



The dopamine receptor D2 agonist bromocriptine inhibits glucose-stimulated insulin secretion by direct activation of the α 2-adrenergic receptors in beta cells

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ARTICLE INFO

Article history:

Received 26 November 2009

Accepted 25 January 2010

Keywords:

Bromocriptine

Insulin secretion

Mice

INS-1E cells

Dopamine receptor

α 2-Adrenergic receptor

ABSTRACT

Treatment with the dopamine receptor D2 (DRD2) agonist bromocriptine improves metabolic features in obese patients with type 2 diabetes by a still unknown mechanism. In the present study, we investigated the acute effect of bromocriptine and its underlying mechanism(s) on insulin secretion both *in vivo* and *in vitro*. For this purpose, C57Bl6/J mice were subjected to an intraperitoneal glucose tolerance test (ipGTT) and a hyperglycemic (HG) clamp 60 min after a single injection of bromocriptine or placebo. The effects of bromocriptine on glucose-stimulated insulin secretion (GSIS), cell membrane potential and intracellular cAMP levels were also determined in INS-1E beta cells. We report here that bromocriptine increased glucose levels during ipGTT *in vivo*, an effect associated with a dose-dependent decrease in GSIS. During the HG clamp, bromocriptine reduced both first-phase and second-phase insulin response. This inhibitory effect was also observed in INS-1E beta cells, in which therapeutic concentrations of bromocriptine (0.5–50 nM) decreased GSIS. Mechanistically, neither cellular energy state nor cell membrane depolarization was affected by bromocriptine whereas intracellular cAMP levels were significantly reduced, suggesting involvement of G-protein-coupled receptors. Surprisingly, the DRD2 antagonist domperidone did not counteract the effect of bromocriptine on GSIS, whereas yohimbine, an antagonist of the α 2-adrenergic receptors, completely abolished bromocriptine-induced inhibition of GSIS. In conclusion, acute administration of bromocriptine inhibits GSIS by a DRD2-independent mechanism involving direct activation of the pancreatic α 2-adrenergic receptors. We suggest that treatment with bromocriptine promotes beta cells rest, thereby preventing long-lasting hypersecretion of insulin and subsequent beta cell failure.

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1. Introduction

Type 2 diabetes, which is often associated with obesity and dyslipidemia, is characterized by insulin resistance, glucose

intolerance and a progressive deterioration of beta cell mass and function [1]. The central and peripheral dopaminergic system is involved in the regulation of whole-body fuel and energy homeostasis [2,3]. Besides its role in the control of complex processes driving feeding behavior, dopaminergic neurotransmission affects both glucose and lipid metabolism. Alterations of this central regulatory pathway have been reported in insulin-resistant rodent models [4–6] and patients with type 2 diabetes [7,8]. Dopamine action is mediated by 5 distinct G-protein coupled receptor subtypes belonging to 2 receptor families according to their effect on target neurons. Activation of dopamine receptors D2, D3 or D4, classified as the D2 family, inhibits adenylyl cyclase activity and cyclic AMP (cAMP) synthesis [9]. By contrast, activation of the receptors belonging to the D1 family (DRD1 and DRD5) leads to stimulation of adenylyl cyclase and cAMP generation [9]. Modulation of DRD2 activity profoundly affects

Abbreviations: α 2-AR, α 2-adrenergic receptors; AMPK, AMP-activated protein kinase; Ca^{2+} , calcium; CREB, cAMP response element binding; DRD2, dopamine receptor D2; ERK1/2, extracellular signal-regulated kinases 1/2; GSIS, glucose-stimulated insulin secretion; HG clamp, hyperglycemic clamp; IBMX, 3-isobutyl-1-methylxanthine; ipGTT, intraperitoneal glucose tolerance test; K_{ATP} -channel, ATP-sensitive potassium channel; KRBH buffer, Krebs–Ringer bicarbonate HEPES buffer; PKA, cAMP-dependent protein kinase A.

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energy homeostasis. Indeed, antipsychotic drugs antagonizing DRD2 enhance appetite and body weight gain in both animals [10] and humans [11]. Conversely, the DRD2 agonist bromocriptine, a semi-synthetic ergot alkaloid used in the treatment of Parkinson's disease and hyperprolactinemia, exerts opposite beneficial effects on energy homeostasis. For instance, prolonged administration of bromocriptine normalizes elevated plasma glucose and insulin concentrations and improves both glucose tolerance and insulin sensitivity in obese insulin-resistant rodents [12,13] and humans [14,15]. Furthermore, long-term treatment with bromocriptine improves pancreatic beta cell function and increases the islet insulin content in obese insulin-resistant mice [12,16,17]. However, the underlying mechanism(s) associated with these beneficial effects remains poorly understood. Interestingly, it has been recently demonstrated that all dopaminergic receptor subtypes are widely expressed on pancreatic beta cells, suggesting that at least some of the metabolic effects of bromocriptine could result from direct interaction with DRD2 on beta cells [18]. Accordingly, dopamine, as well as several synthetic analogues and the DRD2 agonist quinpirole, inhibit insulin secretion in beta cell lines and isolated islets from rodents [18–20].

Here, we hypothesized that one of the early steps involved in the beneficial effects of bromocriptine on glucose homeostasis could be linked to modulation of insulin secretion following direct activation of DRD2 in pancreatic beta cells. To address this issue, the acute effect of bromocriptine on insulin secretion was investigated by means of a glucose tolerance test and hyperglycemic clamp in C57Bl6/J mice and the underlying molecular mechanism(s) was studied in INS-1E beta cells.

2. Materials and methods

2.1. Chemicals

Bromocriptine (2-bromo- α -ergocryptine methanesulfonate salt), domperidone, yohimbine, hGLP-1 7–36 amide and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma–Aldrich (St. Louis, USA). For the *in vivo* experiments, bromocriptine was dissolved in water containing 5% (v/v) DMSO. Domperidone was dissolved in water containing 5% (v/v) DMSO and acidified with HCl until pH 4. Water containing 5% (v/v) DMSO was used as placebo for both drugs.

2.2. Animals

Twelve-week-old male C57Bl6/J mice (Charles River, Maastricht, The Netherlands) were housed in a temperature- and humidity-controlled room on a 12-h light–dark cycle with free access to standard laboratory chow (RM3; Special Diets Services, UK) and water. All animal experiments were performed in accordance with the principles of laboratory animal care and the regulations of Dutch law on animal welfare, and the experimental protocol was approved by the Animal Ethics Committee of the Leiden University Medical Center.

2.3. Experimental design

For determination of the acute effects of bromocriptine on glucose and insulin homeostasis, mice were randomly assigned to groups receiving either bromocriptine (10 or 25 mg/kg BW [12,21]) or placebo. They were first subjected to an intraperitoneal glucose tolerance test (ipGTT) and subsequently, after a wash-out period of 2 weeks, to a hyperglycemic clamp (HG clamp). One hour before the start of the experiments ($t = -60$ min), a baseline blood sample was taken, immediately followed by an i.p. injection of either bromocriptine or placebo. At $t = 0$ min the ipGTT or the HG clamp

was started, as described below. The blood samples taken at $t = -60$ min and $t = 0$ min were used to determine the basal effect of bromocriptine on plasma glucose and insulin levels. Another group of mice was used to determine the impact of the DRD2 antagonist domperidone on bromocriptine-induced metabolic effects. The design for these experiments was similar to the one described above except that domperidone (5 mg/kg BW [22,23]) or placebo was injected 30 min before bromocriptine administration ($t = -90$ min).

