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ORIGINAL ARTICLE

Effects of the atypical antipsychotic clozapine on insulin release *in vitro*

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Abstract

OBJECTIVES: Treatment with the atypical antipsychotic clozapine is frequently associated with metabolic side-effects such as weight gain, lipid abnormalities and diabetes mellitus. Since insulin is a hormone that is involved in both the regulation of body weight, as well as in lipid metabolism and glucose regulation, an effect of clozapine on insulin secretion and/or on insulin action – at least in part – might explain its capability to induce these side-effects. The aim of this study was therefore to examine the influence of clozapine on insulin release *in vitro*.

METHODS: The effect of clozapine in three different concentrations, 10^{-6}, 10^{-5} and 10^{-4} M, was investigated on both basal (i.e. 3.3 mM glucose) and glucose-stimulated (i.e. 16.7 mM glucose) insulin release, using isolated rat islets of Langerhans.

RESULTS: The presence of clozapine in the concentrations of 10^{-6}, 10^{-5} and 10^{-4} M significantly increased basal insulin release compared to the control after 4 h (but not after 1 h) of incubation. As regards the glucose-stimulated insulin release, the presence of clozapine in the concentrations of 10^{-5} and 10^{-4} M, but not in that of 10^{-6} M, significantly inhibited the glucose-stimulated insulin release compared to the control after both 1 and 4 h of incubation.

CONCLUSION: This study demonstrates that the atypical antipsychotic clozapine exerts dual effects on insulin release *in vitro*, through stimulating basal insulin release and inhibiting glucose-stimulated insulin release. Both these effects of clozapine on insulin release may contribute to its disadvantage inducing metabolic side-effects.

INTRODUCTION

The atypical antipsychotic clozapine has been shown to have an antipsychotic effect that is as good as, or even better than, that of classical or other atypical antipsychotics, and is often used in the treatment of psychosis patients who are unresponsive to classical or other atypical agents (Fitton & Heel, 1990; Wagstaff & Bryson, 1995; Chakos *et al*., 2001; McEvoy *et al*., 2006). However, metabolic side-effects such as excessive weight gain, lipid abnormalities and diabetes mellitus have increasingly been recognized with the use of clozapine (Allison *et al*., 1999; Henderson *et al*., 2000; Melkersson & Dahl, 2004), and so far, the mechanisms behind these side-effects are poorly understood.

Interestingly, results from clinical studies show that clozapine treatment is associated with increased insulin levels and insulin resistance (Yazici *et al.*, 1998; Melkersson *et al.*, 1999; Melkersson & Dahl, 2003), whereas insulin levels appear not to be appreciably affected by classical agents like haloperidol, perphenazine and zuclopenthixol (Brambilla *et al.*, 1975; Melkersson *et al.*, 1999). Since insulin is a hormone that is involved in both the regulation of body weight, and in lipid metabolism and glucose regulation (Olefsky, 1997; Woods *et al.*, 1997), an influence of clozapine on insulin secretion and/ or on insulin action at least in part might explain this agent´s capability to induce weight gain, lipid abnormalities and diabetes mellitus. Therefore, it would be worthwhile studying the effect of clozapine also on insulin release *in vitro*.

Previous *in vitro* studies investigating the effect of antipsychotic drugs on insulin release are summarized in Table 1. Taken together, these studies have demonstrated an inhibitory effect of chlorpromazine, pimozide and trifluoperazine at concentrations from 5×10^{-6} to 10^{-4} M on glucose-stimulated insulin release, whereas haloperidol and some other classical agents have shown no consistent effect on glucose-stimulated insulin release, and other atypical agents have not been fully tested (Table 1). Regarding basal insulin release, a stimulatory effect both of clozapine and olanzapine has been demonstrated when tested in a concentration of 10^{-6} M, and of trifluoperazine in concentrations from 10^{-5} to 10^{-4} M, but not of other classical or atypical agents (Table 1). However, the data extant on the effect of antipsychotics on insulin release *in vitro* are still limited, not least for clozapine.

In this light, this study was undertaken to further examine the influence of the atypical agent clozapine on insulin release *in vitro*. We investigated the effect of clozapine in three different concentrations on both basal and glucose-stimulated insulin release from isolated rat pancreatic islets of Langerhans.

