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Effects of $GABA_B$ receptor agonists and antagonists on glycemia regulation in mice

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ABSTRACT

γ-Aminobutyric acid (GABA) inhibits insulin secretion through $GABA_B$ receptors in pancreatic β-cells. We investigated whether $GABA_B$ receptors participated in the regulation of glucose homeostasis *in vivo*. BALB/c mice acutely pre-injected with the $GABA_B$ receptor agonist baclofen (7.5 mg/kg, i.p.) presented glucose intolerance and diminished insulin secretion during a glucose tolerance test (GTT, 2 g/kg body weight, i.p.). The $GABA_B$ receptor antagonist 2-hydroxysaclofen (15 mg/kg, i.p.) improved the GTT and reversed the baclofen effect. Also a slight increase in insulin secretion was observed with 2-hydroxysaclofen. In incubated islets 1.10^{-5} M baclofen inhibited 20 mM glucose-induced insulin secretion and this effect was reversed by coincubation with 1.10^{-5} M 2-hydroxysaclofen. In chronically-treated animals (18 days) both the receptor agonist (5 mg/kg/day i.p.) and the receptor antagonist (10 mg/kg/day i.p.) induced impaired GTTs; the receptor antagonist, but not the agonist, also induced a decrease in insulin secretion. No alterations in insulin tolerance tests, body weight and food intake were observed with the treatments. In addition glucagon, insulin-like growth factor I, prolactin, corticosterone and growth hormone, other hormones involved in glucose metabolism regulation, were not affected by chronic baclofen or 2-hydroxysaclofen. In islets obtained from chronically injected animals with baclofen, 2-hydroxysaclofen or saline (as above), $GABA_{B2}$ mRNA expression was not altered. Results demonstrate that $GABA_B$ receptors are involved in the regulation of glucose homeostasis *in vivo*. Treatment with receptor agonists or antagonists, given acutely or chronically, altered glucose homeostasis and insulin secretion alerting to the need to evaluate glucose metabolism during the clinical use of these drugs.

1. Introduction

Metabotropic γ-aminobutyric acid B ($GABA_B$) receptors are heteromers formed by a $GABA_{B1}$ and a $GABA_{B2}$ subunit (Bettler et al., 2004) and have been shown to form heteromultimers with auxiliary subunits, determining their pharmacology (Schwenk et al., 2010).

GABA is found at high concentrations in Langerhans islets (Gladkevich et al., 2006), similar to brain levels. Glutamate decarboxylase (GAD) and $GABA_B$ receptors have been detected mainly in β-cells and $GABA_A$ receptors in α-cells (Shi et al., 2000). Although a complete GABA system is present in the endocrine pancreas, its role in islet physiology has remained elusive. Some *in vitro* studies postulate an autocrine/paracrine role for GABA in insulin, glucagon and somatostatin secretion (MacDonald et al., 2005). Regulated exocytosis of GABA from β-cells (Braun et al., 2004a; MacDonald et al., 2005) inhibits glucagon release from α-cells (Wendt et al., 2004). Furthermore, insulin sensitizes α-cells to GABA (Xu et al., 2006). GABA inhibits insulin specifically through $GABA_B$ receptors in MIN6 cells and in rat/mouse islets (Braun et al., 2004b; Brice et al., 2002), and *in vivo* in mice (Bonaventura et al., 2008). Furthermore, mice lacking functional $GABA_B$ receptors (Bonaventura et al., 2008) as well as mice overexpressing GAD65 (Shi et al., 2000) show alterations in glucose homeostasis.

The effect of $GABA_B$ analogs on *in vivo* glycemia regulation has been insufficiently evaluated. Some authors report that baclofen does not modify glucose homeostasis in diabetic patients (Quatraro et al., 1986); others demonstrate that baclofen raises growth hormone and insulin in response to a glucose overload, without modifying glycemia in healthy men (Passariello et al., 1982). Conversely, baclofen diminished blood glucose during a glucose tolerance test (GTT) in streptozotocin-diabetic animals, without modifying it in controls (Gomez et al., 1999). To the best of our knowledge, there are no studies describing the effects of $GABA_B$ receptor antagonists on glucose homeostasis.

Several works have analyzed the effect of baclofen on food intake and body weight. Baclofen induced ingestion in satiated pigs (Ebenezer and Baldwin, 1990) and rats (Ebenezer, 1990) acting on central $GABA_B$ receptors (Ebenezer and Patel, 2004). Chronic baclofen

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did not alter food intake or weight gain in rats (Patel and Ebenezer, 2008). Conversely, chronic baclofen reduced ingestion and body weight in diabetic db/db mice and in obese mice on a high-fat diet (Sato et al., 2007).

In clinical practice $GABA_B$ receptor agonists are used to treat anxiety, depression and neuropathic pain (Bowery, 2006). In addition, they improve drug withdrawal syndrome (Sofuoglu and Kosten, 2005) and ameliorate gastro-esophagic reflux (Bettler et al., 2004). $GABA_B$ receptor antagonists improve cognitive performance and have antipsychotic and neuroprotective effects (Bettler et al., 2004; Bowery et al., 2002; Froestl et al., 1995).

Considering the present and future uses of these compounds, little is known regarding their effects on the endocrine environment and particularly on islet function during prolonged treatments. The aim of this work is to evaluate the effect of acute and chronic treatments with $GABA_B$ receptor agonists and antagonists on glucose homeostasis.

2. Materials and methods

2.1. Animals

2 to 4 month-old male BALB/c mice from the Instituto de Biología y Medicina Experimental colony were used. All animals were housed in groups in air-conditioned rooms, with lights on from 0700 to 1900, and given free access to laboratory chow and tap water. Studies were performed according to protocols for animal use approved by the Institutional Animal Care and Use Committee which follows the National Institute of Health (NIH) guidelines. For each experimental design animals were age-matched littermates, and body weights were recorded.

