Pancreatic islets of bank vole show signs of dysfunction after prolonged exposure to high glucose concentrations *in vitro*

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Abstract

Bank voles develop glucose intolerance/diabetes mellitus when kept in captivity. We have characterized β-cell function of glucose intolerant/diabetic animals, and found that this animal model has features of both human type 1 and type 2 diabetes. The aim of this study was to study the functional alterations of islets isolated from glucose tolerant bank voles after a prolonged exposure to various glucose concentrations *in vitro*. For this purpose, pancreatic islets from normal (glucose tolerant) male and female bank voles were cultured at different glucose concentrations (5·6, 11·1 (control), or 28 mM) whereupon islet functions were examined. Overall, islet insulin output was lowered at 5·6 mM glucose, and similar to control, or enhanced after culture in 28 mM glucose. High glucose culture led to decreased insulin contents, but there was no change in islet DNA content and in morphological assessments of cell death, with the latter findings suggesting that the so-called glucotoxicity had not evolved. A slight gender difference was observed in that islets isolated from females exhibited a glucose-regulated (pro)insulin biosynthesis rate and insulin gene expression. In conclusion, we have found that islets isolated from female and male bank voles are affected by glucose concentrations *in vitro* in that some signs of dysfunction were observed upon high glucose exposure. A minor gender difference was observed suggesting that the islets of the females may more readily adapt to the elevated glucose concentration than islets of the male bank voles. It could be that these *in vitro* gender differences observed may represent a mechanism underlying the gender difference in diabetes development observed among bank voles.

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Introduction

It has been postulated that an increased glucose load *in vivo* on islet β-cells may participate in disease progression in type 1 and type 2 diabetes (Weir *et al.* 1986, 2009, Rossetti *et al.* 1990, Wilkin 2001, Chang-Chen *et al.* 2008, Ljungkrantz *et al.* 2008). In type 2 diabetes, an increased glucose concentration damages the β-cell mass in the pancreatic islets, for instance by generation of noxious free oxygen radicals which can affect insulin production (Robertson *et al.* 2003). During the disease progression in type 1 diabetes, the β-cell mass in the pancreatic islets is reduced by immune cell-mediated mechanisms (Hitchcock *et al.* 1988, Bach 1995, Yoon & Jun 2005, Knip & Siljander 2008). An increased functional demand on isolated islets *in vitro* has shown that hyperglycemic-like conditions can alter islet function and be detrimental for the β-cell, but depending on the species of origin, this effect varies (Eizirik *et al.* 1988, 1991, 1992*a*, Sandler *et al.* 1989, Gross *et al.* 1996).

Bank voles develop glucose intolerance/diabetes mellitus both in the wild state and when kept in captivity, and male bank voles seem to a larger extent be affected compared with the females (Schoenecker *et al.* 2000, Niklasson *et al.* 2003*a*,*b*, Blixt *et al.* 2007). We have recently characterized β-cell function of pancreatic islets isolated from normal and glucose intolerant/diabetic bank voles, and found that this animal model has features of both human type 1 and type 2 diabetes (Blixt *et al.* 2007). Moreover, a novel picornavirus (Ljungan virus) was isolated from bank voles (Niklasson *et al.* 1999) and was later found in the pancreas of wild bank voles (Niklasson *et al.* 2003*a*). However, its possible role in the development of diabetes in the bank voles remains to be proven. It has been shown in CD-1 mice that when the mother is inoculated with the Ljungan virus during gestation the offspring develops diabetes (Niklasson *et al.* 2006).

The aim of this study was to study the functional alterations of islets isolated from glucose tolerant bank voles after exposure to a prolonged exposure to various glucose concentrations *in vitro*. Glucose is the main carbohydrate regulator of β-cell activity, and therefore it is of particular interest to investigate the effects of glucose in a novel animal model of diabetes. Herein we report that islets of bank vole display signs of dysfunction after high glucose culture, and that there are slight gender differences suggesting that the islets from male bank voles may have a reduced ability to meet an increased functional demand.