MATERIAL AND METHODS

Isolation and culture of rat pancreatic islets

The study was performed in accordance with guidelines from the Swedish National Board for Laboratory Animals, and approved by the Ethical Committee on Experimental Animal Care. Islets were isolated from pancreata retrieved from 3 month old male Wistar rats weighing 270–350 g (B&K Universal, Sollentuna, Sweden) by a previously described method (Sutton *et al.*, 1986) with minor modifications. In brief, rats were killed by decapitation. A catheter was then inserted into the common bile duct near to the hilus of the animals´ liver, and 7 mL collagenase solution (collagenase type A; 0.9 mg/mL in Hank´s balanced salt solution [HBSS]) was injected into the pancreatic duct system in a retrograde way. Thereafter, each inflated pancreas was removed, cleaned of the fat tissue, and incubated in 3 mL collagenase solution at 37 °C for 24 min. Following incubation, the pancreata were cut into small pieces (3–4 mm in size), gently syringed several times through a 14 FG needle, washed 3 times in HBSS, and passed through a strainer of approximately 500 µm pore size. Islets were then enriched by histopaque gradient centrifugation (800×g; 20 min) and hand picked. After isolation, the islets were cultured overnight at 37 °C in RPMI 1640 supplemented with fetal calf serum (10%), glucose (11 mM), glutamine (2 mM), penicillin (100 IU/mL) and streptomycin (100 µg/mL).

Measurement of insulin release

For measurement of insulin release, static incubations were used. Islets were preincubated for 30 min at 37 °C in Krebs-Ringer Bicarbonate (KRB) buffer (pH 7.4) containing in mM: 115 NaCl, 4.7 KCl, 2.56 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 20 $NaHCO_3$ and 10 HEPES, supplemented with 2 mg/mL bovine serum albumin and 3.3 mM glucose. Batches of three islets were then transferred to tubes (in triplicate), containing 300 µL of KRB buffer with 3.3 or 16.7 mM glucose and the respective clozapine concentration or no clozapine, and incubated for either 1 or 4 h at 37 °C during mild agitation. The incubation was stopped by chilling the samples in ice-water. After centrifugation (200×g; 1 min), the supernatants were collected and stored at –20 °C until assay for insulin.

Clozapine

Pure clozapine substance (Novartis Pharma, Switzerland) was tested in the concentrations of 10^{-6}, 10^{-5} and 10^{-4} M and compared to controls without clozapine. All three clozapine concentrations were investigated after 1 and 4 h of incubation with the islets, regarding both basal (i.e. 3.3 mM glucose) and glucose-stimulated (i.e. 16.7 mM glucose) insulin release.

Materials

Collagenase A was obtained from Roche Diagnostics (Penzberg, Germany), D(+)Glucose from BDH Laboratories Supplies (Poole, UK), HBSS and RPMI 1640 medium from the National Veterinary Institute (Uppsala, Sweden), and HEPES and histopaque 1119 and 1077 from Sigma (St. Louis, MO, USA). All other chemicals were from either Life Technologies (Paisley, Scotland) or Merck (Darmstadt, Germany).

Insulin radioimmunoassay

Rat insulin was measured by a radioimmunoassay (RIA) method, using antibodies against porcine insulin, and charcoal addition to separate antibody-bound and free insulin (Herbert *et al.*, 1965). ^{125}I-labeled porcine insulin was used as tracer and rat insulin as standard (Novo, Bagvaerd, Denmark). The intra- and inter-assay coefficients of variation were both 2.6%. It was also checked that the dissolved clozapine substance used in the study did not interfere with the insulin RIA method.

Statistical analysis

Data are expressed as median with 25th and 75th percentiles. Insulin concentrations in medium from batches treated with different clozapine concentrations were calculated as % of the median in control batches. As the data were not normally distributed, the Mann-Whitney rank sum test was performed to evaluate the effect of each concentration of clozapine compared to controls. A p-value of less than 0.05 was considered statistically significant. All calculations were made with the statistical program Statistica for Windows (Statsoft, Tulsa, OK, USA).

