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CYCAD TOXIN-INDUCED DAMAGE OF RODENT AND HUMAN PANCREATIC β-CELLS

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Abstract—Environmental toxins may be risk factors for some forms of diabetes mellitus and neurodegenerative diseases. The medicinal and food use of seed from the cycad plant (*Cycas* spp.), which contains the genotoxin cycasin, is a proposed etiological factor for amyotrophic lateral sclerosis/Parkinsonism-dementia complex (ALS/PDC), a prototypical neurodegenerative disease found in the western Pacific. Patients with ALS/PDC have a very high prevalence of glucose intolerance and diabetes mellitus (in the range of 50–80%). We investigated whether the cycad plant toxin cycasin (methylazoxymethanol (MAM) β-D-glucoside) or the aglycone MAM are toxic *in vitro* to mouse or human pancreatic islets of Langerhans. Mouse pancreatic islets treated for 6 days with cycasin impaired the β-cell insulin response to glucose, but this effect was reversible after a further 4 days in culture without the toxin. When mouse islets were exposed for 24 hr to MAM/MAM acetate (MAMOAc; 0.1–1.0 mM), there was a dose-dependent impairment in insulin release and glucose metabolism, and a significant decrease in islet insulin and DNA content. At higher MAM/MAMOAc concentrations (1.0 mM), widespread islet cell destruction was observed. Glucose-induced insulin release remained impaired even after removal of MAM and a further culturing for 4 days without the toxin. MAM damages islets by two possible mechanisms: (a) nitric oxide generation, as judged by increased medium nitrite accumulation; and (b) DNA alkylation, as judged by increased levels of O^6-methyldeoxyguanosine in cellular DNA. Incubation of mouse islets with hemin (10 or 100 μM), a nitric oxide scavenger, or nicotinamide (5–20 mM) protected β-cells from a decrease in glucose oxidation by MAM. In separate studies, a 24 hr treatment of human β-islet cells with MAMOAc (1.0 mM) produced a significant decrease in both insulin content and release in response to glucose. In conclusion, the present data indicate that cycasin and its aglycone MAM impair both rodent and human β-cell function which may lead to the death of pancreatic islet cells. These data suggest that a "slow toxin" may be a common aetiological factor for both diabetes mellitus and neurodegenerative disease.

Key words: cycasin; methylazoxymethanol (MAM); nitric oxide; diabetes mellitus; insulin release; pancreatic islets; DNA damage

There are experimental and epidemiological data suggesting that environmental agents play a causal role in diabetes mellitus [1]. Among these agents, chemical toxins have received special attention. Thus, it has been shown that non-lethal doses of SZ§, the *N*-nitrosourea derivative of glucosamide, induces long-lasting functional impairment of rodent β-cells to produce and release insulin [2, 3]. This effect is probably related both to unrepaired damage to nuclear and mitochondrial DNA by alkylation [2, 4–6] and acute generation of NO [7, 8]. There is also circumstantial evidence that *N*-nitroso compounds from smoke-cured mutton contribute to an increased prevalence of IDDM in Iceland [9, 10] and that mutagenic nitroso compounds from Betel-nut are related to increased prevalence of NIDDM in Asians [11]. However, it remains to be proven that environmental toxins of potential relevance to human diabetes mellitus are indeed toxic to rodent and human islets of Langerhans. Furthermore, the cellular and molecular mechanisms responsible for these effects remain to be clarified.

Among the Chamorro people of the islands of Guam and Rota in the Marianas chain of Micronesia (western Pacific area) there is a high prevalence of a prototypical neurodegenerative disorder, consisting of PD and/or ALS/PDC [12]. The Chamorro practice of using the raw or incompletely detoxified seed of the neurotoxic cycad plant (*Cycas circinalis*) for medicine and food, respectively, is a possible etiological factor for ALS/PDC [13]. Cycad seed contains small amounts (0.02%, w/w) of the low-potency excitotoxin BMAA and ~2–4% (w/w) of cycasin, the glucopyranoside of the potent alkylating agent MAM [14]. MAM alkylates nucleic acids, amino acids and proteins [15], and the suggestion that the glucose-transport system permits cycasin to enter and damage CNS cells *in vitro* [16] points to

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§ Abbreviations: PD, parkinsonism-dementia; ALS/PDC, amyotrophic lateral sclerosis/Parkinson-dementia complex; BMAA, β-*N*-methylamino-L-alanine; IDDM, insulin-dependent diabetes mellitus; MAM, methylazoxymethanol; MAMOAc, methylazoxymethanol acetate; NIDDM, non-insulin-dependent diabetes mellitus; NO, nitric oxide; O^6-mdGuo, O^6-methyldeoxyguanosine; SZ, streptozotocin; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; FCS, foetal calf serum; ISB, immuno-slot-blot; NMU, *N*-methylnitrosourea; HRP, horseradish peroxidase; SNP, sodium nitroprusside.

a similarity with the mechanism by which SZ gains access to and damages pancreatic β-cells [17]. These observations, coupled with the high prevalence (60–70%) of glucose intolerance in ALS/PDC patients [18] (30% of them present overt diabetes [19, 20]) raises the possibility that cycasin may be a common etiological factor for both neurodegenerative disease and pancreatic β-cell damage.

We investigated the *in vitro* effects of BMAA, cycasin, MAM and MAM acetate (MAMOAc) on the function and survival of mouse pancreatic islets, and the mechanism(s) responsible for these effects. Additionally, the effect of MAMOAc on human pancreatic islets obtained from organ donors was studied.