2.4. Intraperitoneal glucose tolerance test

Nine mice per group were fasted for 16 h after food withdrawal at 5:00 p.m. At 9:00 a.m. the next day, mice were injected with the drugs as described above and subjected to an ipGTT. This procedure began with a blood sample collection ($t = 0$ min), immediately followed by i.p. injection of 2 g/kg D-glucose, provided as a 20% solution in PBS. Additional blood samples (30 μ l) were taken via tail bleeding at 5, 15, 30, 45, 60, and 120 min after glucose injection for measurement of plasma glucose and insulin concentrations.

2.5. Hyperglycemic clamp

Eleven mice per group were fasted for 16 h after food withdrawal at 5:00 p.m. At 9:00 a.m. the next day, mice were injected with the drugs as described above and then subjected to a HG clamp. During the clamp, mice were anesthetized with a combination of 6.25 mg/kg acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, The Netherlands) and 0.3125 mg/kg fentanyl (Janssen-Cilag, Tilburg, The Netherlands). An initial blood sample ($t = 0$ min) was taken, immediately followed by a primed (30 or 20 mg for placebo and bromocriptine groups, respectively), continuous (10 mg/h) intravenous (i.v.) infusion of a 20% D-glucose solution. To maintain a steady state blood glucose concentration of ~ 20 mM, the continuous glucose infusion rate was adjusted according to the glucose concentration measured via tail bleeding (Accu-chek, Sensor Comfort, Roche Diagnostics GmbH, Germany) at 1, 5, 20, 35, 50, 70 and 90 min after the glucose bolus. Blood samples (30 μ l) for the measurement of plasma glucose and insulin concentrations were taken at 1, 5, 20, 50, 70 and 90 min after the start of the clamp. The blood sampling at 90 min was immediately followed by an i.v. injection of 2.1 mmol/kg L-arginine (Sigma–Aldrich, St. Louis, USA), in order to assess maximal insulin secretion. Two additional blood samples were taken at 5 and 20 min after the injection.

2.6. Analytical procedures

Plasma insulin and glucose concentrations were measured using commercially available kits (insulin: Crystal Chem Inc., Downers Grove, USA; glucose: Instruchemie, Delfzijl, The Netherlands).

2.7. INS-1E cells

The rat insulinoma-derived INS-1E cell line was used as a well differentiated beta cell clone [24] and cultured in a humidified atmosphere containing 5% CO₂ in RPMI 1640 medium (Invitrogen, Breda, The Netherlands) supplemented with 10 mM Hepes, 5% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, 100 units/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Breda, The Netherlands). The maintenance culture was split once a week by gentle trypsinization, and 3×10^6 cells were seeded in 75-cm² Falcon bottles (BD Biosciences, Breda, The Netherlands) with 20 ml

complete medium. For experiments, INS-1E cells were seeded at 2.10^5 cells/1 ml in Falcon 24-well plates (insulin secretion, cell membrane potential and cAMP determination) or 9.10^5 cells/2 ml in Falcon 6-well plates (Western blot) and used 4 days later, with one medium change on day 3.

2.8. Insulin secretion

The response of insulin secretion to glucose or other secretagogues was measured in INS-1E cells between passages 53–67. Before the experiments, cells were maintained for 2 h in glucose-free culture medium. The cells were then washed twice and pre-incubated for 30 min at 37 °C in glucose-free Krebs–Ringer bicarbonate HEPES buffer (KRBH: 135 mM NaCl, 3.6 mM KCl, 5 mM NaHCO_3 , 0.5 mM NaH_2PO_4 , 0.5 mM MgCl_2 , 1.5 mM CaCl_2 , 0.1% BSA and 10 mM HEPES, pH 7.4) in the presence or absence of various drugs, as indicated. Next, cells were washed once with glucose-free KRBH and incubated for 30 min in KRBH with or without various drugs and secretagogues, as indicated. Then, plates were placed on ice and the supernatants were collected for determination of insulin secretion. Cellular insulin content was measured in acid-ethanol extracts [24]. Insulin concentrations were measured after appropriate dilution (1/20 and 1/400 for supernatant and acid-ethanol extract, respectively) using a rat/mouse ELISA kit (Millipore, Nuclilab, Ede, The Netherlands).

2.9. Cell membrane potential and cAMP levels

Cell membrane potential was monitored in INS-1E cells using 100 nM of the fluorescent probe bis-oxonol (bis-(1,3-diethylthio-barbituric acid) trimethine oxonol) (Molecular Probes, Leiden, The Netherlands) in a temperature-controlled (37 °C) plate reader fluorimeter with excitation and emission wavelengths of 544 and 590 nm, respectively [18]. For cAMP measurements, INS-1E cells were incubated in the same conditions as for GSIS experiments, but in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 1 mM). At the end of incubation, cells were lysed following the manufacturer's instructions and cAMP levels were determined using an enzyme immunoassay kit (Amersham Biosciences, Roosendaal, The Netherlands).

2.10. Western blot analysis

INS-1E cells were incubated in the same conditions as for GSIS experiments. At the end of incubation, cells were washed once in PBS and then lysed in 400 μl of a buffer containing 10% (w/v) glycerol, 3% (w/v) SDS and 100 mM Tris–HCl (pH 6.8). The homogenates were immediately boiled for 5 min and then centrifuged (13,200 rpm; 2 min). Protein content of the supernatant was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, USA). Proteins (10 μg) were separated by SDS-PAGE followed by transfer to a PVDF membrane. Membranes were blocked for 1 h at room temperature in TBST buffer (10 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.1% (v/v) Tween 20 containing 5% (w/v) fat free milk) and incubated overnight with primary

antibodies (all from Cell Signalling, Danvers, USA). Blots were then washed in TBST buffer and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. After washing, blots were developed using enhanced chemiluminescence and quantified by densitometry analysis using Image J software (NIH, Bethesda, USA).

