Increased glucose oxidation and contents of insulin and ATP in polyamine-depleted rat insulinoma cells (RINm5F)

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In order to elucidate the role of polyamines in the replication and insulin production of insulin-secreting cells, we have investigated the impact of partial polyamine depletion on the proliferation, metabolism, insulin synthesis and ultrastructure of clonal rat insulinoma cells (RINm5F). For this purpose RINm5F cells were exposed for 4 days to the specific ornithine decarboxylase (ODC) inhibitor difluoromethylornithine (DFMO). This resulted in a profound decrease in ODC activity and cytoplasmic polyamine contents. The polyamine content of cell nuclei was, however, not altered by DFMO. Addition of small amounts of putrescine during culture elevated the intracellular content of this diamine and suppressed ODC activity. The decrease in polyamine contents was accompanied by a pronounced inhibition of the cellular proliferative activity. The rates of glucose utilization, oxygen uptake and activity of the pentose cycle were decreased in DFMO-treated cells, whereas the glucose oxidation rate, oxidation/utilization ratio, ATP content and ATP/ADP ratio were increased. Insulin mRNA content and synthesis of proinsulin, insulin and total protein were not altered by DFMO. In contrast, there was a sizeable increase in the cellular insulin content, despite a lowered total protein content. Electron-microscopic analysis revealed an accumulation of insulin-secretion granules in the DFMO-treated cells. In addition, short-term insulin release was increased after DFMO exposure, but was not rendered glucose-sensitive. It is concluded that polyamines are necessary for the maintenance of rapid insulinoma-cell replication and that DFMO-treated RINm5F cells acquire an enhanced substrate oxidation and increased content of insulin and ATP.

INTRODUCTION

Polyamines are ubiquitous aliphatic cations closely related to cell differentiation and proliferation in numerous mammalian cell systems (reviewed in [1–5]). Studies of the physiological role of polyamines are complicated by the fact that addition of polyamines to cultured cells results in toxic effects due to cell-membrane interaction and formation of noxious aldehydes [6,7]. However, this problem can be obviated by using specific inhibitors of enzymes regulating polyamine biosynthesis. The first and rate-limiting enzyme in the polyamine-biosynthetic pathway is ornithine decarboxylase (ODC, EC 4.1.1.17), which catalyses the formation of putrescine from ornithine. ODC activity is known to be substantially elevated in rapidly proliferating cells [3] and is specifically and irreversibly inhibited by difluoromethylornithine (DFMO) [8]. *S*-Adenosylmethionine (AdoMet) decarboxylase (AdoMetDC, EC 4.1.1.50) brings about the decarboxylation of AdoMet, thereby controlling the formation of spermidine and spermine by supplying aminopropyl moieties.

The aim of the present study was to investigate the role of polyamines in the proliferation, metabolism and hormone production by the insulin-secreting tumour cell line RINm5F. Because of the large amounts of cells available, this cell line is often used for studies of certain aspects of islet β -cell function. However, a considerable drawback is that these cells, in contrast with normal β -cells, are characterized by abnormal glucose metabolism and a low insulin content [9,10].

This work has been presented in part in abstract form [11,12].

MATERIALS AND METHODS

Materials

Culture medium RPMI 1640, fetal-calf serum, L-glutamine,

Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution, benzylpenicillin and streptomycin were from Flow Laboratories, Irvine, Scotland, U.K. Anti-(bovine insulin) serum was supplied by Miles-Yeda, Rehovot, Israel. Crystalline mouse insulin and ¹²⁵I-labelled insulin were from Novo, Copenhagen, Denmark. Trypsin (0.05%)/EDTA (0.02%) solution was supplied by Gibco, Paisley, Scotland, U.K. Putrescine, spermidine, spermine, L-proline, L-ornithine, *S*-adenosyl-L-methionine, pyridoxal phosphate, dithiothreitol, benzamidine, phosphoenolpyruvate and dansyl chloride were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Acrylamide (grade 1) and SDS were from BDH Chemicals, Poole, U.K., and Bromophenol Blue, ammonium persulphate, *NNN'*-tetramethylethylenediamine and *NN'*-methylenebisacrylamide were from Bio-Rad, Richmond, CA, U.S.A. ADP and ATP standards were from Boehringer-Mannheim, Mannheim, Germany. BSA (fraction V) was obtained from United States Biochemical Corp., Cleveland, OH, U.S.A., and Trasylol was delivered by Bayer A.G., Leverkusen, Germany. D-[5-³H]Glucose (21.1 Ci/mmol), D-[6-¹⁴C]-glucose (55 mCi/mmol), D-[1-¹⁴C]glucose (55.9 mCi/mmol), [α -³²P]dCTP (3000 Ci/mmol), L-[1-¹⁴C]ornithine (59 mCi/mmol), *S*-adenosyl-L-[carboxy-¹⁴C]methionine (Ado[¹⁴C]Met; 56 mCi/mmol), L-[4,5-³H]leucine (40 Ci/mmol), Amplify and Hyperfilm-MP were purchased from Amersham International, Amersham, Bucks., U.K. Unisolve was from New England Nuclear, Boston, MA, U.S.A., and Hyamine hydroxide 10-X was provided by Packard Instruments, Downers Grove, IL, U.S.A. CsCl (optical grade) was from Bethesda Research Laboratories, Bethesda, MD, U.S.A. Tissue-culture flasks and culture dishes were obtained from Nunc, Roskilde, Denmark. All other chemicals of analytical grade were obtained from E. Merck, Darmstadt, Germany.