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Materials and Methods

Animals

Bank voles (*Myodes glareolus*) were housed at Astrid Fagreus Laboratory, Karolinska Institute, Stockholm. Animals had free access to water and standard laboratory chew (LABFOR R3, Lactamin, Kimstad, Sweden) with an energy content of 3·01 kcal/g, and were occasionally given pieces of vegetables. The nesting material in the cages was exchanged 2–3 times/week by experienced animal technicians. The experimental procedures were approved by the animals' ethical committee in Stockholm (N248/03, N276/06) and in accordance with international guidelines (NIH publications no. 85-23, revised 1985).

The mean age of the bank voles used was 14·5±1·5 weeks ($n=46$; range 4–40 weeks) for females, and 17·4±1·1 weeks ($n=59$; range 4–42 weeks) for males. All animals were tested with an i.p. glucose tolerance test, where 2 g glucose/kg body weight was injected. Blood samples were taken from the retro-orbital sinus immediately prior to injection (0 min), and at 60 and 120 min post injection. The samples were analyzed with an automated glucose meter (Accu-Chek Aviva; Roche Diagnostics). The animals were anaesthetized with Isoflurane (Abbott Inc.) prior to blood sampling and prior to killing by cervical dislocation. Bank voles with a blood glucose concentration below 11·1 mM at 120 min post injection were classified as normoglycemic.

The pancreas of normoglycemic bank voles was resected and placed in Hanks' balanced salt solution (SBL Vaccine, Stockholm, Sweden) supplemented with 50 U/ml benzylpenicillin and 0·05 mg/ml streptomycin (Sigma–Aldrich).

Islet isolation and pre-culture

Pancreatic islets were isolated by collagenase digestion as described elsewhere (Blixt *et al.* 2007). Islets from individual bank voles were maintained free-floating in separate culture dishes in culture medium RPMI 1640 with 11·1 mM glucose supplemented with 10% v/v FCS, 2 mM glutamine and 50 U/ml benzylpenicillin, and 0·05 mg/ml streptomycin at 37 °C, in humidified air +5% CO_2 (AGA, Stockholm, Sweden) for 5 days. Culture media were exchanged every second day.

Experimental culturing conditions

Islets from two bank voles of the same gender and similar body weight were pooled and distributed into three new culture dishes with 50 islets of equal size in each, and were further cultured for 5 days in culture media supplemented with glucose to a final concentration of 5·6, 11·1, or 28 mM before examination. The culture media were exchanged on days 1 and 3. In separate experiments, islets were cultured in medium containing 11·1 mM D-glucose +16·9 mM L-glucose to explore possible osmotic effects of a high glucose concentration.

Medium insulin and proinsulin accumulation, islet insulin release, and insulin content

Media from the last 48 h of culture were collected for determination of medium insulin and proinsulin accumulation. The insulin release was tested in triplicate groups of ten islets incubated at 1·7 mM glucose in Krebs–Ringer bicarbonate buffer (KRBH; Krebs & Henseleit 1932) supplemented with 2 mg/ml BSA at 37 °C (O_2/CO_2; 95/5%) for 1 h. Subsequently, the buffer was replaced with KRBH supplemented with 16·7 mM and 2 mg/ml BSA followed by incubation for another 1 h. Islet insulin content was measured on islets pooled from the incubation above after disruption in water and extraction overnight at 4 °C using 70% v/v ethanol supplemented with 0·13 mM HCl. The insulin concentrations in the culture media, the incubation buffers, and the insulin extractions were measured with either a rat insulin high range ELISA kit or a rat insulin ELISA kit (Mercodia, Uppsala, Sweden). The proinsulin concentration in the culture media was measured with a rat Proinsulin ELISA kit (Mercodia). No commercial antibodies specific for bank vole insulin or proinsulin are available. This means that absolute values measured herein may be different to actual levels of bank vole insulin or proinsulin.

Islet total protein and (pro)insulin biosynthesis and glucose oxidation rates

The islet total protein and (pro)insulin biosynthesis and glucose oxidation rate were measured on groups of ten islets in duplicates incubated in KRBH containing 16·7 mM glucose and ^{3}H-labeled leucine (protein and (pro)insulin biosynthesis), or D-[U-^{14}C]glucose (glucose oxidation rate), and further analysis was performed as described previously (Blixt *et al.* 2007).