2.11. Statistical analysis

Data is presented as mean \pm SEM. Statistical analysis was conducted with SPSS 16.0 software. The basal effect of treatment on glucose and insulin concentrations was analyzed with a univariate General Linear Model, with pre-injection values as a covariate. Analysis of the remaining *in vivo* data was performed using a one-way ANOVA. Only if the overall *F*-test indicated significant differences between the groups, a Bonferroni post hoc test was used to determine differences between specific groups. Statistical analysis of most of the *in vitro* data was performed using a two-tailed unpaired Student's *t*-test. The membrane potential data was analyzed with a General Linear Model for repeated measures. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Acute effects of bromocriptine on glucose tolerance and insulin secretion in mice

To investigate the effects of bromocriptine on glucose homeostasis, overnight-fasted C57Bl6/J mice received a single i.p. injection of the drug (10 or 25 mg/kg BW) or its vehicle and were subjected to an ipGTT one hour later. As shown in Table 1, bromocriptine induced a dose-dependent rise in basal plasma glucose levels. During the ipGTT, plasma glucose and insulin levels were determined at different time points and their respective areas under the curve (AUC) were calculated (Fig. 1). Glucose levels were significantly elevated by bromocriptine (Fig. 1A and B; +48% and +50% for AUC glucose at 10 and 25 mg/kg BW, respectively; $p < 0.01$), indicating impaired glucose tolerance. Concomitantly, bromocriptine delayed and reduced the rise in plasma insulin levels after the glucose challenge (Fig. 1C). Total insulin release, calculated as AUC, was thus decreased by bromocriptine (Fig. 1D; –19% (NS) and –58% ($p < 0.05$) at 10 and 25 mg/kg BW, respectively).

To further assess the effect of bromocriptine on glucose-stimulated insulin secretion (GSIS), we performed a HG clamp in similar conditions, i.e. one hour after i.p. administration of bromocriptine or placebo in overnight-fasted C57Bl6/J mice. Plasma glucose levels were successfully clamped at 20 mM in both placebo and bromocriptine-injected mice (Fig. 2A). We confirmed that bromocriptine strongly affected GSIS (Fig. 2B). Indeed, insulin release was significantly and dose-dependently decreased during both first-phase (Fig. 2D; –58% ($p = 0.064$) and –79% ($p < 0.01$) for AUC insulin at 10 and 25 mg/kg BW, respectively) and second-phase GSIS (Fig. 2E; –51% and –70% for AUC insulin at 10 and 25 mg/kg BW, respectively; $p < 0.001$).

Table 1

Plasma glucose and insulin levels in fasted mice before and 60 min after a single i.p. injection of bromocriptine or placebo.

	Glucose (mM)		Insulin (ng/ml)	
	Before injection	60 min after injection	Before injection	60 min after injection
Placebo	3.3 \pm 0.2	4.2 \pm 0.2	0.16 \pm 0.04	0.19 \pm 0.05
Bromocriptine 10 mg/kg	3.9 \pm 0.2	5.1 \pm 0.3	0.13 \pm 0.03	0.17 \pm 0.05
Bromocriptine 25 mg/kg	3.7 \pm 0.3	5.8 \pm 0.2**	0.13 \pm 0.04	0.27 \pm 0.06

Data are expressed as mean \pm SEM, $n = 8$ –9 mice per group.

** $p < 0.01$ vs placebo.

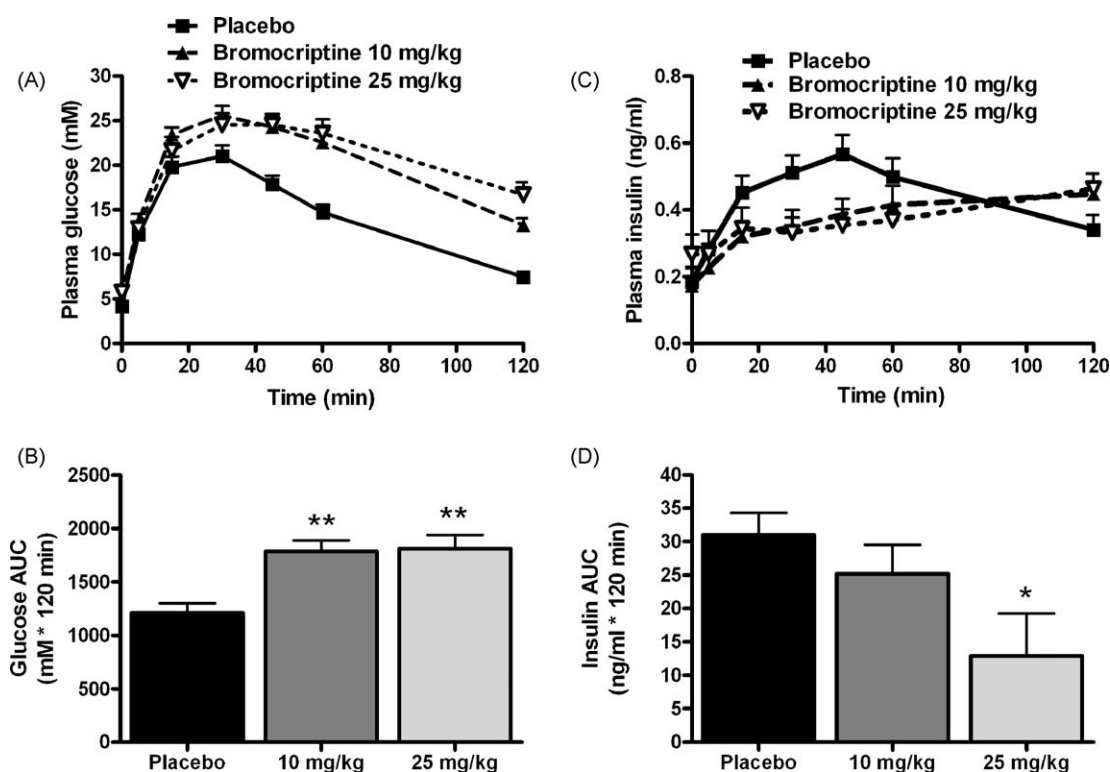


Fig. 1. Effects of acute injection of bromocriptine on plasma glucose and insulin kinetics in mice during an intraperitoneal glucose tolerance test. An ipGTT was performed in overnight fasted C57Bl6/J male mice one hour after a single i.p. injection of bromocriptine (10 or 25 mg/kg BW) or placebo. The glucose (A) and insulin (C) levels were measured and their respective areas under the curve (AUC) were calculated (B and D). Data are expressed as mean \pm SEM, $n = 8-9$ mice per group. *, $p < 0.05$; **, $p < 0.01$ vs placebo.