MATERIAL AND METHODS

Chemicals and toxins. The chemicals were purchased from the following sources: culture medium RPMI-1640, FCS, BSA, DMSO, hemin, nicotinamide and β-glucosidase (Type I from almonds) from Sigma Chemical Co. (St. Louis, MO, U.S.A.); D-[U-^{14}C]glucose (55 mCi/mmol/L) from Amersham International (Amersham, U.K.); collagenase from *Clostridium hystolyticum* from Boehringer-Mannheim (Mannheim, Germany). All other chemicals of analytical grade were obtained from E. Merck (Darmstadt, Germany) or Sigma Chemicals.

BMAA was prepared as described by Vega *et al.* [21]. The purity of the preparation was confirmed by high-voltage paper electrophoresis, HPLC and mass spectrometry as previously described [16, 22].

Cycasin was isolated from frozen cycad seed kernels shipped from Guam; they were homogenized in 80% ethanol, centrifuged and the supernatant ultrafiltered as described in detail elsewhere [23, 24]. The ultrafiltrate, containing cycasin, was sequentially purified over various chromatographic media, and the eluent dried and recrystallized twice in 75% acetone [25]. Authenticity and purity of the toxin were established as previously described [16].

MAM was prepared from methylazoxymethanol acetate (1.0 g, Aldrich) by incubation with porcine liver esterase (3.75 mg/mL, Sigma) in 10 mL of phosphate buffer (0.1 M, pH 7.4) at room temperature [26]. The solution was ultrafiltrated (MWCO 1000), extracted with chloroform, evaporated, and the resulting viscous liquid analysed for purity by HPLC and TLC. Under the incubation conditions described above, all of the MAMOAc was hydrolysed to free MAM as determined by HPLC analysis [16].

Tissue culture and test agents treatment. Pancreatic islets were obtained from male NMRI mice (Anticimex, Sollentuna, Sweden) that had fasted overnight. Islets were isolated from collagenase-digested pancreata and subsequently picked by means of a braking pipette. The islets were maintained free-floating in culture medium for 5–6 days at 37° [27] before treatment with the test agents. The culture medium, which was changed every 48 hr, was RPMI 1640 containing 10% calf serum, benzylpenicillin (60 mg/mL), streptomycin (0.1 mg) and 11.1 mM glucose. Islets were treated for 1–6 days with control media or media supplemented with 0.1–1.0 mM cycasin, BMAA, MAM, MAMOAc. Immediately thereafter, islets were either used for functional studies or transferred to culture medium RPMI 1640 + 10% calf serum and maintained in culture for an additional 4 days, without toxins, before further studies were performed. In a separate series of experiments, islets were exposed for 1 day to MAMOAc in the presence or absence of nicotinamide (5, 10 or 20 mM) or hemin (10 or 100 μM). Hemin was dissolved in DMSO, 0.13 mM.

Islets from five human cadaveric donors were isolated at the Central Unit of the β-Cell Transplant (Medical Campus, Vrije Universiteit Brussel, Brussels, Belgium). The mean age of the donors (±SEM) was 36 ± 8 years (range of 15–58 years). Aliquots of the islet-enriched fraction were examined routinely by electron microscopy, which indicated less than 5% dead cells or exocrine cells in all preparations. The prevalence of insulin- and glucagon-positive cells, as evaluated by light microscopical examination of immunocytochemically stained islets, was 54 ± 6% and 11 ± 2%, respectively. The isolation and culture conditions were as previously described [28]. The islets were subsequently sent by air to Uppsala, where they were cultured in medium RPMI 1640 containing 10% FCS and 5.6 mM glucose. After 5–6 days in culture, islets were treated for 24 hr with control media or media supplemented with different concentrations of MAMOAc and then used for functional studies.

Insulin release, nitrite determination, glucose oxidation and light microscopy. For the insulin-release determinations, triplicate groups of 10 islets each were transferred to sealed glass vials containing 0.25 mL Krebs–Ringer bicarbonate buffer [29] supplemented with 2 mg/mL BSA and 10 mM HEPES (KRBH). During the first hour of incubation, KRBH was supplemented with 1.7 mM glucose. The medium was then gently removed and replaced by KRBH containing 16.7 mM glucose and the incubation continued for an additional hour. The insulin concentration in the incubation medium was measured by radioimmunoassay [30]. After the insulin-release experiments, the islets were pooled and ultrasonically disrupted in 0.2 mL redistilled water. An aliquot of the homogenate was mixed with acidified ethanol and the insulin extracted overnight at 4°. A separate aliquot was taken from the homogenate for determination of DNA content by fluorophotometry [31, 32]. NO production was measured as nitrite accumulation. In aqueous solution, NO reacts with O_2 and accumulates in the culture medium mostly as nitrite. For nitrite determinations, triplicate aliquots of the culture medium (100 μL) were deproteinized, and nitrite measured with the Griess reaction, as previously described [33].