RESULTS

Insulin release in controls

In the controls, the glucose-stimulated insulin release was significantly higher compared to the basal insulin release both after 1 and 4 h of incubation, medians (25^{th} and 75^{th} percentiles) being 860 (663–1 041) versus 201 (152–292) mU/L ($p<0.001$), and 3 102 (1 368–4 746) versus 239 (189–313) mU/L ($p<0.001$) respectively, confirming that the islets used retained an appropriate insulin-secreting responsiveness to glucose.

Basal insulin release in the presence of clozapine

No difference in effect on basal insulin release was found for any of the three concentrations of clozapine (10^{-6}, 10^{-5} or 10^{-4} M) compared to the control after 1 h of incubation (Table 2; Figure 1A). However, after 4 h of incubation, the presence of clozapine in the concentrations of 10^{-6}, 10^{-5} and 10^{-4} M significantly increased basal insulin release compared to the control (Table 2; Figure 1B).

Glucose-stimulated insulin release in the presence of clozapine

The presence of clozapine in the concentrations of 10^{-5} and 10^{-4} M, but not in that of 10^{-6} M, significantly and concentration-dependently inhibited the glucose-stimulated insulin release compared to the control after both 1 and 4 h of incubation (Table 2; Figure 2A and 2B).

DISCUSSION

Main findings

Two main findings came out of this *in vitro* study. The first finding that clozapine in the concentrations of 10^{-5} and 10^{-4} M, but not in that of 10^{-6} M, decreased the glucose-stimulated insulin release in a concentration-dependent manner, suggests that clozapine, not in lower but in higher concentrations, has a direct inhibitory effect on glucose-stimulated insulin release. This inhibitory effect was seen both after 1 and 4 hours of incubation with the islets, with a more pronounced inhibition after 4 hours. To compare, the result is in line with three of four previous *in vitro* studies extant (Melkersson *et al.*, 2001; Melkersson, 2004; Best *et al.*, 2005), showing no effect of the lower clozapine concentration 10^{-6} M, but an inhibition of the higher clozapine concentration 5×10^{-6} M, on glucose-stimulated insulin release. In contrast, in the fourth study (Johnson *et al.*, 2005), no effect of clozapine in the higher concentration 10^{-5} M on glucose-stimulated insulin release was demonstrated. However, this difference in results is probably explained by different experimental conditions (i.e. 8.0 mM glucose-stimulated insulin release, 0.7 hours of incubation and perifused islets) in that study (Johnson *et al.*, 2005) compared with this study and the other three previous studies (Melkersson *et al.*, 2001; Melkersson, 2004; Best *et al.*, 2005).

The second finding that clozapine in all three concentrations (10^{-6}, 10^{-5} and 10^{-4} M) increased basal insulin release with the most pronounced insulin increase at the highest concentration (10^{-4} M), indicates that clozapine in addition may have a direct stimulatory effect on basal insulin release. This result agrees with earlier studies (Melkersson *et al.*, 2001; Melkersson, 2004), demonstrating increased basal insulin release of clozapine as well as of the structurally-related agent olanzapine in the concentration of 10^{-6} M. Since the insulin release in the present as well as in earlier studies (Melkersson *et al.*,