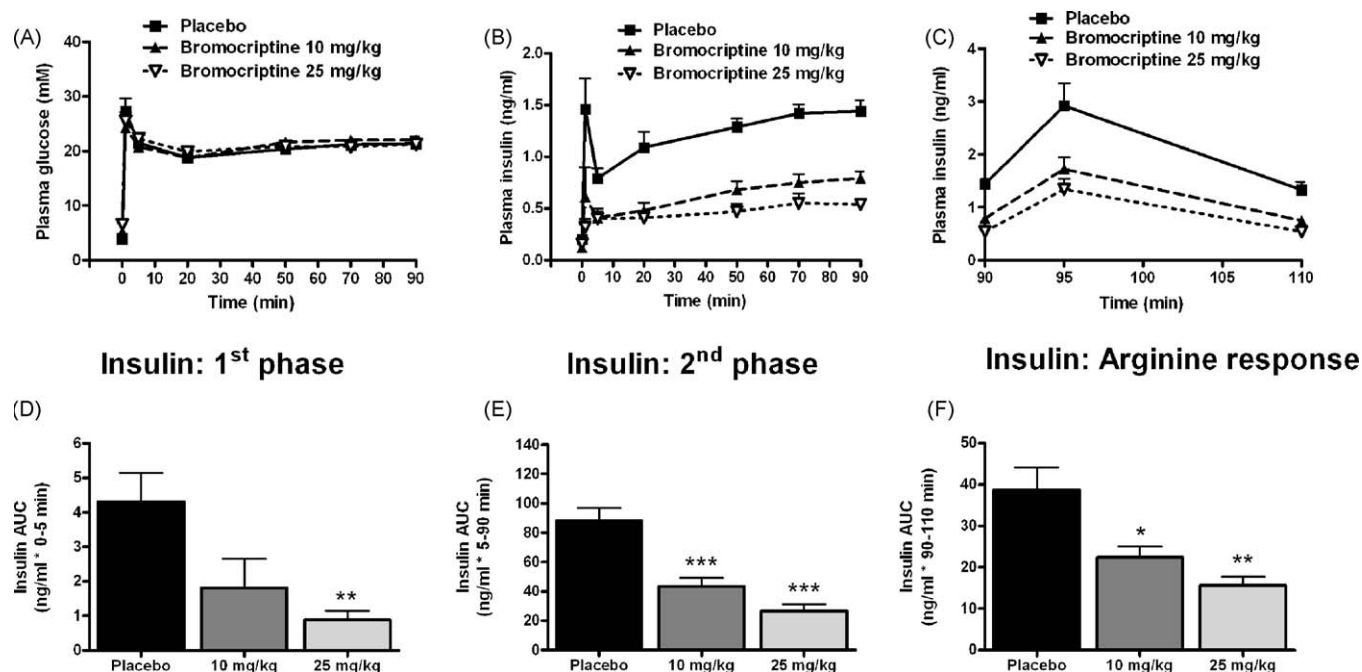


Fig. 2. Effects of acute injection of bromocriptine on glucose and arginine-induced insulin secretion in mice subjected to a hyperglycemic clamp. A HG clamp (~ 20 mM) was performed in overnight fasted C57Bl6/J male mice one hour after a single i.p. injection of bromocriptine (10 or 25 mg/kg BW) or placebo. The glucose (A) and insulin (B) levels were measured at different time points during 90 min and the areas under the curve (AUC) corresponding to the first (D, from 0 to 5 min) and second phase (E, from 5 to 90 min) of insulin secretion were calculated. At $t = 90$ min, an intravenous bolus of arginine (2.1 mmol/kg BW) was administered to the mice and both insulin levels (C) and AUC (F) were determined during the next 20 min. Data are expressed as mean \pm SEM, $n = 8-9$ mice per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs placebo.

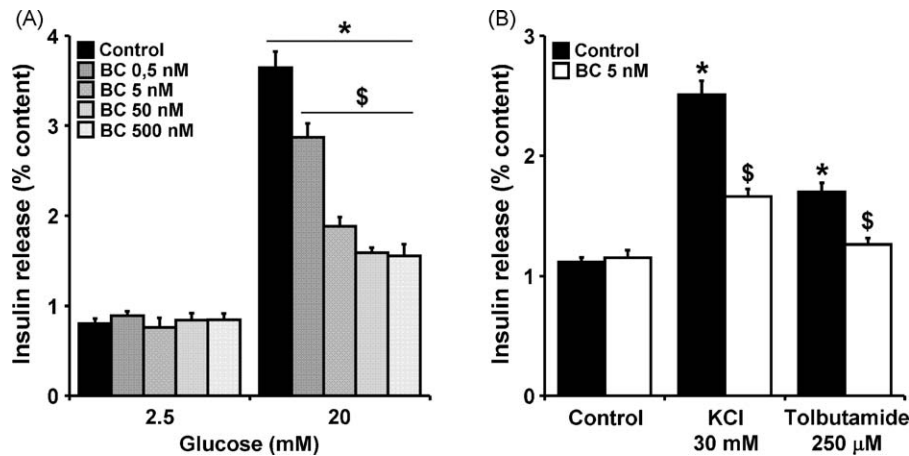


Fig. 3. Effects of bromocriptine on insulin secretion in INS-1E cells. The insulin secretion in response to glucose and non-nutrient secretagogues was determined over a 30-min stimulation period in INS-1E cells pre-incubated for 30 min with various concentrations of bromocriptine (BC) or vehicle (0.05% (v/v) DMSO; black bars). (A) Dose-response effect of BC on insulin secretion in response to basal (2.5 mM) and stimulatory (20 mM) glucose concentrations. (B) Effect of BC (5 nM; open bars) on insulin secretion in presence of 2.5 mM glucose and 30 mM KCl or 250 μM tolbutamide. Data are expressed as mean \pm SEM, $n = 3$ –4 independent experiments performed in triplicate. *, $p < 0.05$ vs 2.5 mM glucose; \$, $p < 0.05$ vs control-vehicle.