2.2. Basal blood glucose titers and glucose tolerance tests

Blood glucose was measured by a One Touch® Ultra™ glucose meter (Lifescan, Scotland Ltd) from tail blood. For the glucose tolerance test (GTT) intraperitoneal (i.p.) glucose (2 g/kg body weight) was injected to overnight fasted mice (15–18 h) and blood glucose levels were evaluated at 0, 30, 60 and 75 min post glucose administration (Bonaventura et al., 2008). The GTTs were only performed until 75 min, even though control animals had not regained basal glucose levels, because in previous preliminary experiments we had determined that the effect of baclofen on glucose levels did not last more than 2 h. As baclofen is injected 20 min before the glucose overload, by 75 min after the glucose administration we were near the end of effect of the drug. This endpoint was then used in all the following experiments.

2.3. Insulin determination and insulin secretion test (IST)

Serum insulin was measured with a mouse insulin ELISA kit (Chrystalchem, Chicago, Il) at 0, 10, 20, 30 and 60 min after the i.p. glucose injection of 2 g/kg body weight or 3 g/kg body weight in mice fasted for 15–18 h, depending on the experiment (Bonaventura et al., 2008).

2.4. Insulin tolerance test (ITT)

ITTs were performed in animals chronically treated with agonists and antagonists of the $GABA_B$ receptor. Blood glucose was measured as above in 2–4 h fasted mice after 0, 10, 20, 30 and 60 min of an i.p. injection of 1 U/kg body weight of human insulin (a gift from Laboratorios Beta, Buenos Aires, Argentina) (Bonaventura et al., 2008).

2.5. Body weight and food intake

Body weight and food intake were monitored in chronically treated animals. Food intake was informed relative to body weight (g of food/g body weight).

2.6. Treatment with $GABA_B$ receptor agonists and antagonists

Acute treatments: baclofen was used as a $GABA_B$ receptor agonist (Lioresal, generously donated by Novartis, Argentina). Baclofen was dissolved in the minimal volume of acetic acid 0.01 M: ascorbic acid 0.1 M (100:1 v/v) and diluted in saline. First, a dose–response curve on the blood glucose response in a GTT was evaluated (2.5, 5.0 and 7.5 mg/kg body weight i.p.). For the following experiments the highest dose was chosen for presenting more consistent results, which in addition is in agreement with previous experiments in mice (Jacobson et al., 2006). CGP55845 ((2*S*)-3-[[(1*S*)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid hydrochloride) and 2-hydroxysaclofen were used as $GABA_B$ receptor antagonists (TOCRIS, Ellisville, MO, USA), the former dissolved in the minimal volume of DMSO and diluted with saline, and the latter was dissolved in saline. Several doses were tested, both alone and prior to baclofen administration (CGP55845: 0.1 and 1 mg/kg body weight; 2-hydroxysaclofen: 10 and 15 mg/kg body weight, both i.p.).

Baclofen was administered 20 min before the glucose overload and the receptor antagonists CGP55845 or 2-hydroxysaclofen 30 min before baclofen. The *per se* effects of these receptor antagonists were also analyzed, administered 30 min before the glucose overload. In all the experiments animals injected with vehicle were used as controls.

Chronic treatments: a group of mice was injected with baclofen administered in a single, daily s.c. dose of 5 mg/kg body weight for 18 consecutive days. Another group of animals was injected with 2-hydroxysaclofen (10 mg/kg body weight s.c. for 18 days), and controls were injected with vehicle.

On days 15 to 17 of treatment, a GTT/IST and an ITT were performed, in the absence of the drug, since the last dose had been injected approximately 20 h before. Evolution of the body weight and food intake was evaluated during the treatment. At the end of treatments, animals were sacrificed by decapitation and blood was collected for hormones determinations.

2.7. Serum hormones determinations

Basal levels of corticosterone, prolactin, insulin-like growth factor I, growth hormone, and glucagon were determined by RIA in serum of animals treated chronically with $GABA_B$ receptor agonists and antagonists. Animals were sacrificed in minimal conditions of stress by decapitation at the end of the treatments, after approximately 20 h of the last drug dose and 48 h after the last *in vivo* test. Glucagon was determined by RIA (Glucagon RIA KIT, Cat# GL-32 K, Millipore, Billerica, MA, EEUU). For corticosterone determination, the samples were extracted with dichloromethane and determined by RIA as previously described (Repetto et al., 2010). Growth hormone and prolactin were also determined by RIA as previously described (Catalano et al., 2005). For insulin-like growth factor I, serum was extracted with acid ethanol and determined by RIA as previously described (Diaz-Torga et al., 2002).

2.8. Glucose stimulated insulin secretion on in vitro incubated Langerhans islets

Pancreatic islets were isolated from 2 to 3 month-old male mice as described previously (Bonaventura et al., 2008). Briefly, 3 ml of collagenase (0.6 mg/ml; Sigma) was injected into the pancreatic duct, and pancreatic tissue was gently removed and digested in collagenase solution at 37 °C for 10–15 min. The digestion was stopped by ice-cold RPMI 1640 supplemented with 10% fetal bovine serum. Islets were then handpicked under a dissecting microscope and incubated overnight in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics.

After the overnight incubation, islets were handpicked into Krebs–Ringer bicarbonate (KRB) buffer containing 2 mM glucose for 1 h

preincubation at 37 °C. They were then transferred to 1.5 ml microcentrifuge tubes (5 islets/tube) containing 500 µl of KRB buffer with 2 mM or 20 mM glucose in the presence or absence of 1.10^{-5} M baclofen or 2-hydroxysalcofen. After 1 h incubation, 300 µl supernatant *per* sample was collected and frozen at −20 °C for insulin determination by RIA.

The microcentrifuge tubes were centrifuged for 10 min at 3000 rpm, the rest of the supernatant was discarded, and acid-ethanol was added (250 µl/sample; ethanol:H_2O:HCl, 150:47:3). The samples were kept overnight at 4 °C, neutralized with an equal volume of Tris.Base 0.85 M, and used for measuring total insulin content in islets.

Insulin was measured by RIA, as previously described (Bonaventura et al., 2008), using human insulin for iodination and standard, provided by Laboratorios Beta, and anti-bovine insulin antibody (Sigma, St. Louis, MO). All samples were evaluated in the same RIA. The minimum detectable concentration was 2 ng, and the intra-assay coefficient of variation was 6.8%.