Abbreviations used: AdoMet, *S*-adenosylmethionine; AdoMetDC, *S*-adenosylmethionine decarboxylase; DFMO, difluoromethylornithine; ODC, ornithine decarboxylase; PBS, phosphate-buffered saline.

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Cell culture

The RINm5F cell line [9] was maintained in tissue culture at 37 °C in medium RPMI 1640 containing 11.1 mM-glucose, 2 mM-L-glutamine, antibiotics (100 units of penicillin/ml and 0.1 mg of streptomycin/ml) and 10 % (v/v) fetal-calf serum. The passage number of the cells used varied, but was routinely > 90. The cells were detached by a brief incubation with trypsin/EDTA in Ca²⁺/Mg²⁺-free Hanks' salt solution. Mycoplasma screening tests were negative.

Cell proliferation

Batches of 10000 cells were seeded in multiwell dishes containing 1 ml of RPMI 1640 in each well supplemented as indicated above and with the addition of the different test substances. Media were changed after 3 days.

For characterization of cell proliferation, duplicate cultures were trypsin-treated at 24 h intervals and cell numbers were counted with a haemocytometer. Cell viability was estimated by Trypan Blue exclusion. In the experiments listed below in which data are expressed on a per-cell basis, the cell number in the same cell suspension was measured in a haemocytometer before the onset of the experiment.

Decarboxylase activities

Cells were dislodged from culture dishes by use of a 'rubber policeman' and sonicated in 50 µl of a lysis buffer containing 50 mM-Tris/HCl (pH 7.4), 0.1 % Triton X-100, 4 mM-EDTA, 5 mM-dithiothreitol, 200000 units of Trasylol/ml, 10 mM-benzamidine and 0.1 mg of BSA/ml. The homogenates were centrifuged at 100000 g for 10 min at +4 °C. For ODC measurements, supernatants were mixed with 50 µl of incubation buffer containing 2 mM-pyridoxal phosphate, 0.4 mM-L-ornithine and 10 µCi of L-[1-¹⁴C]ornithine/ml. The assay was performed in small glass vials inserted into an outer flask tightly sealed with a rubber membrane. After incubation for 60 min at 37 °C in a slowly shaking water bath, reactions were terminated by addition of 100 µl of 5 M-H₂SO₄, whereupon 250 µl of Hyamine was injected into the outer flask to trap liberated ¹⁴CO₂. After further incubation overnight, Unisolve scintillation fluid was added and the radioactivity quantified by scintillation counting. The AdoMetDC assay was carried out similarly to that for ODC, except that the incubation buffer consisted of 5 mM-NaH₂PO₄, 0.4 mM-AdoMet and 4 µCi of Ado[¹⁴C]Met/ml. These assays were linear for at least 60 min at the substrate concentrations used.

Whole-cell polyamine content

The total polyamine content was determined by one-dimensional t.l.c. [13]. For this purpose, groups of (3–4) × 10⁵ cells were scraped off the culture dishes, washed in phosphate-buffered saline (PBS; 154 mM-Na⁺/4 mM-K⁺/140 mM-Cl⁻/10 mM-HPO₄²⁻, pH 7.4) and homogenized by sonication in 20 µl of 0.3 M-HClO₄ at 4 °C. After centrifugation (5 min, 12000 g), the supernatant was made alkaline by addition of 10 µl of 1 M-Na₂CO₃, followed by 75 µl of dansyl chloride (10 mg/ml in acetone). After incubation at room temperature overnight in the dark, excess reagent was made to react with 5 µl of L-proline (250 mg/ml), followed by sonication for 2 min. Dansyl-polyamines were extracted in 100 µl of toluene. The toluene was evaporated and the residue redissolved in 5 µl of toluene for application on t.l.c. plates (h.p.t.l.c. Fertigplatten, Kieselgel 60 F₂₅₄; E. Merck). The dansylated polyamines were separated by one run with ethyl acetate/cyclohexane (1:1, v/v), followed by two runs with diethyl ether/cyclohexane (2:3, v/v). The spots were scraped off the plates and the fluorescence intensities of the supernatants measured in a Perkin-Elmer model LS5 lumin-

escence spectrometer connected to a plate reader at an excitation wavelength of 360 nm and an emission wavelength of 510 nm. This method has, in our hands, an intra-assay variability (S.E.M./mean) of 6 %, an inter-assay variability of 3 % and a sensitivity of about 15 pmol (i.e. an amount of polyamine resulting in a fluorescence intensity 2 s.d. above the blank reading). Standard curves were linear up to at least 1000 pmol and showed correlation coefficients of ≥ 0.99.