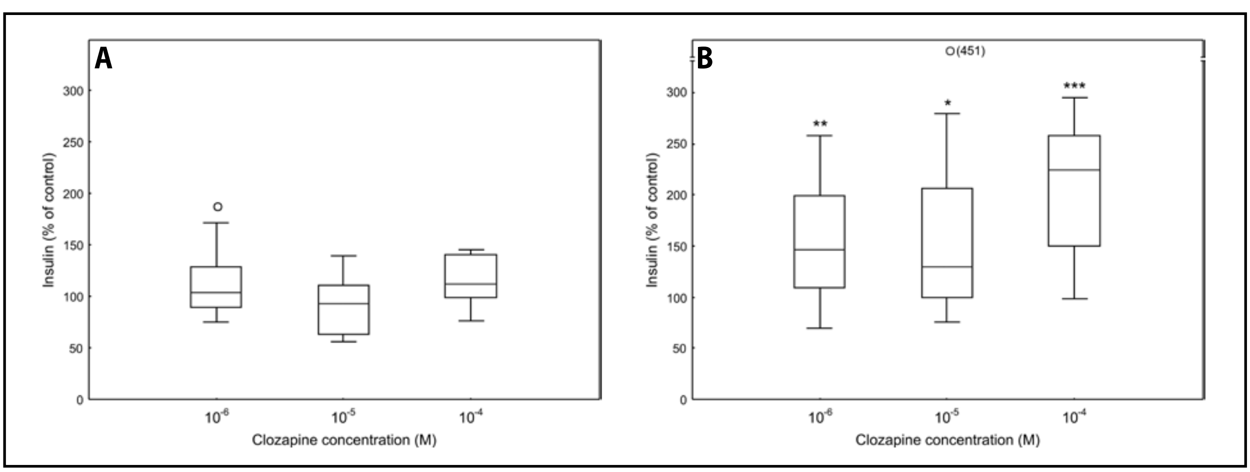

Figure 1. Basal insulin release from isolated rat pancreatic islets after 1 h (**A**) and 4 h (**B**) of incubation and in the presence of clozapine in the concentrations of 10^{-6}, 10^{-5} and 10^{-4} M. The box plots indicate median insulin concentrations in % of control from five experiments with n=3 in each experiment, as well as lower and upper quartiles. The whiskers show 10^{th} and 9^{0th} percentiles and (o) indicates outliers. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to the control.

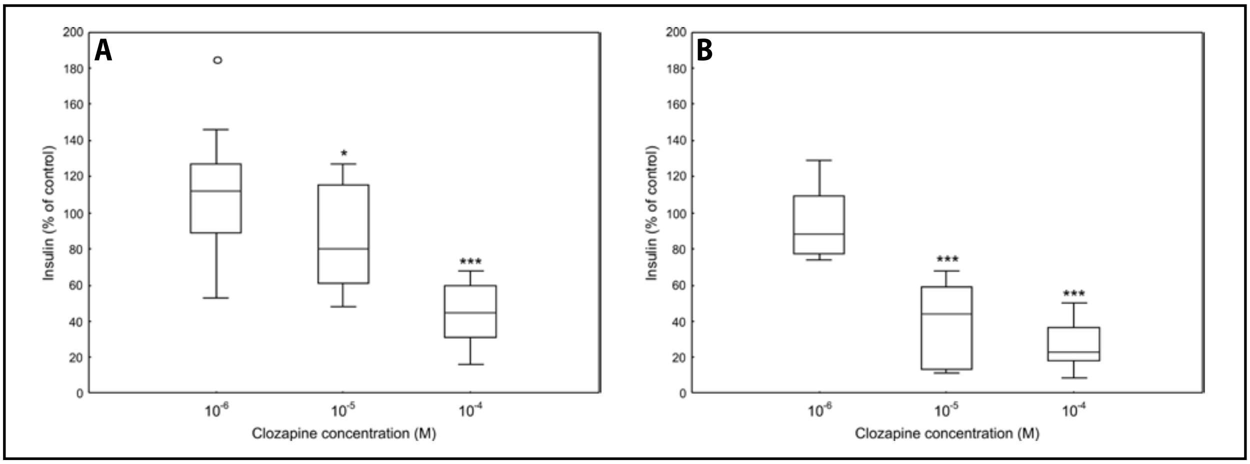

Figure 2. Glucose-stimulated insulin release from isolated rat pancreatic islets after 1 h (**A**) and 4 h (**B**) of incubation and in the presence of clozapine in the concentrations of 10^{-6}, 10^{-5} and 10^{-4} M. The box plots indicate median insulin concentrations in % of control from five experiments with n=3 in each experiment, as well as lower and upper quartiles. The whiskers show 10th and 90th percentiles and (o) indicates outliers. *p<0.05, ***p<0.001 compared to the control.

2001; Melkersson, 2004) occurred after 4 hours and not after 1 hour of incubation, clozapine does not appear to affect the basal insulin release in an acute way.