Insulin results are expressed as the relationship between secreted insulin and insulin content *per* sample.

2.9. RNA isolation and gene expression by semi quantitative RT-PCR

Mouse Langerhans islet pools, following the *in vivo* chronic treatments with baclofen, 2-hydroxysaclofen or saline (as described above) and then isolated (as described above) were immediately handpicked into microtubes containing 800 µl of TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH). Total RNA was isolated according to the manufacturer's protocol. 0.75 µg of RNA was reverse-transcribed in a 20 µl reaction using M-MLV reverse transcriptase (Epicentre, Madison, WI) and random primers (Biodynamics SRL, Buenos Aires, Argentina). For assessment of $GABA_{B2}$ receptor subunit and glyceraldehyde-3-phosphate dehydrogenase (GADPH, control gene) expressions semi-quantitative (sq) RT-PCR was used.

Primers were designed with the Primer Blast program (NCBI): $GABA_{B2}$: forward: GGCTACATCGGAGTGG, reverse: TCGATTCTCTTTCGTATTGCT. GADPH: forward: CCAGAACATCATCCCTGCAT, reverse: GTTCAGCTCTGGGATGACCTT. The densities of bands were measured with the software Scion Image (NIH). Results of $GABA_{B2}$ mRNA quantifications were expressed relative to the control gene in arbitrary units (A.U.).

2.10. Statistical analysis

All results are expressed as means ± S.E.M.. Statistical analyses were performed with Statistica Six Sigma Edition. The differences between means were analyzed by one-way or two-way analysis of variance (ANOVA), followed by Newman–Keuls test or Tukey HSD test for unequal N. For multiple determinations in the same animal, two-way ANOVA with repeated measures design was used, followed by the same post-hoc tests. $P<0.05$ was considered statistically significant.

3. Results

3.1. Acute treatments with agonists and antagonists of $GABA_B$ receptors

3.1.1. Effect of acute administration of baclofen on blood glucose levels

Doses were chosen based on previous works from our laboratory (Rey-Roldán et al., 1997) and from others (Jacobson et al., 2006). Fig. 1 shows that during the i.p. GTTs pre-injection with baclofen, a $GABA_B$ receptor agonist, induced a significant dose-dependent impairment in the recovery of blood glucose baseline levels after the glucose overload, without modifying either basal titers or the maximal glucose levels attained (Fig. 1).

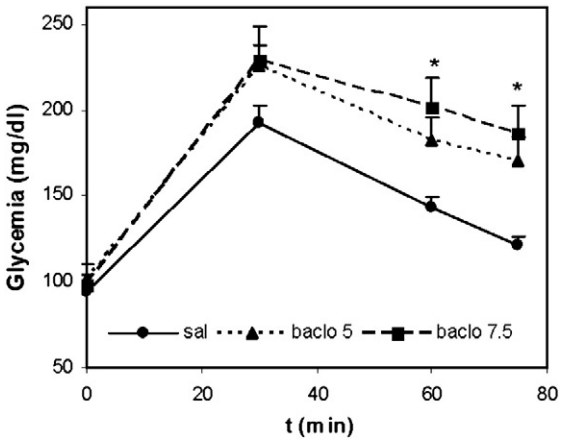

Fig. 1. Dose response curves of the effect of baclofen on glucose tolerance tests (GTT) in BALB/c male mice. Glucose (2 g/kg, i.p.) was injected in fasted mice after i.p. preinjection with baclofen [baclo 7.5 mg/kg (■) and 5 mg/kg (▲)], or saline [sal (●)] 20 min before glucose administration; $n=8$–10 mice per group. Two-way ANOVA with repeated-measures design: interaction, $P<0.01$, * = baclo 7.5 significantly different from saline at 60 ($P<0.02$) and 75 ($P<0.003$) min.

With the 2.5 mg/kg body weight dose of baclofen, no alterations in the glucose curve were observed with respect to saline injected controls (data not shown). With the 7.5 mg/kg body weight dose a sustained alteration in glucose depuration was observed, with significant differences at 60 and 75 min compared to saline injected controls, whereas the 5 mg/kg body weight dose induced a similar pattern of the glucose curve that did not attain statistical significance.

3.1.2. Effect of acute 2-hydroxysaclofen administration on the GTT

In the case of 2-hydroxysaclofen, we observed a clear dose-dependent *per se* effect on the GTT, improving glucose clearance, attaining statistical significance with the higher dose at all time points, as shown in Fig. 2. Furthermore, at 75 min, the animals treated with 15 mg/kg body weight of 2-hydroxysaclofen recovered basal glucose levels while controls were still slightly hyperglycemic (Fig. 2, left panel, a = different from basal, $P<0.05$).

In addition, 15 mg/kg body weight of 2-hydroxysaclofen induced a complete reversion of the hyperglycemic effect induced by baclofen in a GTT (Fig. 2, right panel). CGP55845 did not *per se* modify the glucose curve in the GTT but partially inhibited the baclofen induced glucose intolerance [at 75 min: blood glucose (mg/dl): baclofen: 243 ± 22 (n = 12), baclofen–CGP55845: 208 ± 16 (n = 12) and sal: 181 ± 17 (n = 12); baclofen vs. saline, $P<0.05$; CGP55845–baclofen vs. saline or vs baclofen, ns]. As results with this last receptor antagonist were not as consistent as with 2-hydroxysaclofen, they were not used in the following experiments.

3.1.3. Acute $GABA_B$ analog administration alters glucose-induced insulin secretion

Next we evaluated whether these drugs induced alterations in insulin secretion. Only the doses which provoked clear alterations on GTTs were used. Fig. 3 shows that baclofen induced a significant decrease in 3 g/kg body weight glucose-stimulated insulin secretion at 30 and 60 min post glucose overload, without affecting basal levels (Fig. 3, left panel) [two-way ANOVA with repeated-measures design: interaction: ns, treatment factor, $P<0.01$, baclofen significantly different from saline, ($P<0.02$) or 2-hydroxysaclofen ($P<0.01$)]. The baclofen-induced increase in glucose levels was more marked than when injected with a 2 g/kg overload (Fig. 3, right panel).