Polyamine content of cell nuclei

Cells were scraped off culture dishes, washed in ice-cold PBS and mechanically homogenized in 100 µl of a buffer containing 250 mM-sucrose, 2 % Triton X-100, 2 mM-EDTA and 20 mM-Tris (pH 7.5). After sedimentation of nuclei (12000 g, 10 s), the pellet obtained was suspended once in the homogenization buffer and re-centrifuged. Then 20 µl of 0.3 M-HClO₄ was added and the polyamine content of this nuclear fraction analysed as detailed above. All steps of the fractionation procedure were carried out at 0–4 °C to prevent possible redistribution of polyamines between cellular compartments. Since previous studies have shown that polyamines are found mainly in the insulin-secretion granules [14], it was important to ascertain the purity of the nuclear fraction. For this purpose we have previously determined the insulin content in acid/ethanol extracts of the pellets obtained after HClO₄ extraction [15]. The results revealed that the nuclear fraction contained approx. 1 % of total cellular insulin, demonstrating that the nuclear fraction was essentially devoid of secretion-granule contamination.

Glucose metabolism

Glucose utilization, glucose oxidation and the activity of the pentose cycle were assayed essentially as previously described [16]. Duplicate groups of (1.5–2) × 10⁵ trypsin-treated cells were incubated in small glass vials at 37 °C for 2 h in 100 µl of a bicarbonate buffer [17] supplemented with 10 mM-Hepes, 2.8 mM-or 28 mM-D-glucose and D-[5-³H]glucose (425 µCi/mmol; glucose utilization), D-[6-¹⁴C]glucose (170 µCi/mmol; glucose oxidation) or D-[1-¹⁴C]glucose (170 µCi/mmol; pentose cycle). For glucose-utilization experiments, reactions were terminated by injection of 100 µl of 0.5 M-HCl and vials incubated overnight. The production of ³H₂O was subsequently determined by liquid-scintillation counting. The results were corrected for the recovery of ³H₂O, which was 74 %.

For measurements of glucose oxidation and the pentose cycle, reactions were terminated by injection of 100 µl of 0.05 mM-antimycin A in ethanol. The ¹⁴CO₂ formed was released from incubation media by addition of 100 µl of 0.4 M-Na₂HPO₄ (pH 6.0) and trapped in Hyamine. After incubation overnight, the radioactivity was measured by liquid-scintillation counting. The flux through the pentose cycle was calculated as described previously [16,18]. The validity of this calculation is based on certain assumptions spelled out in ref. [16]. Although these criteria are fulfilled in normal islet cells, this has not been investigated in detail in the RINm5F cells. Therefore the data on pentose-cycle activity should be viewed somewhat cautiously.

Cell respiration and adenine nucleotide contents

RINm5F-cell oxygen-uptake rates were determined as previously detailed [19] by use of the Cartesian-diver technique. For this purpose cultured cells were trypsin-treated and suspensions of 10⁴ cells were allowed to respire first for 60 min at 37 °C in 1.0 µl of a Krebs-Ringer phosphate buffer (ionic composition in mM: Na⁺ 150.8, K⁺ 5.9, Ca²⁺ 1.3, Mg²⁺ 1.2, Cl⁻ 129.0, PO₄³⁻ 17.3 and SO₄²⁻ 1.2) supplemented with 2.8 mM-glucose. The gas phase consisted of ambient air. Respiratory CO₂ evolved was trapped

in KOH. After 60 min the glucose concentration was increased to 28 mM by addition to the medium of a 0.5 μ l side-drop. Throughout the experiment DFMO was present or absent as during culture. The linearity of this assay has previously been demonstrated for a cell-concentration range of $(0.5\text{--}2) \times 10^4/\mu$ l [19].

For determination of the cellular adenine nucleotide content, cell cultures were washed once with PBS, preincubated for 45 min at 37 °C in a bicarbonate buffer [17] containing 10 mM-Hepes, 2 mg of BSA/ml, 2.8 mM-glucose and with or without DFMO. Preincubation media were discarded and replaced by similar media supplemented with 2.8 mM- or 28 mM-glucose and incubated for 60 min. Media were then rapidly discarded, and cultures were quickly washed once with ice-cold PBS and sonicated in ice-cold 0.89 M-HClO₄ + 2 mM-EDTA. The samples were then immediately plunged into liquid nitrogen and kept frozen at –80 °C pending analysis.

ATP was determined by use of a bioluminescence kit [20]. ADP was converted into ATP by addition of pyruvate kinase (170 μ g/ml; EC 2.7.1.40; E. Merck) in a mixture of 0.6 mM-phosphoenolpyruvate, 60 mM-magnesium acetate, 150 mM-potassium acetate, 6 mg of BSA/ml, 2 mM-EDTA, 76 mM-acetic acid and 0.1 M-Tris (pH 7.75). Light signals were quantified at room temperature in a LKB model 1250 luminometer connected to a voltmeter.