Taken together, these two findings point to that clozapine exerts a direct effect on the pancreatic beta cells, affecting the insulin release both during basal and glucose-stimulated conditions. So far, this feature of an effect of antipsychotics on insulin release *in vitro* seems to be limited to clozapine and a few other agents, since besides clozapine, it is only trifluoperazine that in earlier studies has been demonstrated to affect insulin release both during basal and glucose-stimulated conditions, and only olanzapine that has been shown to increase basal insulin release (data on the effect of olanzapine in concentrations of 10^{-5}–10^{-4} on glucose-stimulated insulin is yet limited) (Table 1). Also, even though chlorpromazine and pimozide have been shown to have an inhibitory effect on glucose-stimulated insulin release, no effect of these agents on basal insulin release has been demonstrated (Table 1).

Possible mechanisms behind clozapine´s effects on insulin release

Under physiological conditions, the insulin release from pancreatic beta cells is predominantly regulated by glucose, and to a lesser extent by amino acids, hormones and neurotransmitters (McClenaghan & Flatt, 1999; Meier & Butler, 2006). Briefly, glucose is transported into the beta cell by a membrane-bound transporter (GLUT 1 in humans and GLUT 2 in rodents) (Johnson *et al*., 1990; De Vos *et al*., 1995). Intracellular, metabolism of glucose yields ATP, which induces closure of the ATP-sensitive potassium (K_{ATP}) channels in the cell membrane. The resulting membrane depolarization leads to opening of the voltage-dependent calcium channels, resulting in calcium inflow into the beta cell and an increment of intracellular calcium, which in turn triggers exocytosis and release of insulin. In addition, glucose has been proposed to exert part of its stimulatory effects on insulin secretion through K_{ATP} channel-independent actions. Amino acids, hormones and neurotransmitters, on the other hand, have modulating effects on insulin secretion (McClenaghan & Flatt, 1999; Meier & Butler, 2006). Amino acids can elevate the calcium concentration in the beta cell either through metabolism and ATP generation or direct depolarization of the cell membrane, thereby potentiating the insulin secretion, while hormones and neurotransmitters exert stimulatory or inhibitory modulating effects on insulin secretion through activation of specific cell surface receptors (Jones & Persaud, 1998; McClenaghan & Flatt, 1999; MacDonald *et al*., 2002).

Given this complex insulin release machinery, briefly described above, and the fact that clozapine is an antipsychotic drug targeting multiple receptors (Fitton & Heel, 1990; Wagstaff & Bryson, 1995; Remington, 2003), it seems reasonable to assume that clozapine exerts more than one action on the beta cell. If this is the case, it would explain the dual effects of clozapine on insulin release found in this *in vitro* study. Interestingly, clozapine seems to either promote or inhibit insulin release depending on the actual surrounding glucose and/ or insulin concentrations. Regarding possible mechanisms involved in clozapine´s effects on insulin secretion, it has so far been demonstrated that clozapine can suppress cholinergic-stimulated insulin release from rat pancreatic islets by blocking beta cell muscarinic M_3 receptors (Johnson *et al*., 2005). Furthermore, it has been proposed that changes in beta cell K_{ATP} channel activity contribute to clozapine´s inhibitory effect on glucose-stimulated insulin release (Best *et al*., 2005). However, the exact molecular mechanism(s) behind clozapine´s dual effects on insulin release found in this study remain as yet elusive.

Clinical implications of the present results

The ability of clozapine to affect insulin secretion directly from the beta cells may in part explain this agent´s weight-increasing, lipid-elevating and diabetogenic, effects (Henderson *et al*., 2000, 2005). Treatment

Table 1. Studies regarding effects of antipsychotic substances on insulin release *in vitro*, described in chronological order for each substance.