There was a partial increase in insulin secretion in animals treated with 15 mg/kg body weight 2-hydroxysaclofen, although it did not achieve statistical significance (Fig. 3, left panel). Blood glucose was lower than in controls, particularly at 75 min, where it reverted to

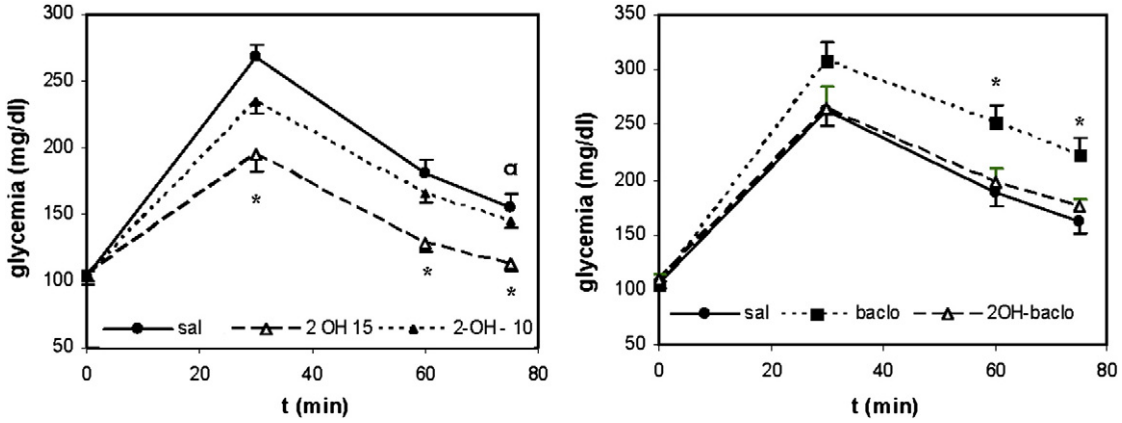

Fig. 2. Left panel: Dose response curves of the effect of 2-hydroxysaclofen on GTTs in BALB/c male mice. Glucose (2 g/kg, i.p.) was injected in fasted mice after i.p. preinjection with 2-hydroxysaclofen [2 OH 15 mg/kg (Δ) and 10 mg/kg (▲)], or saline [sal (●)] 30 min before glucose administration; $n=8$–10 mice per group. Two-way ANOVA with repeated-measures design: interaction, $P<0.01$, * = 2 OH-15 significantly different from saline at 30 ($P<0.01$) 60 ($P<0.01$) and 75 ($P<0.02$) min, a = different from basal in sal ($P<0.01$) and in 2 OH 10 ($P<0.01$). Right panel: Reversion of the effect of baclofen on GTTs by0 pretreatment with 2 OH. Glucose (2 g/kg, i.p.) was injected in all fasted mice after i.p. preinjection with baclofen [7.5 mg/kg body weight, 20 min before, saline-baclo (■)] or 2 OH [15 mg/kg, 50 min before] plus baclofen (as above) [2 OH-baclo (Δ)] or saline, as control [saline-saline, sal (●)]; $n=8$–10 animals per group. Two-way ANOVA with repeated-measures design: interaction, $P<0.01$, * = baclo significantly different from saline at 60 ($P<0.01$) and 75 ($P<0.01$) min.

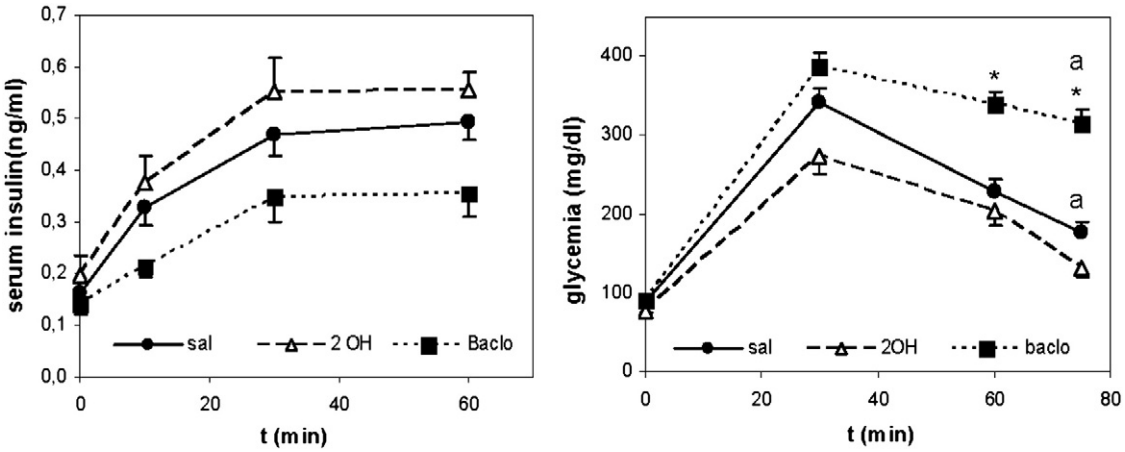

Fig. 3. Effect of baclofen (7.5 mg/kg body weight) and 2 OH (15 mg/kg body weight) on an insulin secretion test (glucose overload: 3 mg/kg i.p.), $n=8$ mice per group. Left panel: insulin secretion, two-way ANOVA with repeated-measures design: interaction: ns, treatment factor, $P<0.01$, baclo (■) significantly different from saline (●) ($P<0.02$) or 2 OH (Δ) ($P<0.01$). Right panel: glucose levels, two-way ANOVA with repeated-measures design, interaction: $P<0.01$. * = baclo at 60 min significantly different from sal ($P<0.01$) and 2 OH ($P<0.01$) and also at 75 min $P<0.01$ and $P<0.01$ respectively, a = significantly different from basal for baclo ($P<0.01$) and saline ($P<0.01$).

basal levels, while in control animals titers were still significantly higher at that time (Fig. 3, right panel).