Insulin production and secretion

For measurements of cellular insulin mRNA, total RNA from 10^6 cells was isolated by the guanidium method as detailed in ref. [20]. The RNA samples were extracted with phenol/chloroform/3-methylbutan-1-ol, and nucleic acids were precipitated with 2 M-potassium acetate in ethanol overnight at –20 °C. The precipitates were washed with 70 % (v/v) ethanol and subsequently treated with 1 M-glyoxal for 1 h at 50 °C, diluted with 3 M-NaCl/0.3 M-sodium citrate and bound to GeneScreen membranes at 80 °C for 2 h. After pre-hybridization for 1 h at 42 °C, the samples were hybridized at 42 °C with the appropriate probe (pRI-7) [21], which had been labelled by using an oligonucleotide-labelling kit. After hybridization, filters were washed and exposed at –70 °C to Hyperfilm-MP with an intensifying screen. The intensities of the spots thus obtained were determined by densitometric scanning and expressed as optical density (o.d.). The assay was linear over the range of concentrations of insulin mRNA tested, and without detectable background hybridization.

For measurements of insulin biosynthesis, RINm5F cell cultures were washed twice in PBS and incubated for 3 h at 37 °C in a 250 μ l of a bicarbonate buffer [17] containing 10 mM-Hepes, 50 μ g of BSA/ml, 28 mM-glucose, 100 μ Ci of [³H]leucine/ml and with or without DFMO. Cells were then washed twice in PBS, lysed by sonication for 15 s in 70 % ethanol/0.18 M-HCl, and a sample of the sonicate preparation was used for measurement of total protein synthesis [20]. The acid/ethanol homogenates were kept overnight at 4 °C for extraction of insulin and proinsulin. Samples of the incubation media were also extracted for insulin and proinsulin by the same protocol. After extraction, a sample was taken for analysis of insulin content by radioimmunoassay [22]. The acid/ethanol of the remaining extract was evaporated and the residue dissolved in 2 % SDS/5 % β -mercaptoethanol/50 mM-Tris (pH 8.8)/10 % sucrose/0.001 % Bromophenol Blue. Proteins were subsequently separated by PAGE as previously described [23], except that a 14–21 % polyacrylamide gradient with 6 M-urea were used. Molecular-mass standards (Rainbow Markers, Amersham) and rat islet-cell homogenates were run in parallel. Gels were treated with Amplify and exposed to Hyperfilm-MP at –80 °C for 2–5 days.

For insulin-secretion experiments, media were removed and the cultures washed once with PBS. They were then preincubated at 37 °C for 45 min in Krebs–Ringer bicarbonate buffer [17] at pH 7.4 containing 2 mg of BSA/ml, 10 mM-Hepes and 2.8 mM-glucose. The preincubation media were then discarded, and cultures were washed once with similar media and subsequently exposed for 30 min to 2.8 mM- or 28 mM-glucose in similar incubation buffer. Samples of the centrifuged incubation media were analysed radioimmunologically for insulin [22].

Electron microscopy

Monolayer cultures of RINm5F cells were fixed in 3 % glutaraldehyde buffered in 0.1 M-sodium phosphate (pH 7.2) at 4 °C for 2 h. While still attached to the bottom of the culture flasks, the cells were washed in buffer and post-fixed with 2 % OsO₄ in veronal acetate buffer at 4 °C. After a brief rinse in distilled water, the cells were treated with aq. 2 % uranyl acetate for 2 h and dehydrated in graded alcohols. The cells were detached from the culture flask with a rinse of propylene oxide and transferred to vials and capsules for final embedding in Polybed 812 (Polysciences Inc., Warrington, PA, U.S.A.). Sections, stained with uranyl acetate and lead citrate, were viewed in a JEOL 100CX transmission electron microscope.

DNA and protein contents

The DNA content of duplicate samples of aqueous cell homogenates was determined fluorimetrically as previously described [24,25]. Protein was measured by the Lowry method [26], with BSA as standard.

Statistical analysis

Means $\pm$ S.E.M. were calculated and groups of data were compared by Student's paired or unpaired *t* test. In cases of multiple comparisons, data were evaluated either by one-way factorial ANOVA or by two-way factorial ANOVA in conjunction with Bonferroni's modified *t* statistics [27] by use of a StatView 512+ (Version 1.0) software package from Abacus Concepts and BrainPower Inc., Calabasas, CA, U.S.A.

RESULTS

Decarboxylase activities and polyamine content

After 4 days of exposure to DFMO, ODC activity was decreased by more than 90 % (Table 1). Putrescine (50 μ M) alone caused a 50 % inhibition of enzyme activity, whereas DFMO

Table 1. Effects of DFMO and putrescine on ODC and AdoMetDC activities

RINm5F cells were cultured for 4 days in media supplemented as indicated below. Decarboxylase activities were assayed by measuring the generation of ¹⁴CO₂ from cell homogenates incubated with L-[¹⁴C]ornithine (for ODC) or Ado[¹⁴C]Met (for AdoMetDC). Values are means $\pm$ S.E.M. for the numbers of observations in parentheses: * (versus control cells), † (versus DFMO-treated cells) and ‡ (versus putrescine-treated cells) denote 99 % multi-comparison significance level using one-way factorial ANOVA.

