Pioglitazone acutely reduces energy metabolism and insulin secretion in rats

Short running title: Pioglitazone and metabolic deceleration

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ABSTRACT

Our objective was to determine if the insulin-sensitizing drug pioglitazone acutely reduces insulin secretion and causes metabolic deceleration \textit{in vivo} independently of change in insulin sensitivity. We assessed glucose homeostasis by hyperinsulinemic-euglycemic and hyperglycemic clamp studies, and energy expenditure by indirect calorimetry and biotelemetry in male Wistar and obese hyperinsulinemic ZDF rats 45 min after a single oral dose of pioglitazone (30 mg/kg). \textit{In vivo} insulin secretion during clamped hyperglycemia was reduced in both Wistar and ZDF rats following pioglitazone administration. Insulin clearance was slightly increased in Wistar but not in ZDF rats. Insulin sensitivity in Wistar rats assessed by the hyperinsulinemic-euglycemic clamp was minimally affected by pioglitazone at this early time point. Pioglitazone also reduced energy expenditure in Wistar rats without altering respiratory exchange ratio or core body temperature. Glucose-induced insulin secretion and oxygen consumption were reduced by pioglitazone in isolated islets and INS832/13 cells. In conclusion, pioglitazone acutely induces whole body metabolic slowing and reduces glucose-induced insulin secretion, the latter being largely independent from the insulin-sensitizing action of the drug. The results suggest that pioglitazone has direct metabolic deceleration effects on the β-cell that may contribute to its capacity to lower insulinemia, and anti-diabetic action.
Major drugs developed to treat type 2 diabetes aim at either increasing insulin secretion or reducing insulin resistance (1-4). Two classes of insulin-sensitizing agents are currently used: the biguanides (metformin) and the thiazolidinediones (TZDs), of which the only one still recommended for use in some countries is pioglitazone (5). TZDs are peroxisome proliferator-activated receptor γ (PPARγ) agonists. They stimulate adipocyte differentiation, relieving other tissues from fat excess, thereby reducing their resistance to insulin (6; 7). TZDs’ beneficial effects are not limited to increased insulin sensitivity but also include preservation of β-cell function (8). It is thought that the beneficial effect of TZDs on β-cell function in vivo is indirect and occurs via a relief of the need for insulin hypersecretion due to their insulin sensitizing action. We should however consider the possibility that the classical anti-diabetic insulin sensitizers pioglitazone and metformin might also have beneficial effects on glucose homeostasis via direct reduction of insulin hypersecretion independently of insulin resistance.

We previously demonstrated in vitro that pioglitazone acutely slows down glucose and lipid metabolism in the β-cell and inhibits glucose-induced insulin secretion (GIIS) primarily at submaximal and much less at maximal glucose concentrations (right shift in the glucose dose response) via a PPARγ-independent mechanism (9). These acute effects of pioglitazone are likely due to complex I inhibition of the electron transport chain (10) and involve reduced glucose oxidation, decreased ATP levels and increased AMPK activation (9). Interestingly, metformin causes similar effects (JL and MP, unpublished). Hence, we proposed the novel concept of “metabolic deceleration” as a mode of action of some anti-diabetic drugs and suggested that the action of pioglitazone to reduce glucose metabolism and insulin secretion in the β-cell may partly explain its beneficial effects (9).
The concept that metabolic deceleration protects the β-cell from both oxidative and endoplasmic reticulum stress has recently been reviewed (11; 12).

In the present study we carried out *in vivo* experiments in normal Wistar and obese ZDF rats to better understand how acute treatment with pioglitazone alters glucose homeostasis, with particular focus on how it reduces hyperinsulinemia. The following questions were asked: 1) can we confirm *in vivo* our previous *in vitro* findings in isolated rat islet and β-cell line that pioglitazone acutely reduces insulin secretion?; 2) is this acute effect of pioglitazone on insulin secretion independent of its effects on insulin sensitivity?; and 3) does pioglitazone acutely slow down whole body energy metabolism?
RESEARCH DESIGN AND METHODS

Materials

Pioglitazone-HCl was suspended either at 5 mg/ml in 0.5% methyl-cellulose prepared in autoclaved tap water or at 10 mmol/l in DMSO, for in vivo and in vitro experiments, respectively. Stock solutions of GW1929 (Alexis Biochemicals, San Diego, CA) and CAY10599 (Cayman Chemical, Ann Arbor, MI) were prepared in DMSO at 1.2 and 15 mmol/l, respectively. Glycemia was monitored using a glucometer. Total plasma insulin, total insulin content and media insulin concentrations were determined by radioimmunoassay using human insulin standards (Linco Research, St. Charles, MO). ELISA was used to determine rat plasma C-peptide concentrations (both I and II; Mercodia AB, Uppsala, Sweden).