RNA isolation, cDNA synthesis, and real-time PCR

RNA was isolated from groups of 50 islets with RNeasy Micro kit (Qiagen) complemented with DNase (Qiagen) and eluted in water. The RNA amount and purity were tested using the Nanodrop ND-1000 system (NanoDrop Technologies, Wilmington, DE, USA). Synthesis of cDNA was performed with Reverse Transcription System (Promega) using 1:2 volume of total RNA and Oligo(dT)15 primer (Promega). The reaction mixtures were incubated at 42 °C in 60 min followed by 99 °C in 5 min.

The LightCycler Instrument (Roche) combined with sequence-independent detection with SYBR Green I was used to amplify and analyze generated cDNA. The sequence of the primers used were: insulin (mouse) forward 5′-CCATCAGCAAGCAGGTTAT-3′ and reverse 5′-GGGTGTGTAGAAGAAGCCA-3′, and β-actin (mouse) forward

5′-CCACCGATCCACACAGAGTACTTG-3′ and reverse 5′-GCTCTGGCTCCTAGCACC-3′ (TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany) PCR amplification of 0·1 μg cDNA sample was performed with 0·2 μM insulin primer and 0·5 μM β-actin primer in SYBR Green JumpStart ready mixture (Roche) and 3·5 mmol/l $MgCl_2$. After an initial 95 °C in 30 s, the following temperatures were cycled 40 times; 94 °C in 5 s, 47 °C in 10 s and 72 °C in 15 s. Cycle threshold (C_t) values were obtained for individual samples with the second derivative maximum method (Gibson *et al.* 1996, Heid *et al.* 1996). The relative mRNA expression was calculated from the formula $2^{-(\Delta C_t)}$.

DNA quantification

DNA content was measured with fluorometric assay using PicoGreen (Molecular Probes, Eugene, CA, USA) labeling system according to the instructions of the manufacturer. The DNA content was measured in water homogenates in conjunction to determinations of the islet insulin content, and total protein and (pro)insulin biosynthesis rates.

Islet viability

Viability of bank vole islet cells was determined using Hoechst 33342 and propidium iodide. In each test, at least ten islets were incubated with 5 μg/ml bisbenzimide and 20 μg/ml propidium iodide for 20 min. After washing in PBS, fluorescence was observed at 461 and 615 nm respectively after u.v. excitation. The islets were photographed in a fluorescence microscope, and the nuclear morphology was later assessed and cell numbers were counted by the observer being unaware of the origin of the specimens.

Statistical analysis

A mean was calculated from each duplicate or triplicate groups of islets, and then considered as one separate observation. Values are expressed as means ± S.E.M., and groups of data were compared using Student's paired *t*-tests. Observed differences were considered statistically significant when $P \leq 0{\cdot}05$. Statistical analysis was performed using SigmaStat (SPSS Inc., Chicago, IL, USA).

Results

The non-fasting blood glucose concentration of the glucose tolerant/normal bank voles used was 6·5 ± 0·3 mM ($n = 59$) for males, and 7·2 ± 0·6 mM ($n = 46$) for females. All islets were precultured for 5 days in RPMI 1640 (11·1 mM glucose), and then the islets were subsequently cultured in media containing 5·6, 11·1, or 28 mM glucose for another 5 days. We have chosen to consider the islets cultured in medium with 11·1 mM glucose as the control group, since this is the regular glucose concentration of the RPMI 1640 medium. Moreover, previous studies from our laboratory have suggested that 11·1 mM glucose may be an optimal glucose concentration for maintenance of insulin production of isolated rodent islets in RPMI 1640 medium (Andersson 1978, Svensson *et al.* 1993).

Medium insulin and proinsulin accumulation during culture

Insulin and proinsulin accumulation in the culture medium was measured in samples obtained from the last 48 h of culture. In comparison with the control islets, the insulin accumulation was reduced in islets isolated from male and female bank voles cultured in 5·6 mM glucose (Table 1). On the other hand, at 28 mM glucose there was an increase in insulin accumulation from islets of both genders compared with the control group.