For the determination of D-[U-^{14}C]glucose oxidation, triplicate groups of 10 islets were transferred to glass vials containing 100 μL KRBH and non-radioactive glucose (final concentration 16.7 mM). Glucose oxidation was subsequently measured in islets as described in detail elsewhere [34]. In these and the above described experiments,

Table 1. Effects of 6 days exposure of mouse pancreatic islets to cycasin or BMAA on islet insulin and DNA content or glucose-stimulated insulin release

Agents (mM)	DNA content (ng/10 islets)	Insulin content (ng/10 islets)	Insulin release (ng/10 islets × 1 hr)		Increase ratio (16.7/1.7)
			1.7 mM	16.7 mM	
Control	272 ± 26	892 ± 37	5.9 ± 1.3	31.0 ± 6.0	5.5 ± 1.0
BMAA 1.0	298 ± 46	948 ± 53	6.0 ± 1.3	23.7 ± 2.9	4.9 ± 0.8
Cycasin 0.1	262 ± 29	971 ± 80	7.3 ± 1.5*	14.0 ± 1.2*	2.3 ± 0.3*
Cycasin 1.0	222 ± 35	882 ± 98	8.5 ± 1.0	35.9 ± 10.7	3.5 ± 0.8*

For insulin release experiments islets were incubated in groups of 10 in KRBH buffer containing 1.7 mM glucose at 37°. After 1 hr, the medium was removed and the islets incubated for another 1 hr in medium containing 16.7 mM glucose. The increase ratio was calculated by dividing the insulin release observed at 16.7 mM glucose by that observed at 1.7 mM glucose in each individual experiment. Values are means ± SEM of six separate experiments, each performed in triplicate. *$P < 0.05$, **$P < 0.01$, respectively, for a chance difference versus control islets; Wilcoxon signed rank test.

the mean of the triplicate observations was considered as one observation.

For light microscopy studies, islets were isolated as previously described, treated with MAMOAc for 24 hr, washed in Hank's balanced salt solution, and fixed in formalin. Tissue was embedded in paraffin, and 7-μm-thick sections were cut and stained with hematoxylin-eosin. To demonstrate insulin-, glucagon- and somatostatin-containing cells, sections were immunostained and observed by light microscopy as previously described [2, 3].

ISB for determination of O^6*-mdGuo.* For determination of O^6-mdGuo, groups of 800–1000 mouse islets were exposed for 3 days (culture conditions as above) to one of the following treatments: (1) control; (2) O^6-benzylguanine, 5 μM (a specific inhibitor of the DNA repair protein alkylguanine alkyltransferase; gift from Dr R. Moschel, NCI-Frederick Cancer Research Facility); (3) MAMOAc, 0.1 mM; (4) MAMOAc, 0.1 mM + O^6-benzylguanine, 5 μM; and (5) SZ, 1.2 mM (used as a positive control; exposure time 24 hr). After exposure to the different treatments, genomic DNA from islets was isolated and purified by homogenizing the tissue in TRI REAGENT™ according to the manufacturer's protocols (Molecular Research Center Inc., Cincinnati, OH, U.S.A.). The pellet was dissolved in water, treated with DNase-free RNase (1 μg/mL) for 1 hr at 37°, the DNA pelleted with ethanol, and the purity of the DNA determined from the 260/280 ratio (range 1.6–1.9).

Purified genomic DNA from mouse islet cells was assayed by ISB for DNA adducts using a monoclonal antibody to O^6-mdGuo (EM-21; a kind gift from Dr M. Rajewsky, University of Essen, Germany) according to the methods of Nehls *et al.* [35]. Briefly, calf thymus DNA was alkylated for 1 hr with 1.0 mM NMU and both non-alkylated and alkylated DNA diluted with water to a final concentration of 30 μg/mL. Alkylated DNA (0–2.7 μg) was further diluted with control calf thymus DNA (to develop a standard curve) and both control and diluted alkylated DNA (final concentration 9.0 μg) loaded onto a nitrocellulose membrane. Membranes were baked at 80° for 2 hr, incubated with blocking solution (0.5% casein, 0.1% deoxycholate in PBS, pH 7.4) at room temperature and incubated overnight with primary antibody (0.2 μg/mL EM-21) in blocking solution at 4°. Specific binding of EM-21 was visualized using an HRP conjugated goat anti-mouse antibody (1 : 1000) and enhanced chemiluminescence (ECL™, Amersham, U.K.) according to the manufacturer's protocols. Membranes were washed four times with buffer, blotted dry, wrapped in plastic wrap and exposed to X-ray film for 15 sec/min. Autoradiographic films were scanned by densitometry (ScanAnalysis™, BioSoft) [36] and the O^6-mdGuo levels in samples determined from a standard curve ($r = 0.97$) of alkylated DNA analysed by HPLC as previously described [37]. Values are expressed as pmol O^6-mdGuo/μg DNA.

Statistical analysis. Data were computed as the mean ± SEM and compared using Wilcoxon signed rank test or Student's *t*-test, as indicated. When multiple comparisons were performed, the data were analysed by analysis of variance (ANOVA).

RESULTS

In initial studies, no significant change in islet cell function was observed in islets treated for 24 hr with 1.0 mM BMAA or cycasin (data not shown). Islets similarly treated with 1.0 mM BMAA for 6 days also failed to affect islet cell function, as indicated by well-preserved islet DNA and insulin content, insulin release in response to glucose (Table 1) and normal glucose oxidation (see below). In contrast, treatment of islets for 6 days with 0.1 or 1.0 mM cycasin impaired the β-cell response to glucose, as judged by a decreased insulin increase ratio (Table 1). This was associated with increased basal insulin release and, at least for 0.1 mM cycasin, an absolute decrease in insulin release at 16.7 mM glucose. The suppressive effects of long-term exposure to cycasin were not paralleled by a decrease in glucose metabolism. The glucose oxidation rate (pmol/10 islets × 90 min; mean ± SEM, N = 8 in all experiments) by control