3.1.4. Effect of Baclofen and 2-hydroxysaclofen on glucose stimulated insulin secretion in islets incubated in vitro

To determine whether the effects of these treatments were exerted directly at pancreatic level, we investigated the effects of $GABA_B$ analogs on isolated islets cultured *in vitro*. Fig. 4 shows that baclofen inhibited 20 mM glucose-stimulated insulin secretion without affecting basal levels. 2-Hydroxysaclofen reversed the effect of baclofen on glucose stimulated insulin secretion, without modifying basal levels (Fig. 4).

3.2. Chronic treatments with $GABA_B$ analogs alter glucose homeostasis

3.2.1. Effect of chronic administration of $GABA_B$ receptor agonists and antagonists on GTTs and insulin secretion

After 15 days of treatment with baclofen or 2-hydroxysaclofen, basal non-fasted glucose levels did not vary among groups [blood glucose (mg/dl): sal: 120 ± 6 ($n=9$), baclo: 124 ± 5 ($n=10$) and 2-hydroxysaclofen: 127 ± 6 ($n=10$), one-way ANOVA: ns]. GTTs performed on overnight-fasted animals are shown in Fig. 5. The last dose of the analog had been administered approximately 20 h before the assay. Both treatments produced significant increases in blood glucose excursion curves post glucose overload compared with saline,

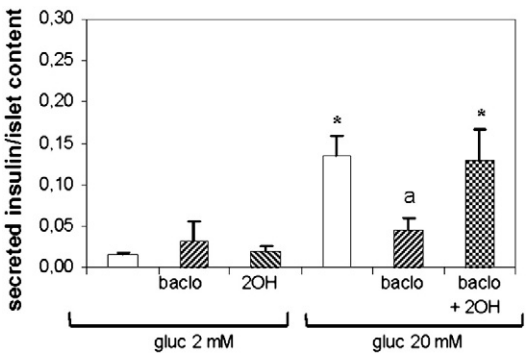

Fig. 4. Effect of baclofen (1.10^{-5} M) and 2 OH (1.10^{-5} M) on insulin secretion in isolated islets incubated *in vitro* ($n=4$–6) under low (gluc: 2 mM) and high glucose (gluc: 20 mM) levels. One way ANOVA: $P<0.01$. * = gluc 2 mM different from gluc 20 mM ($P<0.01$) or gluc 20 mM + baclo + 2 OH ($P<0.01$), a = different from glucose 20 mM ($P<0.02$).

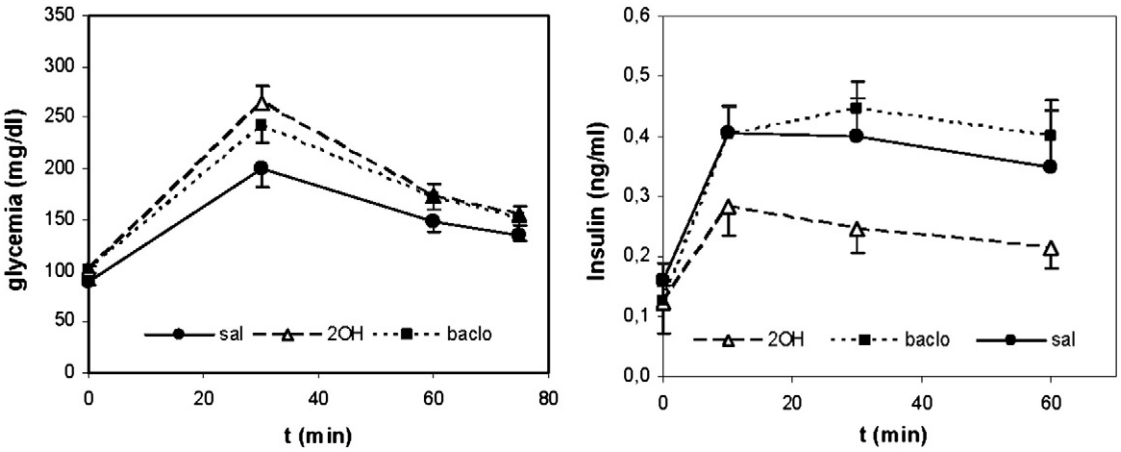

Fig. 5. Effect of chronic treatment with baclofen or 2 OH on an insulin secretion test (glucose overload: 2 mg/kg i.p.). Animals were treated for 15 days with one daily dose of baclofen (5 mg/kg, s.c. ■), 2 OH (10 mg/kg, s.c. Δ) or saline (●), $n = 10$. Left panel: glucose levels, two-way ANOVA with repeated-measures design: interaction: ns, treatment factor: $P<0.03$, 2 OH ($P<0.03$) and baclofen ($P<0.05$) different from saline. Right panel: insulin secretion, two-way ANOVA with repeated-measures design: interaction: ns, treatment factor: $P<0.05$, 2 OH different from saline ($P<0.05$).

without modifying basal titers. The maximum difference was observed at 30 min (Fig. 5, left panel) [two-way ANOVA with repeated-measures design: interaction: ns, treatment factor: $P<0.03$; 2-hydroxysaclofen ($P<0.03$) and baclofen ($P<0.05$) different from saline].

To determine if the alterations observed in GTTs were due to alterations in insulin secretion, this hormone was measured in samples obtained along the GTT, as shown in Fig. 5. There were no significant differences in insulin secretion in baclofen treated animals with regard to controls, while in 2-hydroxysaclofen treated animals the glucose-stimulated insulin secretion was markedly blunted, without affecting basal levels (Fig. 5, right panel) [two-way ANOVA with repeated-measures design: interaction: ns, treatment factor: $P<0.05$; 2-hydroxysaclofen different from saline ($P<0.05$)].

3.2.2. Effects of chronic treatment with $GABA_B$ analogs on insulin sensitivity in peripheral tissues

In Fig. 6 we determined if treatments had effects on peripheral tissue sensibility to insulin. Therefore, ITTs were performed on day 17 on animals treated with $GABA_B$ receptor agonists and antagonists, as well as controls injected with saline. The last dose of the analog had been administered approximately 20 h before the assay. We did not observe significant differences between the treatments at the studied times (Fig. 6).