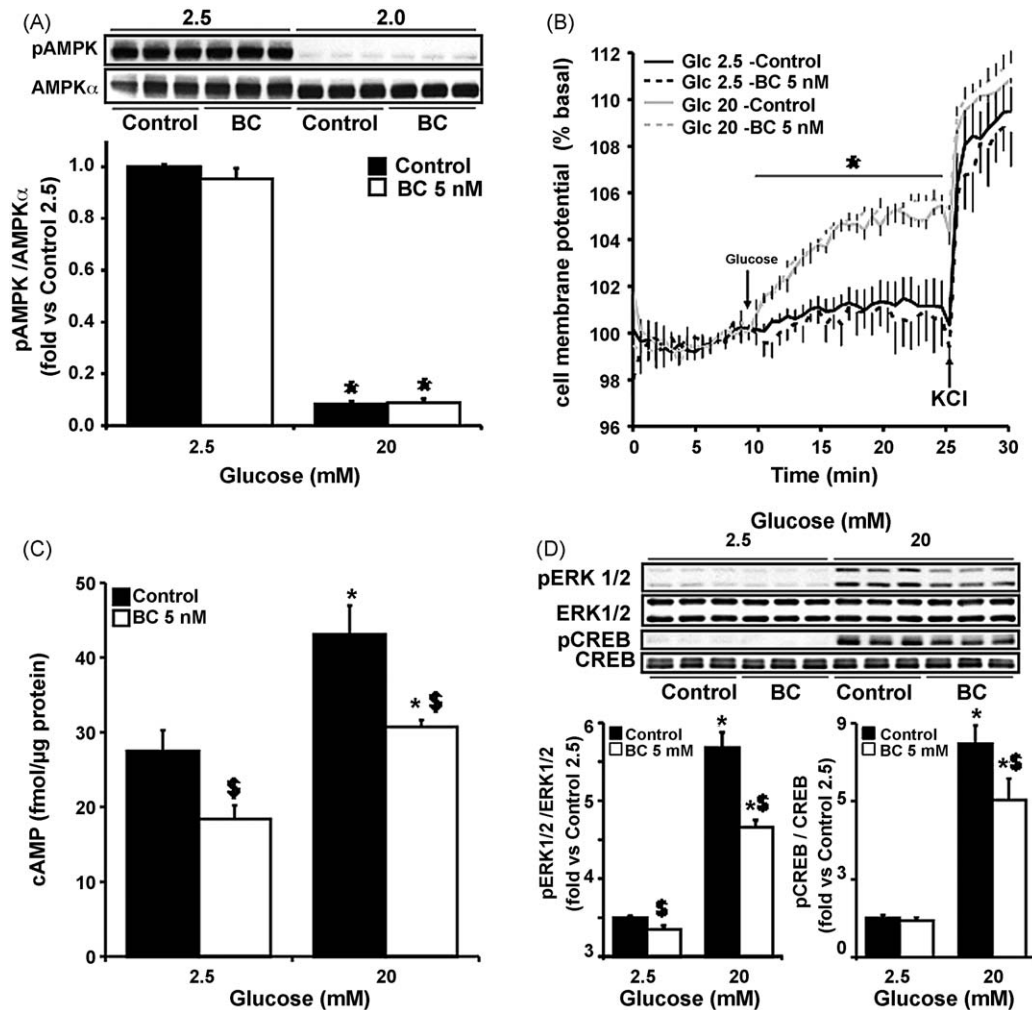


Fig. 4. Effects of bromocriptine on AMPK activity, cell membrane potential and intracellular cAMP levels in INS-1E cells. Various metabolic parameters were determined at low (2.5 mM) and stimulatory (20 mM) glucose concentrations in INS-1E cells treated for 30 min with bromocriptine (BC, 5 nM; open bars) or vehicle (0.05% (v/v) DMSO; black bars). (A) The phosphorylation state of AMPK was assessed by Western blot using anti-phospho-Thr172 antibody. Total AMPK was used as loading control. The quantitative results are expressed in arbitrary units as a ratio over the control-basal glucose group. (B) Cell membrane potential was monitored before and after successive addition of glucose (20 mM) and KCl (30 mM) in INS-1E cells treated from $t = 0$ with BC (5 nM) or vehicle. (C) Intracellular cAMP levels were determined in the same conditions as in panel A except for the additional presence of 1 mM of the phosphodiesterase inhibitor IBMX. (D) The phosphorylation states of the PKA-downstream targets ERK1/2 and CREB were assessed by Western blot using anti-phospho-Tyr202/204 and anti-phospho-Ser133 antibodies, respectively. Total ERK1/2 and CREB were used as loading controls. The quantitative results are expressed in arbitrary units as a ratio over the control-basal glucose group. Data are expressed as mean \pm SEM, $n = 3$ –5 independent experiments. *, $p < 0.05$ vs 2.5 mM glucose; \$, $p < 0.05$ vs control-vehicle.

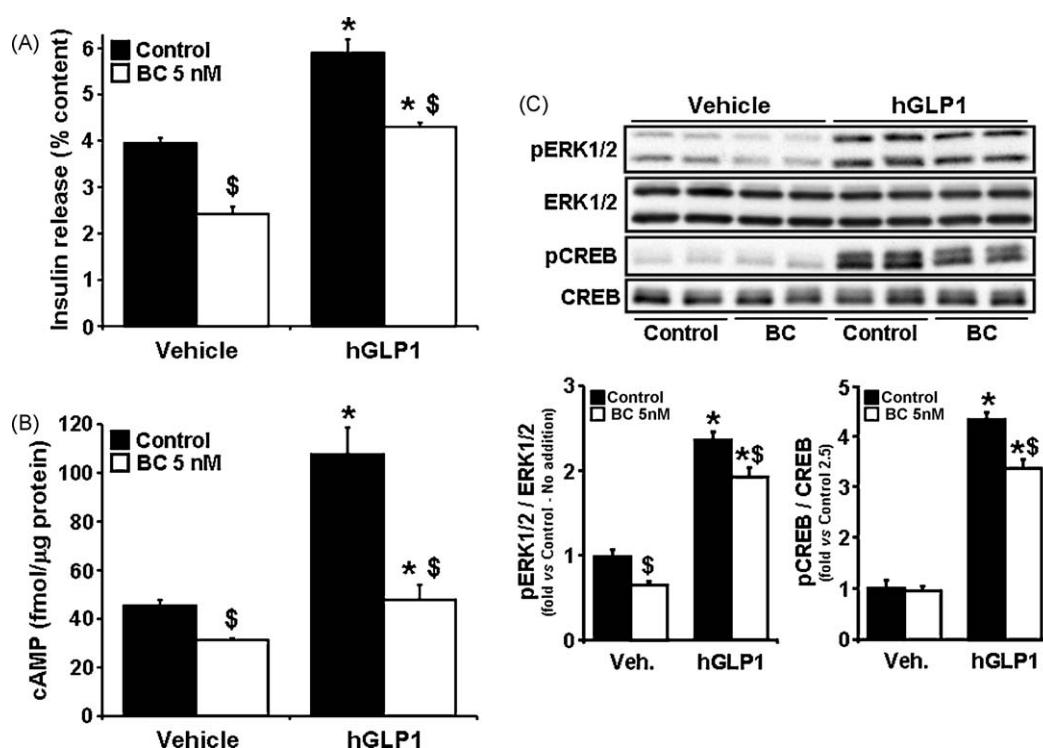


Fig. 5. Effects of bromocriptine on insulin secretion and intracellular cAMP levels in INS-1E cells pre-treated with GLP-1. The insulin secretion was determined in INS-1E cells pre-incubated with hGLP1 7–36 amide (10 nM) or vehicle (KRBH buffer) for 30 min and subsequently challenged with glucose (20 mM) for 30 min in the presence of bromocriptine (BC, 5 nM; open bars) or vehicle (0.05% (v/v) DMSO; control, black bars). (A) Effects of BC (5 nM) on glucose-induced insulin secretion in basal (vehicle) and GLP1-stimulated INS-1E cells. (B) Intracellular cAMP levels were determined in the same conditions except for the additional presence of 1 mM IBMX. (C) The phosphorylation states of the PKA-downstream targets ERK1/2 and CREB were assessed by Western blot as described in Fig. 4D. Data are expressed as mean \pm SEM, $n = 3$ independent experiments performed in duplicate. *, $p < 0.05$ vs vehicle; \$, $p < 0.05$ vs control.

Consistent with reduced GSIS, insulin secretion in response to arginine was still present but decreased in bromocriptine-injected mice (Fig. 2C and F).

3.2. Effects of bromocriptine on insulin secretion in response to glucose and non-nutrient secretagogues in INS-1E cells

To elucidate the underlying molecular mechanism(s), we used the insulinoma-derived INS-1E cell line. The cells were pre-treated for 30 min with increasing concentrations of bromocriptine (from 0.5 to 500 nM) or vehicle (DMSO) before determination of insulin secretion over a 30-min stimulatory period with low (2.5 mM) or high (20 mM) glucose concentration. Fig. 3A shows that addition of high glucose induced a 4.5-fold increase in insulin secretion in vehicle-treated INS-1E cells ($p < 0.05$), in line with previous results obtained with this beta cell line [18,24]. Bromocriptine treatment did neither change cellular insulin content (data not shown) nor basal insulin secretion, but it dose-dependently inhibited GSIS

(from -21% at 0.5 nM to -57% at 500 nM; $p < 0.05$). In vehicle-treated INS-1E cells, insulin secretion was also stimulated by non-nutrient secretagogues eliciting cell membrane depolarization, such as KCl or tolbutamide, leading to a 2.2-fold ($p < 0.05$) and 1.5-fold ($p < 0.05$) increase in insulin release, respectively (Fig. 3B). Bromocriptine also inhibited insulin secretion in these conditions (Fig. 3B; -34% and -26% in KCl- and tolbutamide-stimulated cells, respectively; $p < 0.05$), suggesting that its inhibitory action is located at a step distal to ATP-sensitive potassium (K_{ATP}) channels and cell membrane depolarization.