Proinsulin levels in the media were elevated after culture of islets from males and females in 28 mM glucose, whereas media collected from islets cultured in 5·6 mM glucose displayed a decline. The proinsulin fraction, expressed as percentage of total medium insulin accumulation, was

Table 1 Medium insulin and proinsulin accumulation after culture of bank vole islets at 5·6, 11·1 and 28 mM glucose. The islets were cultured for 5 days and medium was exchanged after 1 and 3 days. Insulin and proinsulin medium accumulation was measured during the last 48 h of culture. Values are means ± S.E.M. for (*n*) observations

	Male islets			**Female islets**		
	5·6 mM glucose	11·1 mM glucose	28 mM glucose	5·6 mM glucose	11·1 mM glucose	28 mM glucose
Culture						
Medium insulin accumulation (ng/10 islets × 24 h)	370 ± 190 (11)*	1220 ± 170 (11)	1580 ± 190 (11)**	220 ± 97 (7)**	1070 ± 150 (7)	1300 ± 170 (7)*
Medium proinsulin accumulation (ng/10 islets × 24 h)	0·5 ± 0·2 (11)†	4·4 ± 0·5 (11)	14·5 ± 2·2 (11)†	0·2 ± 0·03 (7)†	4·0 ± 0·6 (7)	11·5 ± 1·9 (7)**
Proinsulin fraction (% of medium insulin)	0·3 ± 0·1 (11)	0·4 ± 0·04 (11)	1·0 ± 0·1 (11)†	0·1 ± 0·02 (7)**	0·4 ± 0·1 (7)	0·9 ± 0·1 (7)†

Data were compared using Students' paired *t*-test where * denote $P \leq 0{\cdot}05$, ** denote $P \leq 0{\cdot}01$ and † denote $P \leq 0{\cdot}001$ versus islets cultured at 11·1 mM glucose of the same gender.

Table 2 Glucose stimulated insulin secretion, DNA and insulin contents after culture of bank vole islets for 5 days at 5·6, 11·1 and 28 mM glucose. Values are means±S.E.M. for (*n*) observations

	Male islets			Female islets		
	5·6 mM glucose	11·1 mM glucose	28 mM glucose	5·6 mM glucose	11·1 mM glucose	28 mM glucose
Culture						
Insulin release (1·7 mM glucose) (ng/10 islets×60 min)	4·1±1·0 (11)‡	18·5±3·3 (11)	39·5±5·1 (11)‡	1·9±0·1 (7)†	7·9±1·1 (7)	20·4±3·6 (7)†
Insulin release (16·7 M glucose) (ng/10 islet×60 min)	16·9±2·3 (11)‡	45·1±5·1 (11)	33·1±3·1 (11)*	19·2±3·3 (7)‡	35·0±3·4 (7)	26·3±3·3 (7)*
Stimulation index (16·7/1·7)	6·0±1·2 (11)*	3·0±0·5 (11)	0·9±0·1 (11)‡	10·7±1·8 (7)*	4·8±0·7 (7)	1·4±0·1 (7)†
Islet insulin content (ng/10 islets)	216±76 (11)	187±48 (11)	50±16 (11)*	372±129 (6)	182±52 (6)	69±16 (7)
DNA content (ng/islet)	10·8±3·6 (11)	11·9±2·1 (11)	8·0±1·9 (11)	14·7±2·9 (5)	11·2±2·4 (6)	10·5±1·9 (7)
Insulin content (ng insulin/ng DNA)	2·7±1·2 (11)	1·8±0·5 (11)	0·7±0·2 (11)	2·8±0·6 (5)	2·1±0·6 (6)	0·8±0·2 (7)

Data were compared using Students' paired *t*-test where * denotes $P \leq 0{\cdot}05$, † denote $P \leq 0{\cdot}01$ and ‡ denote $P \leq 0{\cdot}001$ versus islets cultured at 11·1 mM glucose of the same gender.

increased in media from both male and female islets cultured in 28 mM glucose, and for the females a decrease was observed at 5·6 mM glucose.

Glucose-stimulated insulin secretion

Islets isolated from both male and female bank voles maintained in 5·6 mM glucose showed a reduced basal insulin secretion at low glucose conditions (1·7 mM glucose), but when islets were cultured in 28 mM glucose, the islet basal insulin secretion was markedly elevated compared with control islets (Table 2). Furthermore, the glucose-stimulated insulin release (16·7 mM glucose) was reduced in islets of males and females after culture in 5·6 mM and after culture in 28 mM glucose compared with islets cultured in 11·1 mM glucose. The stimulation index, the fraction between the insulin release upon a glucose challenge and the basal insulin secretion, was increased in islets isolated from males and females cultured in 5·6 mM glucose compared with control islets, whereas it was lowered for the islets cultured in 28 mM glucose.