Antipsychotic substance	Tissue studied	Incubation time (h)	Effect on basal insulin release [conc. tested; M] (glucose conc.; mM)	Effect on glucose-stimulated insulin release [conc. tested; M] (glucose conc.; mM)	References
Chlorpromazine	Isolated rat islets	1.5	No [10^{-6}, 5×10^{-6}, 10^{-5}, 10^{-4}] (0)	Inhibition [5×10^{-6}, 10^{-5}, 10^{-4}]; No [10^{-6}] (16.7)	Ammon et al. 1973
	Isolated rat islets	1	Not tested	Inhibition [10^{-5}] (8)	El-Denshary & Montague, 1976
	Isolated rat islets	1	Not tested	Inhibition [not described] (8)	El-Denshary et al. 1977
	Perfused rat pancreas	0.4	No [10^{-5}, 10^{-4}] (0)	Inhibition [10^{-5}, 10^{-4}] (20)	Joost et al. 1979
	Isolated mice islets	1	Not tested	Inhibition [10^{-5}, 10^{-4}] (16.7)	Nakadate et al. 1982
	Isolated rat islets	1 and 4	No [10^{-6}] (3.3)	No [10^{-6}] (16.7)	Melkersson et al. 2001
Pimozide	Perfused rat pancreas	1	No [10^{-6}] (0)	Inhibition [10^{-5}]; No [10^{-6}] (10)	Joost et al. 1983
Trifluoperazine	Isolated rat islets	2	Stimulation [5×10^{-5}]; No [2.5×10^{-5}] (0)	Inhibition [2.5×10^{-5}, 5×10^{-5}] (20)	Sugden et al. 1979
	Isolated human islets	2	Not tested	Inhibition [2.5×10^{-5}] (20)	Grant et al. 1980
	Isolated rat islets	0.3	Not tested	Inhibition [10^{-5} to 10^{-4}] (16.7)	Sussman et al. 1983
	Isolated rat islets	0.2	Stimulation [10^{-5}, 10^{-4}]; No [10^{-6}] (2.5)	No [10^{-6}, 10^{-5}, 10^{-4}] (10)	Yasuda et al. 1989
Haloperidol	Rabbit pancreas system	0.3	Not tested	No [10^{-4}] (16.7)	Feldman, 1972
	Perfused canine pancreas	0.2	No [4×10^{-7}, 2×10^{-6}, 10^{-5}] (1.4)	Inhibition [4×10^{-7}, 2×10^{-6}, 10^{-5}] (8), probably due to ethanol that was used as solvent for haloperidol	Hermansen, 1978 Samols & Stagner, 1980
	Isolated rat islets	1 and 4	No [10^{-6}] (3.3)	No [10^{-6}, 1h]; Inhibition [10^{-6}, 4h] (16.7)	Melkersson et al. 2001
	Isolated rat islets	1 and 4	No [10^{-6}] (3.3)	No [10^{-6}] (16.7)	Melkersson, 2004
	INS-1 cells	1 and 4	No [10^{-6}] (0)	Not tested	Melkersson, 2004
	Isolated rat islets	1	No [5×10^{-6}] (4)	No [5×10^{-6}] (16)	Best et al. 2005
Perphenazine	Isolated rat islets	1 and 4	No [10^{-6}] (3.3)	No [10^{-6}] (16.7)	Melkersson et al. 2001
Zuclopenthixol	Isolated rat islets	1 and 4	No [10^{-6}] (3.3)	No [10^{-6}] (16.7)	Melkersson et al. 2001
	Isolated rat islets	1 and 4	No [10^{-6}] (3.3)	No [10^{-6}, 1h]; Inhibition [10^{-6}, 4 h] (16.7)	Melkersson, 2004
	INS-1 cells	1 and 4	No [10^{-6}] (0)	Not tested	Melkersson, 2004
Clozapine	Isolated rat islets	1 and 4	Stimulation [10^{-6}, 4h]; No [10^{-6}; 1h] (3.3)	No [10^{-6}] (16.7)	Melkersson et al. 2001 Melkersson, 2004
	INS-1 cells	1 and 4	Stimulation [10^{-6}, 4h]; No [10^{-6}; 1h] (0)	Not tested	Melkersson, 2004
	Isolated rat islets	1	No [5×10^{-6}] (4)	Inhibition [5×10^{-6}] (16)	Best et al. 2005
	Perifused rat islets	0.7	Not tested	No [10^{-5}] (8)	Johnson et al. 2005
Olanzapine	Isolated rat islets	1 and 4	No [10^{-6}] (3.3)	No [10^{-6}] (16.7)	Melkersson et al. 2001
	Isolated rat islets	1 and 4	Stimulation [10^{-6}, 4h]; No [10^{-6}; 1h] (3.3)	No [10^{-6}] (16.7)	Melkersson, 2004
	INS-1 cells	1 and 4	Stimulation [10^{-6}; 1 and 4 h] (0)	Not tested	Melkersson, 2004
	Perifused rat islets	0.7	Not tested	No [10^{-5}] (8)	Johnson et al. 2005
Quetiapine	Isolated rat islets	1 and 4	No [10^{-6}] (3.3)	No [10^{-6}] (16.7)	Melkersson & Jansson, 2005
Risperidone	Isolated rat islets	1 and 4	No [10^{-6}] (3.3)	No [10^{-6}] (16.7)	Melkersson & Jansson, 2005
Ziprasidone	Isolated rat islets	1 and 4	No [10^{-6}] (3.3)	No [10^{-6}] (16.7)	Melkersson & Jansson, 2005