Additions to medium	ODC activity (fmol of ¹⁴ CO ₂ /30 min per 10 ⁶ cells)	AdoMetDC activity (fmol of ¹⁴ CO ₂ /60 min per μ g of DNA)
Control	400 $\pm$ 26 (7)	97 $\pm$ 11 (4)
DFMO (1 mM)	34 $\pm$ 11 (7)*	1470 $\pm$ 72 (4)*
Putrescine (50 μ M)	210 $\pm$ 43 (7)*†	140 $\pm$ 24 (4)
DFMO + putrescine	32 $\pm$ 14 (7)*‡	170 $\pm$ 21 (4)†

Table 2. Effects of DFMO and putrescine on the total polyamine content of RINm5F cells

RINm5F cells were cultured for 4 days in media supplemented as indicated below. Polyamines were extracted in HClO_4 and measured fluorimetrically after t.l.c. separation of dansylated compounds. Values are means $\pm$ S.E.M. for six observations: * (versus control cells) and † (versus DFMO-treated cells) denote 99% multi-comparison significance level using one-way factorial ANOVA.

Additions to medium	Total polyamine content (pmol/ 10^6 cells)		
	Putrescine	Spermidine	Spermine
Control	63 $\pm$ 21	2540 $\pm$ 160	1910 $\pm$ 160
DFMO (1 mM)	1 $\pm$ 0.5*	56 $\pm$ 10*	570 $\pm$ 120*
Putrescine (50 μM)	460 $\pm$ 71*†	2940 $\pm$ 300†	1640 $\pm$ 160†
DFMO + putrescine	330 $\pm$ 74*†	3200 $\pm$ 160*†	1950 $\pm$ 110†

Table 3. DFMO does not affect the polyamine content of RINm5F-cell nuclei

RINm5F cells were cultured for 4 days in media supplemented as indicated below. Polyamines of a nuclear fraction were extracted in HClO_4 and measured fluorimetrically after t.l.c. separation of dansylated compounds. Values are means $\pm$ S.E.M. for four observations.

Addition to medium	Nuclear polyamine content (pmol/ 10^6 cells)		
	Putrescine	Spermidine	Spermine
Control	7 $\pm$ 1	242 $\pm$ 31	186 $\pm$ 15
DFMO (1 mM)	6 $\pm$ 2	257 $\pm$ 28	199 $\pm$ 23

combined with putrescine inhibited the enzyme to the same extent as DFMO alone. DFMO exposure also resulted in a 15-fold stimulation of AdoMetDC activity, an effect that vanished when putrescine was included.

DFMO diminished the whole-cell putrescine and spermidine contents by approx. 98%, whereas the content of spermine was decreased to one-third of that in control cells (Table 2). When

Fig. 1. DFMO decreases proliferation of cultured RINm5F cells

Cells were counted at 24 h intervals and their viability was assessed by Trypan Blue exclusion. In each panel, the average cell number per well is given on a logarithmic scale on the left ordinate (●), and the percentage viable cells on a linear scale on the right ordinate (○): untreated cells (a) and cells treated with 1 mM-DFMO (b), 50 μM -putrescine (c) and DFMO combined with putrescine (d). Values are means $\pm$ S.E.M. for three or four separate experiments; * [versus (a)] and † [versus (b)] denote 99% multi-comparison significance level using one-way factorial ANOVA.

exogenous putrescine (50 μM) was added, the intracellular content of this diamine increased 5–7-fold, whereas the contents of the other amines were much less affected.

Table 4. Glucose metabolism, respiration and adenine nucleotide contents in DFMO-treated RINm5F cells

RINm5F cells were cultured for 4 days in media supplemented as indicated below. Cells were then preincubated for 45 min in a bicarbonate buffer containing 2.8 mM-glucose and subsequently incubated for another 60 or 120 min in 2.8 mM-glucose. Values are means $\pm$ S.E.M. for four to six experiments. Statistical analysis of differences between groups was performed by two-way factorial ANOVA in conjunction with Bonferroni's modified *t*-statistics: ** and *** denote $P < 0.01$ and $P < 0.001$ versus control cells in 2.8 mM-glucose; †, †† and ††† denote $P < 0.05$, $P < 0.01$ and $P < 0.001$ versus control cells in 28 mM-glucose; ‡ and ‡‡ denote $P < 0.05$ and $P < 0.001$ versus DFMO-treated cells in 2.8 mM-glucose.

Incubation (mM-glucose)...	Cell culture			
	Control		DFMO (1 mM)	
	2.8	28	2.8	28
Glucose utilization (fmol/2 h per cell)	12 $\pm$ 1.5	32 $\pm$ 2.7***	3.2 $\pm$ 0.4***	7.8 $\pm$ 1.5††††
Glucose oxidation (amol/2 h per cell)	140 $\pm$ 4	250 $\pm$ 10***	170 $\pm$ 30	530 $\pm$ 50†††††
Pentose shunt (amol/2 h per cell)	130 $\pm$ 20	190 $\pm$ 20	Not detectable	
Oxygen uptake (nmol of O_2 /h per 10^4 cells)	1.8 $\pm$ 0.14	1.9 $\pm$ 0.18	1.1 $\pm$ 0.10**	1.1 $\pm$ 0.05†
ADP content (pmol/ μg of DNA)	4.7 $\pm$ 0.26	3.8 $\pm$ 0.45	3.92 $\pm$ 0.17	4.10 $\pm$ 0.44
ATP content (pmol/ μg of DNA)	48 $\pm$ 1.8	51 $\pm$ 2.6	99 $\pm$ 2.8***	103 $\pm$ 4.8††
ATP/ADP ratio	10.9 $\pm$ 0.5	15.0 $\pm$ 2.0	25.4 $\pm$ 1.4***	25.4 $\pm$ 2.1†

The nuclear polyamine contents (Table 3) amounted to approx. 10% of the total polyamine contents and were not affected by DFMO.