3.3. Effects of bromocriptine on ATP level, cell membrane potential and cAMP generation in INS-1E cells

We assessed the effects of bromocriptine on basal and glucose-induced changes in cellular energy state, membrane depolarization and cyclic-AMP (cAMP) levels in INS-1E cells. The AMP-activated protein kinase (AMPK) acts as a cellular fuel gauge and is

Table 2

Plasma glucose and insulin levels before and 60 min after a single i.p. injection of bromocriptine or placebo in fasted mice pre-treated with or without domperidone.

	Glucose (mM)		Insulin (ng/ml)	
	Before injection	60 min after injection	Before injection	60 min after injection
Placebo	4.9 \pm 0.3	5.0 \pm 0.2	0.08 \pm 0.01	0.10 \pm 0.00
Bromocriptine 10 mg/kg	4.8 \pm 0.2	6.3 \pm 0.2***	0.13 \pm 0.02	0.22 \pm 0.06
Domperidone 5 mg/kg	5.0 \pm 0.3	5.0 \pm 0.3	0.09 \pm 0.01	0.16 \pm 0.02
Domperidone + bromocriptine	4.8 \pm 0.3	6.1 \pm 0.3**,\$\$	0.10 \pm 0.01	0.19 \pm 0.01

Data are expressed as mean \pm SEM, $n = 9$ mice per group.

*** $p < 0.01$ vs placebo.

**** $p < 0.001$ vs placebo.

\$\$ $p < 0.01$ vs domperidone.

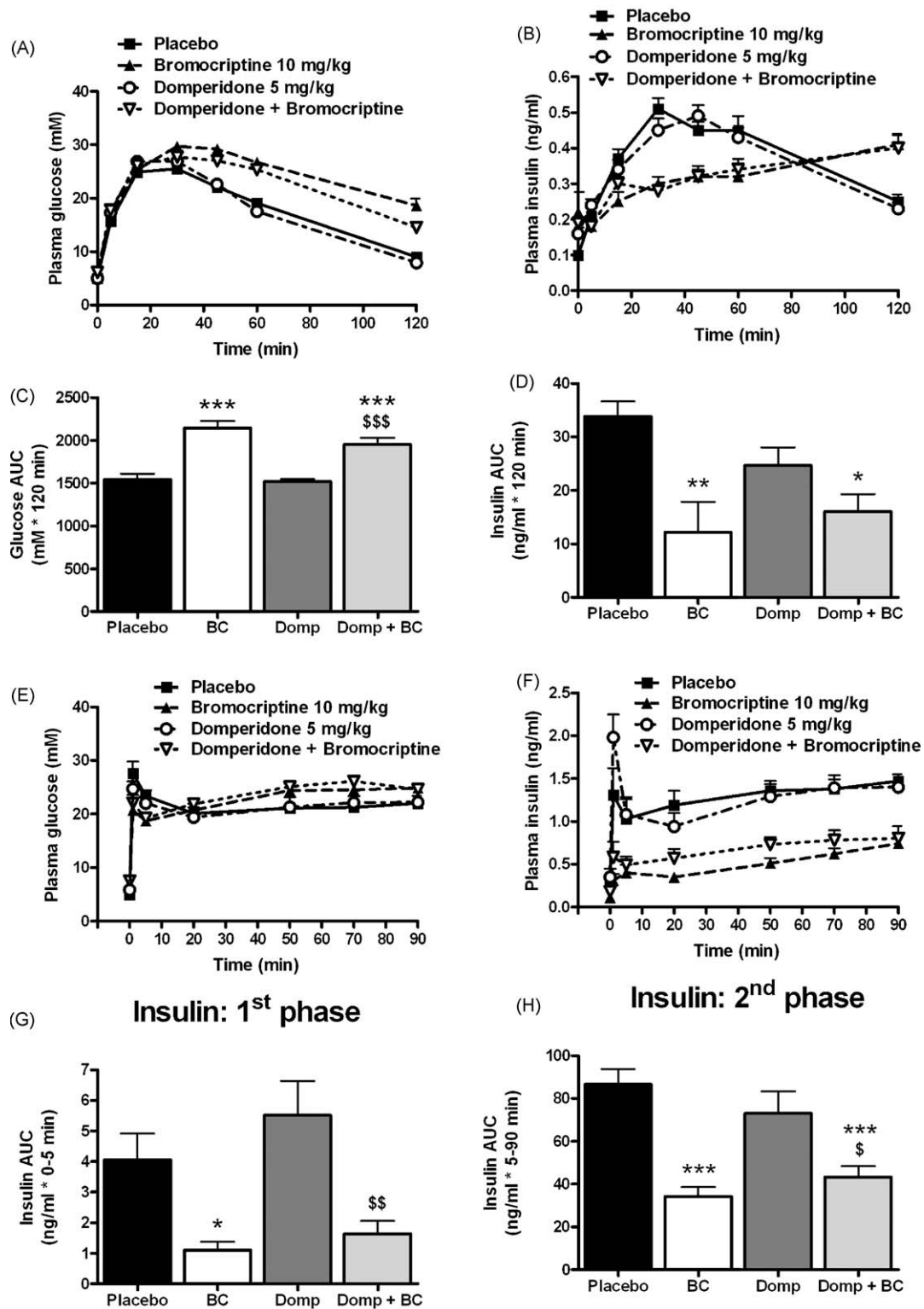


Fig. 6. Effects of the dopamine D2 receptor antagonist domperidone on bromocriptine-induced alterations of glucose tolerance and insulin secretion in mice. An ipGTT was performed in overnight fasted C57BL/6J male mice pre-treated for 30 min with domperidone (i.p., Domp., 5 mg/kg BW) or placebo one hour after a single i.p. injection of bromocriptine (BC, 10 mg/kg BW) or vehicle. The glucose (A) and insulin (B) levels were measured and their respective areas under the curve (AUC) were calculated (C and D). A HG clamp (~20 mM) was performed in the same conditions. The glucose (E) and insulin (F) levels were measured at different time points during 90 min and the areas under the curve (AUC) corresponding to the first (G, from 0 to 5 min) and second phase (H, from 5 to 90 min) of insulin secretion were calculated. Data are expressed as mean \pm SEM, $n = 6-9$ mice per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs placebo and \$, $p < 0.05$; \$\$, $p < 0.01$; \$\$\$, $p < 0.001$ vs domperidone.