Insulin and DNA content

The islet insulin content was measured after the insulin release assay (Table 2). Islets from female and male bank voles were not affected when cultured in 5·6 mM glucose compared with the corresponding controls. However, the insulin content of islets from both genders was markedly reduced after culture in the high 28 mM glucose for male islets, but for female islets this decline did not attain statistical significance ($P=0{\cdot}064$) compared with islets cultured in 11·1 mM glucose. The islet DNA content was not affected by the different glucose concentrations in any of the groups of islets. When the islet insulin content was normalized according to the DNA content, the islet insulin content was still reduced after culture in 28 mM, but the differences did not attain statistical significance (males $P=0{\cdot}052$ and females $P=0{\cdot}067$).

Islet (pro)insulin and total protein biosynthesis rates and insulin mRNA levels

The islet (pro)insulin and total protein biosynthesis rates were measured during a 2-h incubation at 16·7 mM glucose, and normalized according to the DNA content in each observation. Islets isolated from male bank voles displayed no differences in (pro)insulin or total protein biosynthesis rate between any of the groups (Table 3). On the other hand, islets isolated from female bank voles and maintained in 5·6 mM glucose had a reduced islet (pro)insulin biosynthesis rate compared with islets cultured in 11·1 mM glucose. Concerning total protein biosynthesis rates, islets from females cultured in 5·6 mM glucose exhibited a reduction, but at 28 mM glucose, they had an elevated total protein biosynthesis rate compared with the control islets.

Table 3 (Pro)insulin biosynthesis rates and insulin gene expression after culture of bank vole islets for 5 days in culture at 5·6, 11·1 and 28 mM glucose. Values are means±S.E.M. for (*n*) observations

	Male islets			Female islets		
	5·6 mM glucose	11·1 mM glucose	28 mM glucose	5·6 mM glucose	11·1 mM glucose	28 mM glucose
Culture						
(Pro)insulin biosynthesis (dpm/ng DNA×2 h)	149±10 (6)	126±36 (5)	135±14 (5)	279±29 (8)‡	561±58 (8)	732±126 (8)
Total protein biosynthesis (dpm/ng DNA×2 h)	1241±189 (6)	1024±73 (5)	1353±292 (5)	1950±176 (8)*	2406±112 (8)	3618±408 (8)*
(Pro)insulin/total protein×100 (%)	12·9±1·3 (6)	12·0±2·7 (5)	11·2±1·5 (5)	14·6±1·4 (8)‡	23·2±1·9 (8)	20·1±1·6 (8)
Insulin gene (mRNA ($2^{-(\Delta C_t)}$))	90±19 (6)	129±29 (6)	135±32 (6)	37±10 (7)*	84±15 (7)	90±25 (7)

Data are compared using Student's paired *t*-test where * denotes $P \leq 0{\cdot}05$, and ‡ denote $P \leq 0{\cdot}001$ versus islets cultured at 11·1 mM glucose of the same gender.

The (pro)insulin biosynthesis, expressed as percentage of total protein biosynthesis rate, was reduced in islets isolated from female bank voles cultured in 5·6 mM glucose compared with control islets, while no difference in this measurement was found in islets isolated from male animals. The insulin gene expression was not affected by the different glucose concentrations concerning male islets, but for female islets a reduction was observed for islets maintained in 5·6 mM glucose.

Islet glucose oxidation rate

Islet glucose oxidation rates were measured during 90-min incubation at 16·7 mM glucose at the end of the culture period. It was found that the glucose oxidation rates were reduced after culture in 5·6 mM glucose for both genders; however, no change could be observed in islets cultured in 28 mM glucose compared with controls (Fig. 1).

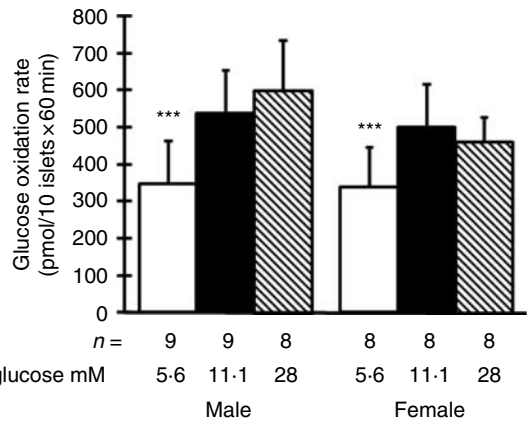

Figure 1 Glucose oxidation rates of male and female bank vole islets cultured in 16·7 mM glucose after culture in 11·1 mM glucose for 5 days and subsequently cultured in 5·6, 11·1, or 28 mM glucose for 5 days. Values are means±S.E.M. for (*n*) observations as shown below the bars. Data were compared using Student's paired *t*-test where *** $P \geq 0{\cdot}001$ versus those obtained from islets cultured in 11·1 mM glucose of the same gender.