Cell proliferation and viability

As illustrated in Figs. 1(a)–1(d), DFMO exerted pronounced effects on the cell number of the culture as well as the population doubling time. After 6 days the cell number in the cultures treated with DFMO (1 mM) was approx. 10% of that in control cultures. The population doubling time was calculated to be 20 h for untreated cells in exponential growth phase (days 2–5) and 60 h for DFMO-exposed cells during the same period of time. The content of DNA per cell was not altered by DFMO (14.3 ± 2.3 pg/cell in control cells and 16.2 ± 2.1 pg/cell in DFMO-treated cells; $n = 4$). DFMO-treated cultures also exhibited a modest decline in cell viability, which, however, always exceeded 90% of the viability in control cells. All the DFMO-induced alterations in cell growth and viability were prevented by addition of 50 μ M-putrescine.

Glucose metabolism

As demonstrated in Table 4, the glucose utilization rate was significantly stimulated in response to an increased glucose concentration in both untreated and DFMO-treated RINm5F cells. DFMO treatment resulted in a marked decrease in glucose utilization, to approximately the same extent in cells incubated at 2.8 mM- and 28 mM-glucose. The glucose oxidation rate was increased in control as well as DFMO-treated cells incubated at 28 mM-glucose as compared with 2.8 mM-glucose. This increase in response to glucose was, however, significantly greater in DFMO-treated cells than in controls. The oxidation/utilization ratio was approx. 1% in control cells irrespective of the glucose concentration. This value was increased to 5–10% by DFMO, the greatest increase occurring in high glucose.

The activity of the pentose cycle in control cells was not significantly affected by 28 mM-glucose, as compared with 2.8 mM-glucose, but was decreased to undetectable levels after DFMO treatment.

Addition of putrescine (50 μ M) with DFMO completely reversed the effects of the inhibitor on glucose metabolism (results not shown).

Cell respiration and adenine nucleotide contents

As shown in Table 4, the rate of oxygen uptake was not enhanced when the glucose concentration was increased from 2.8 mM to 28 mM, in either control or DFMO-treated cells. However, respiration in DFMO-treated cells was suppressed by approx. 40%, irrespective of the glucose concentration.

After 4 days of culture, cells exposed to 1 mM-DFMO contained 2-fold more ATP than control cells (Table 4). The ADP content was not altered by DFMO, resulting in an elevated

Fig. 2. DFMO does not affect insulin synthesis or release of newly synthesized insulin in cultured RINm5F cells

Cultured RINm5F cells were pulse-labelled for 3 h with [³H]leucine in a bicarbonate buffer. Cells were washed and sonicated in acid/ethanol. Sonicated preparations and incubation media were extracted for proinsulin and insulin in acid/ethanol. The ethanol was evaporated and extracts dissolved in SDS sample buffer [23] and boiled for 5 min. Samples (20 μ l) were applied to 14–21% gradient gels and electrophoresed. Extracts of labelled rat pancreatic islets and molecular-mass markers were run in parallel. The gel obtained was treated with Amplify and autoradiographed. Lane 1, extract of control cells; lane 2, extract of DFMO-treated cells; lane 3, incubation medium from control cells; lane 4, incubation medium from DFMO-treated cells. The positions of molecular-mass markers (kDa) are indicated to the left. Arrows indicate (from top to bottom) the positions of proinsulin and insulin B- and A-chains. The gel shown is typical for experiments performed with three different cell preparations.

ATP/ADP ratio. The content of the different adenine nucleotides was not influenced by increasing the glucose concentration from 2.8 mM to 28 mM. Addition of putrescine (50 μ M) with DFMO completely reversed the effects of the inhibitor on the ATP content (results not shown).

Insulin, proinsulin and total protein biosynthesis, contents of insulin mRNA, insulin and protein, and insulin release

As shown in Fig. 2, analysis by SDS/PAGE of labelled RINm5F-cell proteins revealed that DFMO did not affect the synthesis of either proinsulin or insulin. DFMO also did not affect the amount of newly synthesized proinsulin or insulin released into the incubation medium. In addition, no differences in total protein biosynthesis between controls and DFMO-

Table 5. Contents of insulin mRNA, protein and insulin and protein biosynthesis in DFMO-treated RINm5F cells

RINm5F cells were cultured for 4 days in media supplemented as indicated below. Values are means $\pm$ S.E.M. for four to ten experiments: * (versus control cells) and † (versus DFMO-treated cells) denote 99% multi-comparison significance level using one-way factorial ANOVA. ‡‡‡ denotes $P < 0.001$ versus control cells using Student's unpaired t test. Abbreviations: o.d., optical density; N.D., not determined.

