similar *in vitro* and *in vivo*, and approximately similar ratios were also obtained for the three *in vitro* groups (Table 3).

Pi Transport in Isolated Liver Mitochondria

The swelling technique applied disclosed no effect of cAMP or IBMX on mitochondrial Pi transport. Thus, the swelling curves for 0.01 mM, 0.1 mM and 1 mM cAMP were not significantly different from that for Pi without cAMP (Fig. 1), and the curves for 1 mM and 10 mM IBMX were not significantly different from that for Pi without IBMX (Fig. 2). The swelling curve (not

Fig. 1 Effect of adenosine 3':5'-cyclic monophosphate (cAMP) and mersalyl on the swelling of isolated mouse liver mitochondria. Downward deflexion representing swelling. Isolated mitochondria suspended in 0.25 M sucrose, 5 mM Tris and 1 mM EDTA, pH 7.4, are incubated at room temperature for 2 min with 0.01 mM (a), 0.1 mM (b) and 1 mM (c) cAMP, with no added cAMP (d), and with mersalyl (m). Then about 50 μ l of the solutions are transferred to a cuvette and mitochondrial swelling is monitored spectrophotometrically at room temperature in 5 mM TES, 0.1 M Pi, and 1 μ g rotenone, pH 7.4. The protein concentration is 0.3 mg/ml.

presented in figure) for 1 mM cAMP after preincubation for 15 sec was similar to the other curves for cAMP. The control experiments with mersalyl showed a complete inhibition of Pi transport (Figs. 1 and 2). No effect on Pi transport was seen in control experiments with plain isolation medium and plain DMSO (not presented in figures).

Table 3 Surface-to-Volume Ratios

	<i>In vitro</i>			<i>In vivo</i>
	Glucose	Glucose + IBMX	IBMX	
S_{mit}/V_{mit} (m^2/cm^3)	10.2	9.7	9.8	10.4

Surface-to-volume ratios (S_{mit}/V_{mit}) for the B cell mitochondria of isolated islets incubated for 1 hr in the presence of 3 mM glucose, 3 mM glucose and 1 mM 3-isobutyl-1-methylxanthine (IBMX) or 1 mM IBMX. For comparison, *in vivo* data are given for the surface-to-volume ratio of the B cell mitochondria from islets of mice injected with saline (data from Lorentzon and Boquist, 1979).

DISCUSSION

In a preceding study 1 mM IBMX was found to increase the islet content of cAMP, and to potentiate the glucose-induced insulin secretion in C57BL-6J-db^{2J}/db^{2J} mice, but not in C57BL-KsJ-db/db mice (8). The stereological determinations in the present study did not disclose any differences between the groups of mitochondria, with the exception that the numerical density (N_{mit}) was significantly higher in the glucose group than in the glucose plus IBMX and IBMX groups. The increase in numerical

Fig. 2 Effect of 3-isobutyl-1-methylxanthine (IBMX) and mersalyl on the swelling of isolated mouse liver mitochondria. Details as in Fig. 1. Incubations were carried out with 1 mM (a) and 10 mM (b) IBMX, with no added IBMX (c), and with mersalyl (m).

density, in the mitochondria exposed to glucose alone, might seem surprising in the absence of increased parameters for mitochondrial volume and for surface area per unit volume of tissue. However, some of the B cell mitochondria may under the presently used experimental conditions be complex, curved and branching structures, like those in rat liver (10). This might explain an increase only in the parameter for the number of mitochondria per unit volume of tissue. It should thus be stressed that the average number of profiles per unit section area not only depends on the numerical density, but also on the shape and size of the mitochondria (17). The combined stereological data indicate that there are no quantitative structural alterations in the B cell mitochondria of mouse islets incubated for 1 hr in the presence of 1 or 10 mM IBMX.

The *in vitro* data for B cell mitochondria exposed to low glucose (3 mM) were for all stereological parameters investigated significantly higher than the *in vivo* data for B cell mitochondria of mice injected with saline (15). Since the B cells and their organelles are known to respond rapidly to different stimuli and different experimental conditions this is not an

unexpected finding. It emphasizes that interpretations based upon comparison between *in vitro* and *in vivo* data should be made with caution.

Different explanations may be given for the observed differences. Thus, apart from the self-evident fact that the experimental conditions are not identical *in vitro* and *in vivo*, e.g. the collagenase technique used for isolation of islets might affect the mitochondria.

One may wonder whether the increase in the values for the stereological parameters *in vitro* represents a non-specific swelling. The surface-to-volume ratios, however, suggest that this is not the case. In general, the surface-to-volume ratio is related to the geometric shape of a certain structure (18). Swelling of the mitochondria, resulting in a more spherical shape, leads to a decrease in the surface-to-volume ratio. Thus, this ratio for B cell mitochondria is decreased from 10.4 ten min after alloxan administration to 5.9 one hour following alloxan (15), when there is also qualitative ultrastructural evidence for swelling (2).

In the present *in vitro* study, no obvious difference in the surface-to-volume ratio was found between the three groups of mitochondria which all showed an approximately similar ratio as normal mitochondria do *in vivo* (15). Therefore, the observed increase in mitochondrial volume and surface densities and in individual mitochondrial parameters for B cell mitochondria exposed to low glucose concentration *in vitro*, compared with the parameters for B cell mitochondria from mice injected with saline, was most probably due to a hypertrophy of the mitochondria. Our observations are in agreement with those of Borg *et al.* (9) who obtained stereological data indicating a hypertrophy of B cell mitochondria cultured in low-glucose medium for 7 days. In contrast, our present stereological data are in general not in agreement with those of Dean (12) for islets incubated in the presence of 2.8 mM glucose. The mitochondrial values of that study were very close to those obtained by us *in vivo* (15) and by Borg *et al.* (9) during high-glucose conditions.

Our studies with swelling technique gave no evidence for an effect of either cAMP or IBMX on the Pi-induced swelling of isolated mouse liver mitochondria. The swelling curves obtained in the presence of cAMP and IBMX were similar to those found in the absence of these compounds, whereas inhibition of Pi transport was observed for mitochondria exposed to mersalyl which is a well-known inhibitor of Pi transport in liver

mitochondria. Thus, with the presently used techniques and intervals no evidence could be obtained for an effect of cAMP on mitochondrial volume and Pi transport in mouse B cells and liver.

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