Test of osmotic effects

In a separate set of experiments ($n=4$), possible osmotic effects of the increased glucose concentration in the culture medium (28 mM) were tested on islets obtained from both females and males. Isolated islets were cultured in media containing 11·1 mM D-glucose or 11·1 mM D-glucose +16·9 mM L-glucose. The results did not reveal any differences between the groups when tested for the insulin release (ng/10 islets×60 min) at 1·7 mM glucose (11·1 mM D-glucose, 28·8±3·9; and 11·1 mM D-glucose +16·9 mM L-glucose, 26·0±2·2) at 16·7 mM glucose (11·1 mM D-glucose, 53·6±9·0; and 11·1 mM D-glucose +16·9 mM L-glucose, 52·3±5·3), the insulin content (ng/10 islets) (11·1 mM D-glucose, 251±33; and 11·1 mM D-glucose +16·9 mM L-glucose, 235±26), or the medium insulin accumulation (ng/10 islets×24 h) (11·1 mM D-glucose: 975±82; and 11·1 mM D-glucose +16·9 mM L-glucose, 984±20) or the medium proinsulin accumulation (ng/10 islets×24 h) (11·1 mM D-glucose, 2·6±0·4; and 11·1 mM D-glucose +16·9 mM L-glucose, 2·4±0·5).

Islet viability

In some experiments, islets were collected for assessment of islet cell viability following staining with Hoechst 33342 and propidium iodide. For female islets, ($n=6$) the percentage of non-viable islet cells were 4·7±1·0, 6·3±1·2, and 8·0 ±3·1% at 5·6 mM glucose, 11·1 mM glucose, and 28 mM glucose respectively. For male islets ($n=8$), the percentage of non-viable islet cells were 6·3±1·0, 5·3±1·1, and 3·6 ±0·4% at 5·6 mM glucose, 11·1 mM glucose, and 28 mM

glucose respectively. No statistical difference was observed in the extent of islet cell death when female or male islets were compared with those cultured in 11·1 mM glucose, using Student's paired *t*-test.

Discussion

In the present study, we have shown that the function of bank vole islets was markedly affected by long-term exposure to a high glucose concentration *in vitro*. The functional changes observed share features of both rodent and human islets maintained at similar culture conditions (Eizirik *et al.* 1988, 1992*a*). We maintained islets isolated from male and female bank voles, classified as glucose tolerant, for a long-term culture period *in vitro*. Different glucose concentrations were used in order to assess if glucose affects β-cell function. In comparison to the control group (11·1 mM glucose), glucose-induced changes were observed in islets of both male and female origin, and we could also observe some differences in islet response between genders.

At the lower glucose concentration (5·6 mM), islets isolated from males and females displayed a down-regulation of both basal and glucose-stimulated insulin release, whereas at high glucose (28 mM), a markedly elevated basal insulin secretion was observed while the glucose-stimulated insulin release was moderately reduced compared with controls. The stimulation index value revealed that the relation between basal and glucose-stimulated insulin release was changed depending on the different glucose concentrations. Thus, the islets maintained in 28 mM glucose seem to have an impaired ability to respond to the high glucose stimulation.