Additions to medium	Insulin mRNA (o.d./10 ⁶ cells)	Protein (μ g/10 ⁴ cells)	Insulin (ng/ μ g DNA)	Protein synthesis (c.p.m./10 ⁵ cells)
Control	70 $\pm$ 7	3.0 $\pm$ 0.1	13 $\pm$ 1	31 900 $\pm$ 3700
DFMO (1 mM)	68 $\pm$ 5	0.9 $\pm$ 0.2‡‡‡	82 $\pm$ 11*	31 700 $\pm$ 3400
Putrescine (50 μ M)	79 $\pm$ 26	N.D.	15 $\pm$ 2†	N.D.
DFMO + putrescine	90 $\pm$ 28	N.D.	14 $\pm$ 2†	N.D.

Fig. 3. Accumulation of insulin-secretion granules in DFMO-treated RINm5F cells

(a) shows the cytoplasm of a typical untreated RINm5F cell, with very few secretion granules. In contrast, (b) shows numerous secretion granules (arrow) in the area of the Golgi complex in cells treated with 1 mM-DFMO. Magnification $\times 29\,000$.

treated cells were noted (Table 5). DFMO-treated cells contained similar amounts of insulin mRNA as control cells, whereas these cells contained 6 times more immunoreactive insulin than untreated cells (Table 5). Despite the increased insulin content, the total protein content was actually lowered in DFMO-treated cells (Table 5).

The short-term insulin release was not altered by raising the glucose concentration from 2.8 mM to 28 mM irrespective of whether the RINm5F cells had been treated with DFMO or not. On the other hand, insulin release from DFMO-treated cells was approx. 2-fold higher than that from control cells ($P < 0.01$; two-way factorial ANOVA with Bonferroni's transformation). Thus insulin release (pg/30 min per μg of DNA; $n = 4$) was 410 ± 83 in control cells at 2.8 mM-glucose, 430 ± 62 in control

cells at 28 mM-glucose, 990 ± 88 in DFMO-treated cells at 2.8 mM-glucose, and 970 ± 105 in DFMO-treated cells at 28 mM-glucose.

Ultrastructure

Fig. 3 shows the ultrastructure of RINm5F cells as analysed by electron microscopy. Fig. 3(a) shows the cytoplasm of a typical untreated RINm5F cell with very few secretion granules. In contrast, Fig. 3(b) shows numerous secretion granules in the area of the Golgi complex in cells treated with 1 mM-DFMO.

DISCUSSION

The present paper demonstrates that partial depletion of the polyamine content of RINm5F cells caused marked inhibition of

cell proliferation, glucose utilization, pentose cycle and cell respiration, but enhanced glucose oxidation. These effects were paralleled by an elevated ATP content, unchanged insulin mRNA content and increased cellular contents of insulin and insulin-secretion granules.

The content of putrescine in the RINm5F cells was relatively low when compared with that in islets of adult mice [20] and rats [14] and fetal rats [15], suggesting a more rapid conversion of the diamine into spermidine and spermine. This observation agrees with the notion that spermidine and spermine are more important than putrescine for rapid cell proliferation [5]. Also notable in this context is the recent finding of a more rapid turnover rate for putrescine and of higher ODC activity in tumoral islet cells than in normal islet cells [28]. These features may provide the tumoral cells with a large amount of polyamines necessary for the rapid proliferation of these cells. We also found that the content of spermine in DFMO-treated cells was much less altered than that of the other amines. This is most probably due to the continued synthesis of spermine from spermidine and putrescine and also inhibition of spermine degradation [5]. Further characterization of the subcellular polyamine distribution disclosed that the nuclear polyamine content was not decreased by DFMO. Thus it appears that the RINm5F cell is equipped with a mechanism for translocating polyamines into the nucleus when inhibition of polyamine synthesis occurs. This may serve as a means by which the cell nucleus can maintain a sufficient polyamine content for the cell to function, despite a decrease in the cytoplasmic polyamine content.

The observed inhibitory action of putrescine on ODC activity in this study is concordant with similar findings in other cell systems and may in part be explained by a feedback inhibition due to phosphorylation and inactivation of ODC by polyamine-dependent casein kinase II [29]. There was also a powerful enhancement of AdoMetDC activity by polyamine-synthesis inhibition, a finding noted in other cell systems and which is likely to reflect relief of the spermidine- and spermine-mediated feedback inhibition exerted on this enzyme [30].

The high glucose utilization and low glucose oxidation rates observed here in untreated RINm5F cells confirm previous findings of aberrations in tumour-cell carbohydrate metabolism [31]. The further observation of a glucose-induced increase in glucose utilization, taken to reflect the activity of the glycolytic pathway [32], confirms previous findings in the RINm5F cell line [10]. However, we, and others [33], were unable to reproduce findings in a previous report [32] that suggested a decreased rate of glucose oxidation and oxygen uptake in response to an increase in the extracellular glucose concentration. The activity of the pentose cycle, which generates ribose 5-phosphate utilized in nucleic acid biosynthesis, was decreased to undetectable levels after DFMO exposure. This suggests that this branch of RINm5F-cell glucose metabolism is selectively shut off as DNA replication ceases. Alternatively, a decrease in the activity of the pentose cycle in DFMO-treated cells could represent a primary event which causes decreased replication by limiting the supply of ribose 5-phosphate. Our findings moreover indicate that the flux through the glycolytic pathway was decreased, and the glucose oxidation rate in response to a high glucose concentration was increased, after DFMO treatment, thus resembling the situation in normal β -cells [32]. It can be calculated that the increase in ATP generation emanating from the enhanced glucose oxidation is less than the decrease in ATP production due to the inhibited glucose utilization. Thus it appears as if the elevated cellular content of ATP in DFMO-treated cells, also noted in other cell systems [34], results from a decreased consumption of ATP, owing to cessation of cell proliferation. This rise in ATP is also liable to explain the decrease in respiration and glucose