Table 2. Insulin release in % of control, described as median (25th and 75th percentiles).[a]

Clozapine concentration (M)	1 h of incubation		4 h of incubation	
	3.3 mM glucose	16.7 mM glucose	3.3 mM glucose	16.7 mM glucose
10^{-6}	105 (89–128)	113 (89–127)	147**(110–199)	88 (77–109)
10^{-5}	94 (63–111)	80*(61–115)	129*(100–207)	44***(13–59)
10^{-4}	112 (99–141)	45***(31–60)	224***(150–258)	23***(18–36)

[a]Results are based on five experiments with n=3 in each experiment
*Significantly different compared to the control, p<0.05
**Significantly different compared to the control, p<0.01
***Significantly different compared to the control, p<0.001

with clozapine will increase the non-glucose-stimulated insulin secretion (Melkersson *et al*., 1999; Melkersson and Dahl, 2003), with increased appetite and weight gain as possible consequences (Woods *et al*., 1997). The diabetogenic and lipid-elevating effects of clozapine, in turn, may be related to both its inhibitory effect on glucose-stimulated insulin secretion, as indicated in this *in vitro* study, and its interference with insulin action, which is supported by others´ *in vitro* data, demonstrating decreased glucose uptake in muscle cells by this agent (Ardizzone *et al*., 2001).

A clozapine concentration of 10^{-6} M, which was the lowest concentration tested in this study, is close to clozapine serum concentrations found in patients on therapeutic doses (Perry *et al*., 1991; Melkersson & Hulting, 2001; Melkersson & Dahl, 2003). Using isolated rat pancreatic islets, we could demonstrate an effect on basal insulin release of all three clozapine concentrations 10^{-6}, 10^{-5} and 10^{-4} M, but an effect on glucose-stimulated insulin release only of the higher clozapine concentrations 10^{-5} and 10^{-4} M. However, it is well documented in animal studies that clozapine accumulates in the brain as well as in other tissues such as liver, spleen and lungs to levels that are up to 50 times higher than the levels in serum (Gardiner *et al*., 1978; Baldessarini *et al*., 1993). Although the pancreas has not been studied in this respect, the clozapine concentration in the pancreatic tissue also might be several times higher than in serum. If this is the case, patients treated with higher doses and/ or having higher serum concentrations of clozapine would seem to be at increased risk of developing diabetes mellitus and hyperlipidemia.

To compare, among classical antipsychotics, it is primarily chlorpromazine together with a few other phenothiazines that in clinical studies have been reported to induce hyperglycemia and diabetes mellitus (Schwarz & Munoz, 1968; Thonnard-Neumann, 1968). These phenothiazines also has been proposed to affect glucose-insulin homeostasis in two ways (Catanese & Kahn, 2001), both by inhibiting glucose-stimulated insulin secretion from pancreatic beta cells (Table 1) and by decreasing insulin sensitivity and glucose uptake in peripheral tissues (Rafaelsen 1961; Jori & Carrara, 1966; Guarner *et al*., 1993).

CONCLUSIONS

In summary, this study demonstrates that the atypical antipsychotic clozapine exerts dual effects on insulin release *in vitro*, through both stimulating basal insulin release and inhibiting glucose-stimulated insulin release. This feature of an effect of antipsychotics on insulin release from pancreatic beta cells seems to be unique for clozapine and a few related agents, and may in part explain its (and the related agents´) disadvantage inducing metabolic side-effects.

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