activated in response to drop in ATP levels or increase in AMP/ATP ratio [25]. As shown in Fig. 4A, challenging the cells with 20 mM glucose, which results in increased intracellular ATP levels, is associated with a significant decrease in AMPK activation, i.e.

phosphorylation on its Thr172 residue. Bromocriptine did not change the AMPK phosphorylation state in both basal and glucose-stimulated conditions (Fig. 4A), suggesting that inhibition of GSIS by the drug is not mediated by impairment in glucose-induced

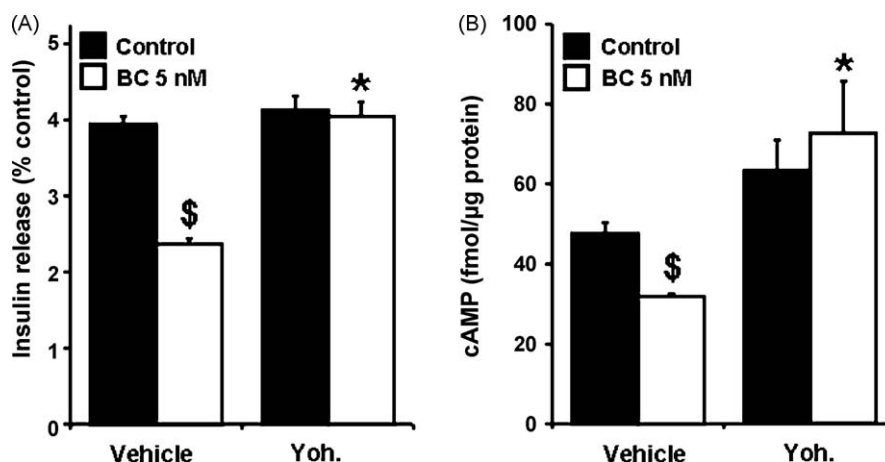


Fig. 7. Effects of the α_2 -adrenergic receptor antagonist yohimbine on bromocriptine-induced decrease in intracellular cAMP levels and insulin secretion in INS-1E cells. The insulin secretion was determined in INS-1E cells pre-incubated with the α_2 -adrenergic receptor antagonist yohimbine (1 μ M) or vehicle (KRHB buffer) for 30 min and subsequently challenged with glucose (20 mM) for 30 min in the presence of bromocriptine (BC, 5 nM; open bars) or vehicle (0.05% (v/v) DMSO; black bars). (A) Effects of BC (5 nM) on glucose-induced insulin secretion in vehicle- and yohimbine-treated INS-1E cells. (B) Intracellular cAMP levels were determined in the same conditions except for the additional presence of 1 mM IBMX. Data are expressed as mean \pm SEM, $n = 3$ independent experiments. *, $p < 0.05$ vs vehicle; \$, $p < 0.05$ vs control.

mitochondrial ATP generation. In line with our results showing that the inhibitory effect of bromocriptine is still present when insulin secretion is triggered by KCl and tolbutamide (see above), we confirmed that the drug did not affect glucose- and KCl-induced cellular membrane depolarization measured using bis-oxonol fluorescence (Fig. 4B). Because cAMP is a key player in the amplifying pathway involved in insulin secretion by beta cells [26], we also measured intracellular cAMP levels in INS-1E cells. As expected, upon a 30-min stimulatory period with 20 mM glucose in the presence of the phosphodiesterase inhibitor IBMX, intracellular cAMP levels were increased compared to 2.5 mM glucose (Fig. 4C; 28 ± 3 fmol/ μ g vs 43 ± 4 fmol/ μ g protein, respectively; $p < 0.05$). Bromocriptine at 5 nM significantly decreased cAMP levels at both 2.5 and 20 mM glucose (Fig. 4C; -33% and -29% respectively; $p < 0.05$). Accordingly, the phosphorylation state of two of the main cAMP-dependent protein kinase (PKA) downstream protein targets, CREB-Ser133 and ERK1/2-Thr202/Tyr204, were also significantly reduced by bromocriptine (Fig. 4D) in a dose-dependent manner (Supplementary Fig. 1).

3.4. Effects of bromocriptine on GLP-1-stimulated insulin secretion in INS-1E cells

GLP-1 is among the most effective agents potentiating glucose-dependent insulin secretion in beta cells. It exerts its stimulatory effect via an increase in cAMP levels resulting from G-protein-coupled receptor-mediated activation of adenylyl cyclase [27]. As expected, GLP-1 increased intracellular cAMP content and phosphorylation of CREB and ERK1/2, leading to amplified GSIS (Fig. 5A–C). In this condition, addition of bromocriptine reduced GLP-1-induced increase in cAMP level and GSIS, suggesting that the drug could interact with some G-protein-coupled receptors inhibiting adenylyl cyclase activity, such as DRD2 [28].

3.5. Effects of the DRD2 antagonist domperidone on bromocriptine-induced inhibition of GSIS in mice and INS-1E cells

We therefore tested whether the *in vivo* effects of bromocriptine on glucose tolerance and insulin secretion could be prevented by administration of the peripheral DRD2 antagonist domperidone in C57Bl6/J mice. Pre-treatment with domperidone did not prevent the rise in basal plasma glucose level induced by bromocriptine (Table 2). During the ipGTT, domperidone did neither affect the bromocriptine-induced impairment of glucose disposal (Fig. 6A

and C) nor its inhibitory effect on glucose-stimulated insulin response (Fig. 6B and D). In addition, domperidone did not prevent the bromocriptine-induced decrease in first- and second-phase GSIS during the HG clamp (Fig. 6E–H). Accordingly, we found that domperidone only modestly rescued the inhibitory effect of bromocriptine on GSIS in INS-1E cells whatever the antagonist concentrations used (-41% for bromocriptine alone vs -29% and -33% when pre-treated with 10 and 25 nM domperidone, respectively; $p < 0.05$; Supplementary Fig. 2), suggesting that a DRD2-independent signaling pathway(s) plays a more prominent role. Since it has been suggested that bromocriptine, in addition to its D2-dopaminergic activity, can stimulate the G-protein-coupled α_2 -adrenergic receptors (α_2 -AR) [29], we investigated the effect of yohimbine, a selective α_2 -AR antagonist, on cAMP levels and GSIS in INS-1E cells. Interestingly, yohimbine completely prevented the bromocriptine-induced decrease in intracellular cAMP levels and GSIS (Fig. 7). Taken together, our results indicate that the major effect of bromocriptine on insulin secretion results from modulation of the α_2 -AR rather than direct activation of the pancreatic DRD2.

4. Discussion

Here we report for the first time that a single administration of the DRD2 agonist bromocriptine inhibits GSIS in C57Bl6/J mice at concentrations in the range of those used for chronic treatment in rodents [12,16,17,21]. This acute effect was also observed in INS-1E beta cells at therapeutic plasma concentrations, suggesting that bromocriptine affects insulin secretion through a direct effect on pancreatic beta cells. We further demonstrated that the underlying molecular mechanism of bromocriptine is not mediated by DRD2 activation but rather by modulation of the α_2 -AR.