When comparing these findings with previous studies using the RPMI 1640 medium for culture of islets from other species, the insulin release behavior of bank vole islets much resemble normal mouse pancreatic islets of various strains (Eizirik *et al.* 1988, Svensson *et al.* 1993). Another interesting rodent in this context is the sand rat (*Psammomys obesus*), which can develop a type 2 diabetes-like condition in the laboratory when given certain diets (Gadot *et al.* 1994, 1995). Moreover, in a study where islet cells from normal *P. obesus* were explanted *in vitro* and cultured as monolayer, it was found that an elevated glucose concentration (33·3 mM) for 10 days caused a pronounced reduction in glucose-induced insulin secretion (Gross *et al.* 1996). Culture of normal rat islets at high glucose, depending on the selection of culture media, may either cause a decrease in glucose-stimulated insulin secretion the so-called desensitization (Bolaffi *et al.* 1988) or have no obvious harmful effect (Sandler *et al.* 1989). In comparison to human islets, the bank vole islets display a similar response in stimulation index and in insulin content with increasing glucose concentration in the culture medium (Eizirik *et al.* 1992*b*). Interestingly, we recently found that bank vole islets responded to proinflammatory cytokines *in vitro* according to a pattern more resembling human islets than that seen in rodent islets (Blixt *et al.* 2009).

The reduced insulin content in bank vole islets cultured in 28 mM glucose suggests that the islets were not able to comply with the increased functional demand but rendered them to be depleted of insulin. Furthermore, the increased proinsulin amount in the culture medium supplemented with 28 mM glucose may suggest that the islet insulin production capacity was exceeded. This would be in line with observations in *P. obesus* (Gadot *et al.* 1995) and in a model of glucose infusion in rats (Alarcon *et al.* 1995). We also observed that in 11·1 mM glucose the insulin accumulated in the medium was similar to that in 28 mM glucose, and this may suggest that the islets maintained at these culture conditions were facing a too great functional demand on the β-cells. These findings suggest that the optimal culture condition for bank vole islets is not the standard 11·1 mM glucose, but is closer to 5·6 mM glucose level. This is similar to that of human islets for which the optimal culture condition using RPMI 1640 medium is lower than 11·1 mM glucose (Eizirik *et al.* 1992*b*). Based on these findings, we hypothesize that the bank vole islet resembles the human islet more than previously characterized rodent islets.

The islet total protein and (pro)insulin biosynthesis rates showed glucose-induced changes when islets of females were studied, whereas islets from males did not exhibit any glucose-dependent alterations in these functions. We interpret this possible gender difference that the female islets are more prone, than male islets, to adapt their (pro)insulin biosynthesis rates and may also be total protein biosynthesis rates to the ambient glucose concentration. Besides for the translational level, this adaption might also be regulated on the transcriptional level since the insulin mRNA expression also displayed glucose dependence in female islets. The islet glucose oxidation rates were not currently affected after culture in high glucose which suggests that an altered glucose metabolism did not mediate glucose-induced impairments in islet function. Furthermore, osmotic effects due to high glucose concentrations are unlikely to have influenced the islet functions investigated.

The Ljungan virus is difficult to isolate in tissue culture. Virus isolation is therefore not used as a diagnostic method. The humoral response is weak. Persistently infected individuals show no or a very weak antibody response, while individuals that clear the infection have detectable antibodies. Serology can therefore not be used to diagnose past or present infection in an individual. Ljungan virus infection often results in a chronic persistent or long-lasting infection. RT-PCR can be used to detect viral RNA in persistently infected animals (Donoso Mantke *et al.* 2007). PCR positive animals are often found several months after the acute infection. However, the RNA copy number at this time is often low. We have also found several examples where animals have been PCR negative and are still proven infectious. This means that the diagnostic arsenal presently available only allow us to state that our bank vole colony is Ljungan virus infected. We cannot determine with accuracy if an individual with disease is Ljungan virus infected or not, and we cannot determine what proportion of the animals in the colony carry the virus.

Thus, to what extent each individual animal used herein is infected cannot be determined. If so, how this might have influenced our present findings and if a gender difference in viral susceptiblity has contributed to our current findings is unclear.

Previously we have shown that bank voles developing glucose intolerance/diabetes display features that are to some extent different between genders (Blixt *et al*. 2007). Herein we have found that islet isolated from female and male bank voles are affected by glucose concentration *in vitro* in that some signs of dysfunction were observed upon high glucose exposure, although this did not cause direct toxicity and cell death. A minor gender difference was observed suggesting that the islets of the females may more readily adapt to the elevated glucose concentration than islets of the male bank voles. It could be that these *in vitro* gender differences observed may represent a mechanism underlying the difference in diabetes development observed among bank voles (Blixt *et al*. 2007).

Declaration of interest

BN is CEO and an owner of Apodemus AB, which holds several patents relating to the Ljungan virus. MB and SS declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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