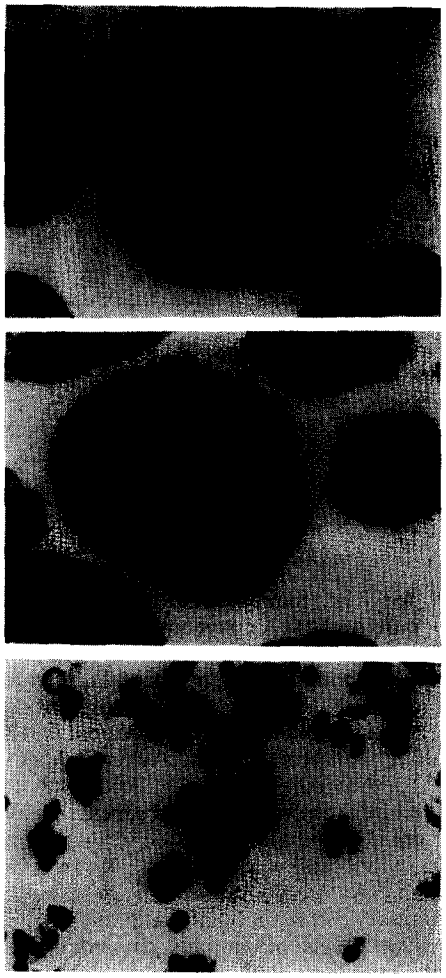

Fig. 1. Mouse pancreatic islets were fixed after a 24 hr treatment with control media (A), or media supplemented with 0.1 mM MAMOAc (B) or 1.0 mM MAMOAc (C). In panels (A) and (B), the islet structure and the majority of the islet cells appear well preserved. Occasionally there was mild degranulation of MAMOAc-treated islets, but the vast majority of the islets contained well granulated insulin-containing cells. In islets treated with 1.0 mM MAMOAc (C), most islets are disintegrating and several cells have pyknotic nuclei. Sections were immunostained for insulin and counterstained with haematoxylin and eosin. Original magnification ×600.

islets was 288 ± 17, and for islets exposed to BMAA 1.0 mM, cycasin 0.1 mM and cycasin 1.0 mM was 237 ± 53, 272 ± 29 and 266 ± 54, respectively. In three separate experiments where islets were treated for 6 days with cycasin (0.1 or 1.0 mM) and then maintained in culture for an additional 4 days without the toxin, there was a complete recovery of insulin release in response to glucose. Thus, insulin release at 16.7 mM glucose (ng/10 islets × 1 hr; mean ± SEM) was 17.7 ± 3.6 in control islets, and 33.8 ± 9.2 and 16.1 ± 3.0 for islets previously exposed to 0.1 or 1.0 mM cycasin, respectively.

In a subsequent series of experiments, islets were exposed for 24 hr to the aglycone form of cycasin, MAM/MAMOAc. High concentrations of MAM or MAMOAc (1.0 mM) induced widespread islet cell death, as judged by: (a) the disintegration of islet morphology and presence of nuclear pyknosis in several islet cells (Fig. 1); (b) the marked decrease in DNA and insulin content; and (c) values of glucose oxidation approaching zero (Table 2). Lower concentrations (0.1 mM) of MAM or MAMOAc induced a less marked effect; while there were no obvious islet morphological changes, except for a mild degranulation in the insulin-positive cells (Fig. 1), there was a 20–30% decrease in DNA and insulin content and a 50–60% inhibition of glucose metabolism (Table 1). Well-granulated glucagon- and somatostatin-containing cells were located mainly in the periphery of control or MAMOAc (0.1 mM)-treated islets (data not shown), suggestive of a preserved islet architecture in both groups. In islets treated with 1.0 mM MAMOAc, glucagon- and somatostatin-positive cells were apparently also affected, some of them showing nuclear pyknosis and loss of cell contact with the other islet cells (data not shown).

The severe islet damage induced by 1.0 mM MAM or MAMOAc precluded the evaluation of glucose-induced insulin release in these islets. Indeed, under these conditions there is passive insulin leakage from dying β-cells, leading to very high basal (1.7 mM glucose) insulin "release" and no further increase in response to 16.7 mM glucose. This passive loss of insulin was probably also present to a minor extent in islets treated with 0.1 mM MAM or MAMOAc, as judged by the significant increase in basal insulin release and diminished insulin increase ratio in response to 16.7 mM glucose (Table 3). A significant decrease in the absolute insulin release at 16.7 mM glucose and medium insulin accumulation were present in islets treated with 0.1 mM MAMOAc, but not in those exposed to MAM.

To determine if MAM/MAMOAc-induced β-cell damage was reversible, islets were treated for 24 hr with the test agent (same groups as presented in Tables 2 and 3) and maintained in culture for an additional 4 days without MAM or MAMOAc. Note that in four experiments there was extensive islet damage, precluding the retrieval of enough islets to perform the 4 day follow-up. After the 4 day period, there was no significant decrease in DNA content in islets previously exposed to MAM or MAMOAc (Table 4). Since adult β-cell proliferation is minimal under *in vitro* conditions [38], this apparent recovery of islet DNA content is probably due to experimental variations and to a potential bias induced by excluding the four experiments where islet damage was most severe (see above). Medium insulin accumulation returned to values similar to those observed in control islets (Table 4), but there was still a significantly small decrease in islet insulin content in cells previously treated with MAMOAc.