A single i.p. injection of bromocriptine acutely elevated fasting plasma glucose levels in mice. This is in agreement with several other studies reporting that short-term administration of L-DOPA, dopamine or dopamine agonists induces hyperglycemia in rodents [30–33]. The exact underlying mechanism remains unknown, although stimulation of glycogenolysis and/or gluconeogenesis through modulation of sympatho-adrenal axis might be involved [19,32]. We also found that administration of bromocriptine acutely impairs glucose tolerance. This finding is in apparent contradiction with the improvements of fasting plasma glucose and insulin levels, glucose tolerance and insulin sensitivity observed in obese insulin-resistant animals and humans treated

with bromocriptine [12,14,34]. However, this discrepancy might be explained by the difference between single/acute and multiple/chronic treatment and also possibly by the use of healthy/lean C57Bl6/J mice instead of insulin-resistant/obese animals in the present study. Thus, it is interesting to note that the pancreatic K_{ATP} activator diazoxide, which also decreases insulin secretion, also exerts opposite effects on glucose tolerance in lean and obese Zucker rats [35]. Whether acute administration of bromocriptine differentially affects glucose tolerance and insulin secretion in rodent models of insulin-resistance/obesity requires further investigation.

Our most striking finding is the clear demonstration that bromocriptine acutely inhibits GSIS in both mice and INS-1E beta cells. This confirms previous *in vitro* reports showing a negative impact of dopamine and several dopaminergic agonists on insulin secretion [18,20,36]. GSIS from beta cells is primarily controlled by metabolism-secretion coupling [26]. Following an increase in its circulating levels, glucose rapidly equilibrates across the plasma membrane owing to GLUT2 and is phosphorylated by glucokinase to enter the glycolysis pathway. Subsequently, mitochondrial metabolism generates ATP, which promotes the closure of K_{ATP} channels and, as a consequence, depolarization of the plasma membrane. This leads to intracellular calcium (Ca^{2+}) influx through voltage-gated Ca^{2+} channels resulting in increased free cytosolic Ca^{2+} levels, which ultimately triggers insulin exocytosis [26]. Although this triggering pathway is essential for GSIS, activation of additional metabolic signals involved in the so-called amplifying pathway, are also required to fully stimulate insulin secretion [26].

Interestingly, it has been shown that all dopamine receptor subtypes, including DRD2, are expressed in rat, mouse, and human islets as well as in INS-1E cells [18]. In addition to the early demonstration that dopamine inhibits GSIS in various cellular models [18,20], a role for dopaminergic receptors in the regulation of insulin secretion was supported by a recent large-scale compound screening showing that a substantial number of dopaminergic agonists and antagonists were able to modulate GSIS [37]. Furthermore, the siRNA-mediated knockdown of DRD2 in INS-832/13 beta cells enhances GSIS, which is consistent with the involvement of this receptor in the negative regulation of insulin secretion [37].

In the present study, we showed that the inhibition of GSIS by bromocriptine is apparently not a consequence of impaired triggering signal since neither intracellular energy state nor glucose- and KCl-induced cell membrane depolarization are affected in INS-1E cells. By contrast, bromocriptine significantly decreases intracellular cAMP levels, affecting one of the main metabolic amplifying pathways involved in GSIS, suggesting that activation of G-protein-coupled DRD2 receptors could mediate its effect. However, pre-treatment with domperidone, a peripheral DRD2 antagonist which does not cross the blood brain barrier [38], did not prevent the inhibition of insulin secretion or the impairment of glucose tolerance induced by bromocriptine *in vivo*. Although we can not exclude that part of the bromocriptine-induced inhibition of GSIS is mediated by a DRD2-dependent central effect *in vivo*, the experiments performed in INS-1E cells confirmed that domperidone only marginally prevented inhibition of GSIS, pointing to a DRD2-independent mechanism.

Our *in vitro* results provide clear evidence that activation of G-protein-coupled adrenergic receptors primarily accounts for the inhibition of GSIS by bromocriptine since yohimbine, a α 2-AR antagonist, completely prevented its action. Interestingly, activation of α 2-AR has already been shown to inhibit insulin secretion by a still incompletely understood effect on the exocytosis machinery [39] following receptor-coupled inhibition of adenylyl cyclase and decrease in cAMP synthesis [36,40–43]. However, this

effect on cAMP-mediated pathways seems to be ascribed to selective activation of the α 2A-, but not α 2C-AR, in isolated islets [44], suggesting that part of the inhibition of GSIS by bromocriptine could also be mediated by cAMP/PKA-independent effects through α 2C-AR activation. Further studies in receptor-specific α 2-AR knock-out mice would be crucial to clarify this point.

Strikingly, overexpression of α 2-AR results in impaired insulin secretion and glucose intolerance [45,46], whereas α 2-AR knock-out mice display higher basal insulin levels and improved glucose tolerance [47]. Finally, an α 2-AR-mediated inhibition of GSIS by bromocriptine is further supported by *in vitro* binding studies showing that the drug exhibited high affinity for α 2-AR [48] and that yohimbine can counteract some of its effects on other biological systems [29,49]. It is also tempting to speculate that the reduction of blood pressure observed in patients treated with bromocriptine could be ascribed to its peripheral α 2-adrenergic action [15].

Although counterintuitive, the ability of bromocriptine to acutely suppress insulin secretion might be at the basis of its beneficial effect on glucose homeostasis observed after long-term treatment in obese insulin-resistant rodents and humans [13,15,34]. Indeed, it has been suggested that the progressive deterioration of beta cell mass and function in patients with type 2 diabetes might not only be the consequence of hyperglycemia *per se*, but could also be due to the compensatory insulin secretion itself. Thus, inhibition of GSIS could avoid long-lasting hyperinsulinemia and therefore prevent subsequent development of insulin resistance and beta cell failure [50,51]. Accordingly, it has been shown that chronic increase of insulin release using tolbutamide impaired GSIS in insulin-sensitive rats [52], whereas long-term pharmacological inhibition of insulin secretion improves beta cell glucose responsiveness in hyperglycemic rats [53] and patients with type 2 diabetes [54,55]. While several mechanisms have been proposed to explain the beneficial impact of beta cell rest on glucose homeostasis [50,51], future studies will have to clarify this point.

In conclusion, we report here that acute administration of bromocriptine inhibits insulin secretion both *in vivo* and *in vitro* mainly by a DRD2-independent mechanism involving direct activation of the pancreatic α 2-AR. Taking into account that long-term treatment with bromocriptine has been shown to improve glucose homeostasis in patients with type 2 diabetes, we suggest that inhibition of GSIS by the drug could be one of its early effects, preventing long-lasting hypersecretion of insulin and subsequent beta cell failure.

Acknowledgements

The authors are grateful to Jan Kriek for his technical assistance and to Ron Wolterbeek for his help in statistical analysis. This work was supported by grants from the Dutch Diabetes Foundation (project 2002.01.005, to J.E.d.L.v.W. and H.P.), TI Pharma (project T2-105, to B.G., D.M.O., H.P., L.M.H. and J.A.R.) and the Swiss National Science Foundation (to P.M.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.01.029.

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