utilization rates, whereas the cause of the enhanced glucose oxidation remains elusive at this stage. The oxygen-uptake rate observed here is in close agreement with that in a previous study of this cell line [19]. At a first glance, the lack of glucose stimulation of respiration and ATP content despite an increased glucose oxidation appears conflicting. However, since for each glucose molecule oxidized 6 oxygen molecules are consumed, it can be calculated that the fraction of total oxidative metabolism contributed by glucose represents no more than approx. 1 %. It is therefore not surprising that a doubled glucose oxidation rate does not give rise to a detectable increase in respiration and ATP.

The unaltered insulin mRNA content and insulin synthesis and elevated insulin content in DFMO-treated RINm5F cells suggest that partial polyamine depletion in insulinoma cells has effects on the insulin production which are different from those observed after corresponding treatment in normal mouse islet cells [20]. In these latter cells, polyamine deficiency was associated with impaired insulin synthesis and inhibition of cell replication. However, it should be noted that, although elevated, the cellular contents of ATP and insulin in the DFMO-treated RINm5F cells were still much lower than in normal rat islets [35,36]. The unaltered insulin mRNA content and insulin synthesis in association with increased ATP contents in DFMO-treated cells is at variance with the situation in normal rat β -cells, in which increased cellular ATP contents coincide with stimulation of insulin production [37]. This finding may reflect the importance of polyamines for transcription, stabilization of RNA and translation [4,38]. The unaltered insulin production may thus be the result of the dual effect exerted by DFMO on the contents of polyamines and ATP in the RINm5F cells. In contrast, the large increase in cellular insulin content could be due to a gradual build-up of the secretion-granule pool during the 4-day culture period, and should not be viewed as being contradictory to the unaltered insulin synthesis. It should also be recognized that the insulin content depends not only on insulin synthesis but also on degradation and secretion of the hormone. The basal insulin release was increased approx. 2-fold after DFMO exposure, whereas the cellular insulin content was elevated 6-fold. Thus the fractional insulin release (i.e. the ratio between secreted insulin and the insulin content) was actually decreased in DFMO-treated cells, which may explain the build-up of the secretion-granule pool observed ultrastructurally. The present findings also point out an effect of DFMO exclusively on secretion of stored insulin, since the release of newly synthesized hormone was found to remain unaffected by DFMO. Although not investigated in detail in this study, it could thus be that DFMO imposes a change in the secretory pattern of the insulinoma cells, normally characterized by predominance of a constitutive secretory pathway, towards the regulated secretion found in native β -cells. It should be recognized that, compensatory to DFMO treatment, polyamine-uptake systems are activated [2]. These transporters not only carry polyamines but may also enhance the uptake of various extracellular molecules [2]. It thus cannot be excluded that the apparent lack of effect of DFMO on protein synthesis, despite a decreased protein content, reflects artefactually elevated uptake rates of labelled leucine. Consequently, insulin biosynthesis and secretion of newly synthesized insulin should be considered relative to the total radioactivity recovered in trichloroacetic acid precipitates. Finally, the decreased protein content in DFMO-treated cells should not be taken to reflect toxicity of DFMO, since the contents of insulin and ATP were elevated. Instead, the lowered protein content is likely to be due to a decreased cell size.

In conclusion, the present data show that polyamines are necessary for the rapid proliferation of the RINm5F cells, and that partial polyamine depletion induces increased insulin and

ATP contents and changes in the aberrant oxidative metabolism of these tumoral cells towards that of normal β -cells. These improvements in cellular functions increase the suitability of the RINm5F cell line for studies of normal β -cell function.

DFMO was kindly given by Dr. Peter P. McCann, of the Merrell Dow Research Center, Cincinnati, OH, U.S.A. We thank Dr. Michael Welsh and Dr. Stellan Sandler for constructive criticism and valuable suggestions. Skilful technical assistance by Astrid Nordin, Eva Forsbeck and Margareta Engkvist, and secretarial assistance by Agneta Snellman, is gratefully acknowledged. Financial support was received from the Swedish Medical Research Council (12X-109), the Medical Faculty at Uppsala University, the Swedish Diabetes Association, the Swedish Society of Medicine, the Clas Groschinsky Memorial Foundation, the Helge Ax:son Johnson Foundation, the Torsten and Ragnar Söderberg Foundations, the Royal Swedish Academy of Sciences, the Nordic Insulin Fund, the Hoechst Diabetes Foundation, the Lars Hierta Memorial Foundation and the Swedish Society for Medical Research.

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Received 10 January 1991/7 March 1991; accepted 19 March 1991