Following the 4 day culture period without toxin, basal insulin release returned to control values (Table 5). However, there was still a 30% decrease in absolute insulin release at 16.7 mM glucose and, at least for islets exposed to MAM, a significant decrease in insulin increase ratio in response to

Table 2. Effects of 24 hr exposure of mouse pancreatic islets to MAM or MAMOAc on islet insulin and DNA content and glucose oxidation

Agents (mM)	DNA content (ng/10 islets)	Insulin content (ng/10 islets)	Glucose oxidation (pmol/10 islets × 90 min)
Control	327 ± 24 (12)	765 ± 64 (12)	414 ± 29 (8)
MAM 0.1	235 ± 30 (12)*	621 ± 93 (12)**	168 ± 41 (8)**
MAM 1.0	158 ± 6 (3)**	235 ± 42 (3)*	2 ± 1 (3)**
MAMOAc 0.1	243 ± 34 (12)*	549 ± 115 (12)*	187 ± 38 (8)**
MAMOAc 1.0	171 ± 11 (3)***	179 ± 36 (3)*	4 ± 2 (3)**

Rates of glucose oxidation were measured in groups of 10 islets for 90 min in KRBH buffer (without albumin) supplemented with D-[U-^{14}C]glucose and 16.7 mM nonradioactive glucose. The results are means ± SEM of the number of experiments given in parentheses. *$P < 0.05$, **$P < 0.01$, respectively, for a chance difference vs control islets; Wilcoxon signed rank test or paired *t*-test (experiments with 1.0 mM MAM or MAMOAc).

Table 3. Effects of 24 hr exposure of mouse pancreatic islets to MAM or MAMOAc on glucose-stimulated insulin release and insulin accumulation into the culture medium

	Insulin release (ng/10 islets × 1 hr)			
Agents (mM)	1.7 mM	16.7 mM	Increase ratio (16.7/1.7)	Medium insulin (μg/100 islets × 24 hr)
Control	8.5 ± 1.5	52.6 ± 11.5	6.3 ± 0.8	3.7 ± 0.6
MAM 0.1	20.8 ± 7.1*	50.9 ± 13.2	3.3 ± 0.4***	2.7 ± 0.3
MAMOAc 0.1	21.1 ± 7.2**	45.4 ± 10.1*	3.6 ± 0.5**	2.4 ± 0.2*

For insulin release experiments islets were sequentially exposed to 1.7 and 16.7 mM glucose, as described in Table 1. The results are means ± SEM of 12 separate experiments, each performed in triplicate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, respectively, for a chance difference versus control islets; Wilcoxon signed rank test.

Table 4. Islet insulin and DNA content and insulin accumulation into the culture medium of mouse pancreatic islets exposed for 24 hr to MAM or MAMAc and then cultured for 4 additional days without the toxins

Agents (mM)	DNA content (ng/10 islets)	Insulin content (ng/10 islets)	Medium insulin (μg/100 islets × 24 hr)
Cont—Cont	334 ± 33	1193 ± 115	1.9 ± 0.4
MAM 0.1—Cont	260 ± 47	988 ± 128	1.8 ± 0.3
MAMOAc 0.1—Cont	312 ± 44	880 ± 177*	2.1 ± 0.2

Mouse pancreatic islets were precultured in the presence of MAM or MAMOAc for 24 hr, and then maintained in culture for an additional 4-days without the toxin. Control (Cont—Cont) islets were cultured for the whole 5-day period without MAM or MAMOAc. Medium insulin accumulation was determined during the last 24 hr of culture. The results are means ± SEM of seven to eight separate experiments. *$P < 0.05$ for a chance difference versus control islets; Wilcoxon signed rank test.

glucose (Table 5). Taken together, the data presented in Tables 4 and 5 suggest that there is an improvement, albeit not a total recovery, in the function of mouse islets treated for 24 hr with MAM/MAMOAc and then cultured for an additional 4 days without toxin.

In order to investigate the mechanisms involved in MAM-induced β-cell damage, we measured nitrite accumulation in culture medium (RPMI + 10% FCS) incubated with MAMOAc. After 24 hr, there was no detectable nitrite in the control medium, but in medium containing 0.1 mM MAMOAc there was 1.64 ± 0.04 nmol nitrite/5 mL (N = 4) and at 1.0 mM MAMOAc there was 16.8 ± 0.7 nmol nitrite/5 mL (N = 4). In a second series of experiments, we tested if hemin, a NO scavenger [39, 40], could protect mouse islets against MAMOAc. In these series measurement of glucose-induced insulin release was

Table 5. Insulin release of mouse pancreatic islets exposed for 24 hr to MAM or MAMOAc and then cultured for 4 additional days without the toxin

Agents (mM)	Insulin release (ng/10 islets × 1 hr) 1.7 mM	16.7 mM	Increase ratio (16.7/1.7)
Cont—Cont	3.3 ± 1.1	37.1 ± 7.2	17.4 ± 4.1
MAM 0.1—Cont	3.5 ± 1.2	24.5 ± 7.3*	10.8 ± 3.0*
MAMOAc 0.1—Cont	2.6 ± 0.8	28.8 ± 8.3	17.3 ± 4.4

Mouse pancreatic islets were precultured in the presence of MAM or MAMOAc for 24 hr, and then maintained in culture for an additional 4-days without the toxin. Control (Cont) islets were cultured for the whole period without MAM or MAMOAc. At the end of this 5-day period, the sequential insulin release in response to 1.7 and 16.7 mM glucose was performed as described in Table 1. The results are means ± SEM of seven to eight separate experiments, each performed in triplicate. *$P < 0.05$ for a chance difference versus control islets; Wilcoxon signed rank test.