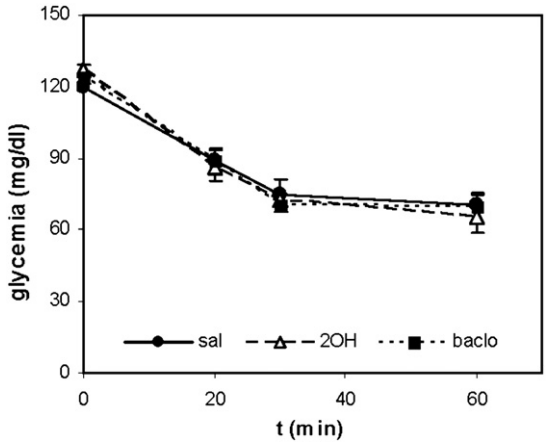

Fig. 6. Effects of chronic treatment with baclofen (■), 2 OH (Δ) or saline (●) on insulin sensitivity (ITT), $n = 10$ mice per group. Two-way ANOVA with repeated-measures design: ns.

This result indicates that the treatments had no adverse effect on the peripheral sensibility to insulin since insulin mediated glucose depuration was not altered. They also confirm that alterations in glucose homeostasis observed with $GABA_B$ analogs are mainly due to alterations in the secretion and/or regulation of hormones related to glucose homeostasis control.

3.2.3. Effects of chronic treatments on body weight and food intake

There are contradictory results in the literature related to the role of the $GABA_B$ receptor in the regulation of food intake. We evaluated if chronic treatments were altering the above-mentioned parameter. We observed no significant differences in food intake between groups during the period studied (Fig. 7, left panel). Body weight was also monitored during treatments, but no differences were observed, in agreement with results on food intake (Fig. 7, right panel).

3.2.4. Basal hormonal serum levels

Taking into account that alterations in glycemia in baclofen chronically-treated animals were not related to alterations in insulin secretion or insulin sensitivity, we determined whether this was due to alterations in other hormones involved in glucose homeostasis regulation. It has been described that glucocorticoids produce hyperglycemia and that activation of $GABA_B$ receptors may alter the corticotropic axis (Hausler et al., 1993; Marques and Franci, 2008), so we evaluated basal corticosterone secretion in animals chronically treated with $GABA_B$ analogs. Despite not attaining statistical significance, there was a tendency in baclofen-treated animals to have higher basal serum corticosterone values (Fig. 8, panel A).

Glucagon, the main anti-insulin hormone, is known to be regulated by GABA through $GABA_A$ receptors in α cells, but, as prolonged treatments with GABA analogs may change GABA content as well as the expression of its receptors at the endocrine pancreas, we evaluated basal glucagon levels. We found no differences in basal glucagon serum levels among treatments (Fig. 8, panel B). We also determined serum levels of other hormones related to glucose homeostasis that are known to be regulated by $GABA_B$ receptors, such as growth hormone (Muller et al., 1999; Passariello et al., 1982) and prolactin (Brelje et al., 2004; Ferreira et al., 1998; Rey-Roldán et al., 1996), and we found no differences in basal levels of these hormones after chronic treatments (Fig. 8, panel C and D). Finally, we also evaluate serum insulin growth factor-I, which is known to promote islet cell growth and maintenance (Guo et al., 2005) and has also been described to be altered by chronic treatment with baclofen in humans (Bauman et al., 2006). We found no differences in basal levels of this hormone in our experimental model (Fig. 8, panel E).

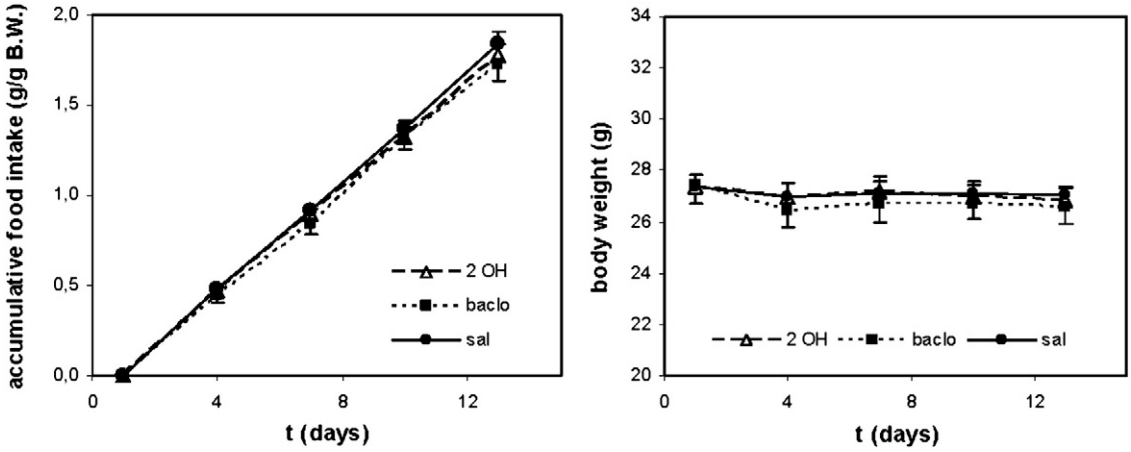

Fig. 7. Accumulative food intake (left panel) and body weight (right panel) in chronically treated animals with baclo (■), 2 OH (Δ) or saline (●), $n = 10$ mice per group. One-way ANOVA with repeated-measures design: ns in both cases.

3.2.5. Islet $GABA_{B2}$ receptor subunit mRNA expression after chronic treatments with $GABA_B$ analogs

To determine whether chronic exposure to $GABA_B$ receptor agonists or antagonists had an effect on $GABA_B$ receptor expression, the $GABA_{B2}$ subunit mRNA was evaluated by sq RT-PCR. In islets obtained from chronically injected animals with baclofen, 2-hydroxysaclofen or saline, $GABA_{B2}$ mRNA expression was not altered ($GABA_{B2}$ mRNA/GADPH mRNA (AU): saline: 0.36 ± 0.02 (n = 5), baclofen: 0.31 ± 0.03 (n = 4), 2-hydroxysaclofen: 0.35 ± 0.03 (n = 5), ANOVA: ns).