hampered by leakage of insulin from islets damaged by MAMOAc. Thus, islet glucose oxidation was used as a parameter of islet function. The rate of glucose metabolism (Table 6) was decreased by 30% in the presence of MAMOAc plus DMSO (the solvent used to dilute hemin and also included in other experimental groups), an effect counteracted by hemin (10 or 100 μM). Interestingly, DMSO alone induced partial but significant protection against MAMOAc, as indicated by a higher glucose oxidation rate in islets exposed to MAMOAc + DMSO compared to islets exposed to MAMOAc ($P < 0.001$; Table 6). To test if the protective effect of DMSO was related to NO scavenging, mouse pancreatic islets were exposed for 24 hr to SNP (0.2 mM), a chemical NO donor, in the absence or presence of DMSO (0.13 mM or 0.26 mM). Glucose oxidation ratio by control islets was 560 ± 33 pmol/10 islets × 90 min (mean ± SEM; N = 8 in all experimental groups), and this ratio was not modified by DMSO alone (data not shown). In the presence of SNP, glucose oxidation was reduced to 156 ± 23 pmol/10 islets × 90 min ($P < 0.001$ versus control; ANOVA), and this effect was partially counteracted by 0.13 mM DMSO (glucose oxidation: 216 ± 13 pmol/10 islets × 90 min; $P < 0.05$ versus SNP and $P < 0.001$ versus control) or 0.26 mM DMSO (glucose oxidation: 242 ± 16 pmol/10 islets × 90 min; $P < 0.01$ versus SNP and $P < 0.001$ versus control). Taken together, these results suggest that DMSO has protective effects against NO, and that DMSO and the NO scavenger hemin protect mouse islets against MAMOAc.

Table 6. Islet glucose oxidation rate at 16.7 mM glucose of mouse pancreatic islets cultured for 24 hr in the presence of MAMOAc (0.2 mM) and/or hemin (10 or 100 μM) or DMSO (0.13 mM)

Agents	Glucose oxidation (pmol/10 islets × 90 min)
Control	580 ± 36 (10)
MAMOAc	444 ± 38 (10)*
Hem 10	525 ± 53 (12)
Hem 10 + MAMOAc	525 ± 43 (12)**
Hem 100	603 ± 30 (12)
Hem 100 + MAMOAc	568 ± 43 (12)**
Control without DMSO	528 ± 37 (8)
MAMOAc without DMSO	96 ± 30 (8)†‡

Rates of glucose oxidation were measured in triplicate groups of 10 islets for 90 min in KRBH buffer (without albumin) supplemented with D-[U-^{14}C]glucose and 16.7 mM nonradioactive glucose. Except when indicated, all groups were exposed to 0.13 mM DMSO, used as a vehicle to dissolve hemin. Results are means ± SEM of the number of experiments indicated in parenthesis. *$P < 0.05$ versus Control; **$P < 0.05$ versus MAMOAc, †$P < 0.001$ versus Control without DMSO; ‡$P < 0.001$ versus MAMOAc (ANOVA).

Another potential mechanism of MAMOAc-induced β-cell damage is by direct DNA alkylation. To investigate this possibility, mouse pancreatic islets were exposed for 3 days to 0.1 mM MAMOAc in the absence or presence of 5 μM O^6-benzylguanine, an inhibitor of the DNA repair protein alkylguanine alkyltransferase [41]. Genomic DNA isolated from these islets was analysed by ISB using a monoclonal antibody to O^6-mdGuo (Fig. 2) to determine the extent of DNA damage. A significant amount of DNA damage was detected in islet cells treated with MAMOAc alone or in combination with BG (~0.9 or 0.6 pmol O^6-mdGuo/μg DNA, respectively). Moreover, MAMOAc-induced DNA damage in β-islets was approximately three times higher than islets treated for 24 hr with 1.2 mM SZ.

A subsequent series of experiments addressed the putative protective effects of nicotinamide against MAMOAc-induced inhibition of glucose metabolism. Since the higher concentration of nicotinamide induced a trend for decreased glucose oxidation (Table 7), the data are presented both in absolute values and as a percentage of the respective controls. The rate of glucose metabolism was reduced by 65% when islets were treated with MAMOAc (0.2 mM) (Table 7). All three concentrations of nicotinamide tested (5, 10 or 20 mM) prevented this decrease in glucose metabolism ($P < 0.01$ versus controls).

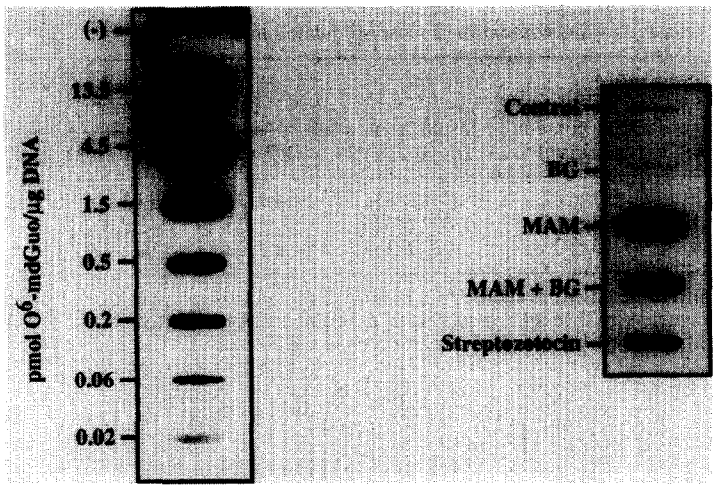

Fig. 2. Murine β-islet cell cultures were treated for 3 days with control media or media supplemented with 5.0 μM O^6-benzylguanine (BG) (a DNA repair inhibitor), 0.1 mM MAMOAc, 0.1 mM MAMOAc + 5.0 μM BG or 1.2 mM streptozotocin (24 hr) and analysed by immuno-slot-blot for DNA damage. Genomic DNA from control (2.8 μg) and treated (1.3–3.2 μg) islets was blotted onto a nitrocellulose membrane (*right blot*) and incubated with a monoclonal antibody (mAb) to O^6-mdGuo (EM-21, provided by Dr M. Rajewsky). Constant amounts of control (−) or NMU-alkylated calf thymus DNA were similarly blotted on the same membrane (*left blot*) and used to determine the amount of O^6-mdGuo in DNA isolated from islets. Bound mAb was visualized using an HRP conjugated goat anti-mouse antibody (1:1000) and enhanced chemiluminescence (ECL™, Amersham) according to the manufacturer's protocols. Autoradiograms were evaluated by densitometry.