4. Discussion

The presence in the endocrine pancreas of a complete GABAergic system has been amply documented. Nevertheless, an in-depth comprehension of the importance of pancreatic GABA in the control of glucose homeostasis is still lacking. As mentioned before, recent studies have pointed to an autocrine inhibitory role of β-cell-secreted GABA on insulin secretion by acting on $GABA_B$ receptors (Braun et al., 2004b; Brice et al., 2002; Gu et al., 1993). An intra-islet paracrine inhibitory role for GABA on glucagon secretion by acting on $GABA_A$ receptors in α cells was also reported (Gilon et al., 1991; Rorsman et al., 1989; Wendt et al., 2004; Xu et al., 2006). Although these *in vitro* studies point to a specific role for GABA in the control of insulin/glucagon release, the importance of this neurotransmitter in the *in vivo* condition, where complex signals regulating glucose homeostasis converge, remains to be elucidated.

Previous results from our laboratory show that the absence of functional $GABA_B$ receptors produced profound alterations on glucose homeostasis *in vivo* in $GABA_{B1}$ knockout mice (Bonaventura et al., 2008). Therefore, we decided to study the effects of $GABA_B$ receptor activation with pharmacological acute and chronic treatments with receptor agonists and antagonists on glucose homeostasis *in vivo* in normal mice.

Acutely administered baclofen did not affect basal or maximal glucose levels attained in a GTT but dose-dependently inhibited the reinstatement of basal serum glucose titers 60 and 75 min after the glucose challenge, similar to what we had previously observed with only one dose of the receptor agonist (Bonaventura et al., 2008). Other groups had shown a lack of effect of baclofen on blood glucose in a GTT, though a single low dose and a single time point were evaluated (Gomez et al., 1999); in addition, those results were obtained in rats and species differences may occur. Furthermore, we observed an acute dose-dependent *per se* effect of the $GABA_B$ receptor antagonist 2-hydroxysaclofen, diminishing glycemia on a GTT, without altering basal levels. In addition, the 15 mg/kg body weight 2-hydroxysaclofen exerted a pronounced reversion of the hyperglycemic effect of baclofen on the GTT, thus demonstrating that the effect of baclofen was specific by acting on peripheral $GABA_B$ receptors since 2-hydroxysaclofen does not cross the blood brain barrier. Nevertheless, a central action of baclofen cannot be discarded.

Baclofen induced an inhibition of glucose-stimulated insulin secretion in mice, in agreement with other *in vitro* models (Braun et al., 2004b; Brice et al., 2002; Gu et al., 1993). 2-hydroxysaclofen increased insulin secretion in the insulin secretion test, although it did not attain statistical significance; in this experimental condition insulin is maximally stimulated by glucose and it is therefore difficult to increase its levels. To our knowledge there are no other reports studying the *in vivo* effects of 2-hydroxysaclofen on glycemia or insulin secretion. Braun et al. showed that addition of CGP55845 (another $GABA_B$ receptor antagonist) resulted in enhanced glucose-stimulated insulin secretion in rat islets (Braun et al., 2004b), which is in agreement with our findings. Given these results, we performed an *in vitro* assay with mice islets stimulated with glucose in presence or absence of baclofen and 2-hydroxysaclofen. Basal insulin secretion in the presence of 2 mM glucose was unaffected by either treatment. Baclofen suppressed 20 mM glucose-stimulated insulin secretion and this inhibitory action was fully antagonized by 2-hydroxysaclofen. These results demonstrate that the compounds used exert their effects directly on islet $GABA_B$ receptors, in agreement with previous reports (Braun et al., 2004b; Brice et al., 2002; Gu et al., 1993), and confirming our *in vivo* observations.

In view of these results, we studied the effects of prolonged treatments with these compounds on glucose homeostasis, since the inconsistencies surrounding chronic treatments with $GABA_B$ receptor agonists or antagonists have remained largely unresolved.

Chronic administration of baclofen has been shown to decrease the number of $GABA_B$ receptors associated to the pharmacological effects of this agent (Enna et al., 1998; Malcangio et al., 1993) and also to induce the desensitization of the receptor without altering the mRNA expression of the $GABA_B$ subunits (Sands et al., 2003), while others reported lack of effects in different brain areas (Motohashi, 1992). Pratt et al. suggested an increase in the number of $GABA_B$ receptors in prefrontal cortex lamina I after repeated oral administration of 10 mg/kg baclofen, although not achieving statistical significance (Pratt and Bowery, 1993). This discrepancy may point to different sensitivities between $GABA_B$ receptors from different cell types or sub-classes of $GABA_B$ receptors (Schwenk et al., 2010), as well as the contribution of the experimental model, administration routes or doses used, and merits further analysis.

Controversy also exists regarding the effect of receptor antagonists on $GABA_B$ receptor expression. Some works point to an up-

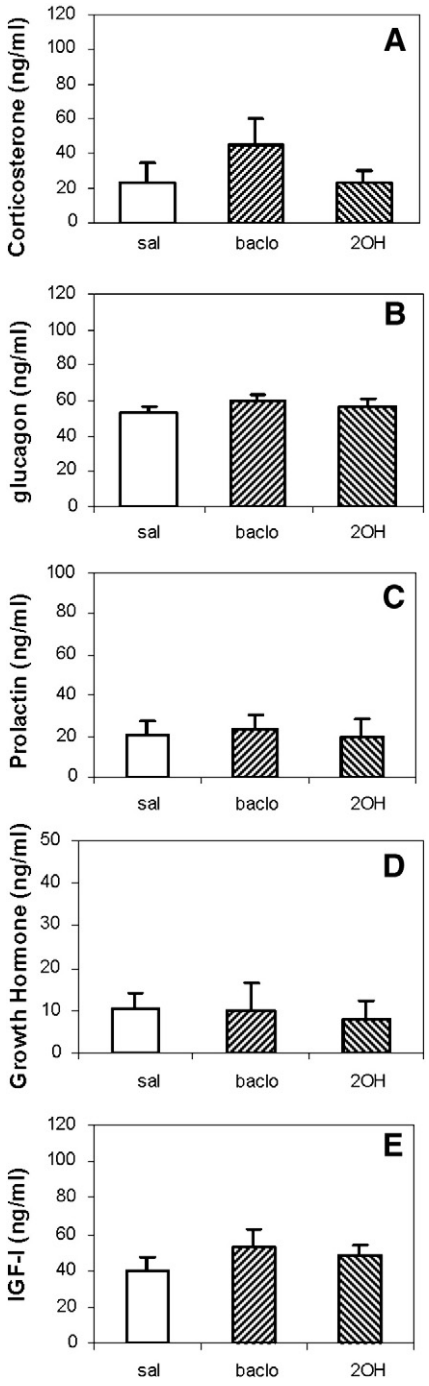

Fig. 8. Basal levels of serum corticosterone, glucagon, prolactin, growth hormone and insulin growth factor-I (IGF-I) in chronically treated animals with baclo (▨), 2OH (▧) or saline (□), $n = 10$ mice per group. One-way ANOVA with repeated-measures design: ns in both cases.