Table 7. Islet glucose oxidation rate at 16.7 mM glucose of mouse pancreatic islets cultured for 24 hr in the presence of MAMOAc and/or nicotinamide (Nic)

Agents (mM)	Glucose oxidation (pmol/10 islets × 90 min)	Glucose oxidation % of the control
Control	455 ± 23	—
Nic 5	423 ± 66	—
Nic 10	419 ± 21	—
Nic 20	337 ± 22	—
MAMOAc 0.2	164 ± 27*	37 ± 6
MAMOAc 0.2 + Nic 5	344 ± 52	87 ± 10**
MAMOAc 0.2 + Nic 10	348 ± 56	85 ± 14**
MAMOAc 0.2 + Nic 20	336 ± 53	103 ± 16**

Rates of glucose oxidation were measured in triplicate groups of 10 islets for 90 min in KRBH buffer (without albumin) supplemented with D-[U-^{14}C]glucose and 16.7 mM nonradioactive glucose. The % of the control values was determined in each individual experiment by expressing glucose oxidation of islets exposed to MAMOAc or MAMOAc + Nic as % of the respective controls (i.e. Control, Nic 5, Nic 10 or Nic 20). The results are means ± SEM of seven separate experiments. *$P < 0.01$ for a chance difference versus control islets (ANOVA); **$P < 0.01$ for a chance difference versus islets exposed to MAMOAc (unpaired *t*-test).

Moreover, all groups treated with MAMOAc in the presence of nicotinamide exhibited higher glucose oxidation rates ($P < 0.05$) than in islets exposed to MAMOAc alone.

In a final series of experiments, we determined the effect of MAMOAc on human pancreatic islet cell function. Note that since it is very difficult to obtain human islet preparations, we could only perform a limited number of experiments. Control human islet insulin and DNA content, and insulin release in response to glucose (Table 8), were comparable to values obtained in mouse pancreatic islets (Tables 1–5). The lower concentration of MAMOAc (0.1 mM) did not affect human islet function, but 1.0 mM MAMOAc induced a significant decrease both in islet insulin content and insulin

Table 8. Effects of 24 hr exposure of human pancreatic islets to MAMOAc on islet insulin and DNA content or glucose-stimulated insulin release

Agents (mM)	DNA content (ng/10 islets)	Insulin content (ng/10 islets)	Insulin release (ng/10 islets × 1 hr) 1.7 mM	16.7 mM	Increase ratio (16.7/1.7)
Control	237 ± 42	789 ± 335	4.4 ± 1.5	39.3 ± 12.0	11.7 ± 4.2
MAMOAc 0.1	236 ± 55	859 ± 376	5.9 ± 2.0	43.3 ± 16.9	12.1 ± 5.9
MAMOAc 1.0	183 ± 38	439 ± 167*	4.2 ± 1.6	11.5 ± 3.1***	3.6 ± 1.2**

Sequential insulin release in response to 1.7 and 16.7 mM glucose was performed as described in Table 1. Values are means ± SEM of five separate experiments, each performed in triplicate. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, respectively, for a chance difference versus control islets; paired t-test.

release (Table 8). However, there was no significant decrease in islet DNA content (Table 8) or evidence of islet cell damage under the stereomicroscope.

DISCUSSION

The present data suggest that cycasin, and its aglycone MAM/MAMOAc, impair mouse β-cell function and may lead to islet cell destruction. The same is not true for the excitotoxin BMAA. The cycad toxins cycasin and BMAA are considered to be potential etiological candidates for western Pacific ALS/PD [13, 16]. Chamorros with PD from Guam and Rota have an unusually high prevalence of glucose intolerance and diabetes mellitus, in the range of 50–80% [19, 20]. Increased prevalence of glucose intolerance has also been reported in other neuromuscular disorders [42, 43], but the observations are conflicting [44], and in these studies the number of patients with overt diabetes mellitus was much lower than that observed in PD patients from Guam and Rota. In light of the present observations, it is conceivable that at least one of the toxins present in the cycad plant, i.e. cycasin and its derivative MAM, may contribute to the observed *in vivo* human β-cell dysfunction. In support, extensive islet cell damage was observed in pancreatic tissue obtained from primates chronically intoxicated (up to 18 years) with various regimens of cycad meal, cycasin and/or MAMOAc (Dr U. Thorgeirsson, personal communication).

The observation that MAMOAc impairs the function of human pancreatic islets is of special relevance. Indeed, to our knowledge, this is the first demonstration that an environmental toxin with a potential role for inducing diabetes mellitus in a large population is toxic to human β-cells under controlled *in vitro* conditions. Interestingly, human islets are less sensitive than mouse islets to the toxic effects of MAMOAc (present observations). Thus, while 0.1 mM MAMOAc was clearly toxic to mouse islets, human islets were only affected by the highest concentration of MAMOAc (1.0 mM). The same pattern has been observed with another alkylating agent and NO generator, the diabetogenic drug SZ. Thus, while SZ induces diabetes in rodents, it fails to do so in humans [45]. Moreover, there are recent data suggesting that while mouse and rat islet function is inhibited by 1–2 mM SZ, higher concentrations (12 mM) are needed to inhibit human islet function [46]. Similar observations were made with combinations of cytokines [47], alloxan or sodium nitroprusside [46], suggesting that human islets present a pattern of susceptibility and/or resistance to noxious agents different from that observed in rodent islets. It remains to be determined if this is related to the activation of more efficient and/or different cell-repair mechanisms [48].

It has been proposed that cycasin enters brain tissue as the intact glucoside, perhaps via a glucose transporter [16]. CNS tissue contains β-glucosidases capable of hydrolyzing cycasin to the toxic aglycone MAM [16]. In fact, β-glucosidase activity in rodent brain tissue is approximately 500 times higher than in other organs (e.g. liver, heart, kidney) [26]. Pancreatic β-cells also possess an active glucose transport system. Moreover, β-glucosidase activity in cultured murine islets is similar to that observed in brain tissue [49]. Thus, it may be that the toxic agent cycasin gains access to β-cells by a mechanism similar to that described for the CNS. Note that the isoform and cellular compartmentalization of β-glucosidase on pancreatic β-cells remains to be characterized. Thus, the observations on the toxic effects of MAM on pancreatic islets (see below) should be extrapolated with caution to the potential toxic effects of cycasin on these cells. Once in contact with the β-cell, our present data suggest that MAM may induce cell toxicity either by releasing NO or by alkylating DNA. In line with this hypothesis, we observed that hemin, a NO scavenger [39, 40], protects β-cells against MAMOAc. Moreover, nicotinamide, an agent previously shown to protect rodent pancreatic islets both against NO donors [50] and alkylating agents [17, 45, 51], also prevented MAMOAc-induced islet dysfunction. This effect of nicotinamide, especially at concentrations below 10 mM, is probably related to inhibition of the enzyme poly(ADP-ribose)polymerase, which is activated following NO exposure [52] or DNA alkylation [45, 51] and the subsequent formation of DNA strand breaks. Excessive activation of the enzyme may lead to a critical depletion in cellular NAD/NADH, contributing to cell death [17, 51, 52]. If indeed NO generation is responsible for part of cycasin's deleterious effects to neurons and β-cells

in vivo, then the present data are one of the first indications that environmental toxins may act via NO production. It is noteworthy that high concentrations of MAM also induced cell damage to glucagon- and somatostatin-positive cells, arguing against a specific effect of the toxin on insulin-producing cells. However, considering that cycasin probably gains access to the β-cells via glucose transport (see above), it is conceivable that β-cells will accumulate more cycasin and MAM than the other islet endocrine cells.

MAM alkylates nucleic acids with the formation of N^7-methylguanine and O^6-methylguanine adducts [26, 53, 54; present data]. O^6-Methylguanine damage to DNA is proposed to induce tumourigenesis in highly proliferative tissue, but it is unclear how this DNA lesion affects cells that are post-mitotic (neurons) or have a low capacity for proliferation (β-cells) [13]. It is conceivable that nucleic acid alkylation may represent an irreversible epigenetic change, leading to defective protein expression and eventual cell degeneration [13]. Indeed, pancreatic islets exposed *in vitro* to SZ present a long-lasting impairment in the expression of nuclear-encoded [2] and mitochondrial-encoded genes [5, 6]. Against this, the present data suggest that β-cells may partially recover their function after short-term treatment with MAMOAc. Whether reversibility would be demonstrable after repeated exposure for a prolonged period is unknown. This is relevant to the Chamorro lifestyle in which cycasin-containing foodstuffs would have been consumed over many years, each tortilla containing approximately 1.25 mg of cycasin (estimated minimum daily intake 2.5 mg/70 kg person) [14]. Conceivably, this type of low-level exposure to cycasin coupled with short-term additional exposure to raw cycad seed (used as a poultice for open wounds) would lead to repeated sub-clinical damage to the genome of central nervous system and β-cells. It is noteworthy that in inherited genetic diseases affecting the mitochondrial DNA, neurological dysfunction and diabetes mellitus are common clinical outcomes, even though all tissues have the genetic defect [55, 56]. This suggests that these two tissues may share a common vulnerability to some forms of genetic damage, deletions and/or mutations.

The form of diabetes described in ALS/PDC patients resembles NIDDM: it is late in onset and mild in nature, with few patients presenting ketonuria or acidosis [16]. If we accept the concept that repeated toxic insult may be related to the etiology of hyperglycemia in these patients (see above), it is puzzling why they do not present a more severe form of diabetes, similar to IDDM. Indeed, when some strains of mice are exposed to repeated sub-diabetogenic doses of SZ, they develop insulitis and a severe IDDM-like syndrome [51]. However, this only occurs in the presence of a particular genetic background [51], and most mouse strains will not present the autoimmune component of this experimental model of IDDM. The prevalence of IDDM in Asia and the Pacific region is much lower than that observed in the Western countries [57], and the form of diabetes reported in these areas is of late onset and mostly resembles NIDDM [58]. In this context, it is likely that the genetic background of the Chamorro population does not predispose them to develop IDDM. Thus, it may be speculated that repeated exposure to the alkylating agent cycasin/MAM will not trigger an autoimmune assault against the β-cell, but will lead to a progressive decrease in the ability of β-cells to produce and release insulin. This, plus other contributing factors which increase insulin resistance (obesity, lack of exercise, etc.), may eventually lead to hyperglycaemia. Considering that prolonged exposure of human pancreatic islets to high glucose levels impairs β-cell function [28], this would lead to a vicious circle culminating in overt diabetes mellitus.

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