regulation of $GABA_B$ binding after chronic treatment with the receptor antagonist SCH50,911 ((2*S*)-(+)-5,5-Dimethyl-2-morpholineacetic acid), indicating that persistent blockade of $GABA_B$ receptors leads to their compensatory up-regulation, and suggest that $GABA_B$ receptors are tonically activated by endogenous GABA (Pibiri et al., 2005). Others have observed increased binding or lack of effect depending on the receptor antagonist used (Pratt and Bowery, 1993). The studies named above were performed in the CNS; few works provide an analysis of the effects of $GABA_B$ receptor agonists and/or antagonists on receptor expression and/or function in peripheral tissues (Blandizzi et al., 1995; Piqueras and Martinez, 2004; Reis and Duarte, 2006).

For evaluation of long-term effects, the last dose was administered approximately 20 h before the functional tests were performed, so it can be assumed that the plasma concentration at the moment of the assay was negligible (Saulino and Jacobs, 2006).

The chronic treatment with 2-hydroxysaclofen produced glucose intolerance observed on the GTTs, where the glucose overload produced a higher increase in blood glucose in treated animals compared to controls, without modifying basal levels; in these animals insulin secretion was impaired, explaining the GTTs observed. It is interesting to note that these effects were opposite to those observed with the acute administration of the receptor antagonist. A possible explanation for these results could be the up-regulation of β-cell $GABA_B$ receptors in response to chronic exposure to the receptor antagonist. In response to glucose overload, β-cells secrete insulin as well as GABA, having the latter an autocrine effect inhibiting insulin secretion through $GABA_B$ receptors. The presence of a higher number of $GABA_B$ receptors on β-cells could enhance this inhibition, diminishing serum insulin levels and explaining the response to the glucose overload in these animals.

Our results show that chronic administration of the $GABA_B$ receptor agonist, baclofen, or the receptor antagonist, 2-hydroxysaclofen, did not modify $GABA_{B2}$ subunit mRNA expression in isolated islets. Since $GABA_B$ receptor modulation may occur during prolonged exposure to $GABA_B$ analogs (Pibiri et al., 2005; Pratt and Bowery, 1993), our results suggest that this regulation is primarily due to nongenomic mechanisms, as proposed by Sands et al.(2003).

Surprisingly, chronic baclofen administration also produced glucose intolerance, altering the GTT, but to a lesser degree than the receptor antagonist. In fact, this alteration was not accompanied by alterations in insulin secretion or sensitivity. In this context it is important to take into account that 2-hydroxysaclofen does not cross the blood–brain barrier, so it can be inferred that its effects are exclusively peripheral. On the contrary, baclofen can cross the blood–brain barrier and therefore the observed effects could be attributed to the integration of multiple actions on peripheral and central tissues. Therefore, the alterations observed in the GTTs of baclofen-treated animals must be attributed to other hyperglycemic factor/s.

Since several authors propose the activation of hypothalamic–pituitary–adrenal axis by peripheral baclofen administration (Hausler et al., 1993), one possible hyperglycemic factor involved could be corticosterone. We observed a small increase in serum corticosterone in baclofen-treated animals, although it did not achieve statistical significance, possibly because of high serum variability due to its circadian rhythm of secretion (Ambrogini et al., 2002). No alterations in corticosterone levels were observed in 2-hydroxysaclofen-treated animals.

In addition, glucagon, growth hormone, insulin growth factor-I and prolactin, all hormones related to glucose homeostasis regulation, were not altered in baclofen or 2-hydroxysaclofen chronically-treated animals.

Our results suggest that the alterations observed with chronic $GABA_B$ analogs administration were due to their actions on hypo/hyperglycemic factors, since ITTs were not altered, indicating that the treatments did not modify insulin sensitivity at peripheral targets.

It has been reported that baclofen increases short-term food intake but does not modify accumulative food intake in rats (Patel and Ebenezer, 2010), while 3-APPA ($GABA_B$ receptor agonist that does not cross the blood–brain barrier), did not alter food intake, suggesting that the effect is exerted at CNS (Ebenezer and Patel, 2004). In contrast in humans baclofen and gabapentin, another widely used $GABA_B$ receptor agonist (Parker et al., 2004), have shown anorexigenic effects (Guardia et al., 2011; Rutecki and Gidal, 2002). Given this controversy, we evaluated body weight and food intake during the chronic treatments with $GABA_B$ receptor agonists and antagonists but no differences among treatments were found.

5. Conclusion

Our observations demonstrate that peripheral $GABA_B$ receptors are involved in the regulation of glucose homeostasis *in vivo*. Furthermore, these results alert to the potential side effects of $GABA_B$ drug administration on glucose homeostasis, and point to the need to evaluate the metabolic state of patients, particularly regarding the diabetic or prediabetic states. Both agonists and antagonists of $GABA_B$ receptors are in use or are being evaluated for future clinical uses. The former, such as baclofen, are used e.g. in the treatment of spasticity and trigeminal neuralgia and are being evaluated for the treatment of drugs of abuse dependence, as baclofen has been shown to decrease craving for various drugs. The latter, such as the receptor antagonist SGS742 (3-aminopropyl-*n*-butyl phosphinic acid), are in advanced clinical trials for cognitive impairment (Bowery, 2006).

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