Animals and pioglitazone administration

All procedures were approved by the CRCHUM Institutional Committee for the Protection of Animals. 300-350 g-male Wistar rats (Charles River, St-Constant, QC, Canada) and 10-wk-old male obese Zucker Diabetic Fatty (ZDF) rats (Charles River, Kingston, NY) were housed under controlled temperature on a normal 12-h light–dark cycle with unrestricted access to water and standard chow. For glucose clamp procedures, animals underwent catheterization of the jugular vein and carotid artery as described (13) and recovered for 6 days. For indirect calorimetry studies, rats were acclimatized for 36 h in acrylic chambers bedded with hardwood chips. For biotelemetry studies, C50-PT probes (Data Sciences International, St. Paul, MN) were implanted with a catheter inserted through the femoral artery up to the abdominal aorta and the telemetry device body fixed to the muscle wall as described (14). Rats were allowed 10-13 days to recover
and received 5 mg/kg carprofen subcutaneous in the first 3 days. On experimental days, food was withdrawn at 07:00 and pioglitazone (30 mg/kg) or vehicle was administered by gavage (6 ml/kg) at 09:00. All animals were sacrificed at the end of procedures by pentobarbital overdose.

**Hyperglycemic clamps (HGC)**

Conscious rats were subjected to one-step (ZDF) or two-step (Wistar) HGC followed by an arginine bolus, in a technique modified from (13). The clamp procedure was started 45 min after the rats were gavaged with pioglitazone or vehicle. Blood glucose was clamped either at 16.7 mmol/l for 120 min (one-step) or at 8.3 mmol/l for 30 min and 16.7 mmol/l for another 30 min (two-step). A bolus of arginine (174 mg/kg) was injected at 121 min to assess total β-cell secretory capacity. Plasma samples (150 µl of blood, 11 samples) were collected from the carotid artery for insulin and C-peptide determinations before glucose infusion, during hyperglycemia and, 1 and 10 min after arginine injection. Insulin clearance was estimated by the C-peptide/insulin molar ratio (15). HGC with ZDF rats were all performed in 10-wk-old animals.

**Hyperinsulinemic-euglycemic clamps (HIEC)**

On the day of the experiment, the jugular vein catheter was connected to two syringes containing either insulin (Humulin-R®; Eli Lilly) or 20% dextrose, installed on a Harvard 33 Twin Syringe Pump. Insulin was prepared as follows: 20 µl of insulin 100 U/ml was added to 20 µl of rat plasma and diluted to 200 mU/l with saline. The clamp procedure started 45 min after the gavage of pioglitazone or vehicle in conscious animals by an insulin bolus (time 0 min) of 75 mU/kg/min for 45 s and 37.5 mU/kg/min for another 45 s, and the insulin infusion was then reduced to 5 mU/kg/min for the rest of the
procedure. After 5 min, a blood sample was collected and a 20% dextrose infusion was started to clamp blood glucose at pre-clamp level (6.5 mmol/l). Glycemia was monitored from samples collected every 5 min. Plasma samples (150 µl of blood) were collected from the carotid artery for insulin determination at 0 (before any infusion), 60, 90 and 120 min. The M/I index of insulin sensitivity was calculated by dividing the average glucose infusion rate during the last 60 min of the clamp (“M” expressed in µmol/kg/min) by the average circulating insulin value (“I” expressed in pmol/l) during the same time period (15).

**Indirect calorimetry**

Gas exchanges were measured for 6 h following gavage by pioglitazone or vehicle in an open circuit calorimeter with ambulatory activity monitoring using PhysioScan and VersaMax systems (AccuScan Instruments, Columbus, OH). Oxygen and carbon dioxide concentrations were analyzed in each cage by sequential readings from gas samples continuously withdrawn from cages at a rate of 2.0 l/min. Gas samples were compared to ambient air composition for VO$_2$ and VCO$_2$ calculations. The oxygen analyzer was calibrated before each experiment by adjusting respiratory quotient (VCO$_2$/VO$_2$) readings to 0.7 using two 24 h-fasted rats (not included in the study). Ambulatory activity was monitored based on infrared beam interruptions in horizontal and vertical positions. Gas fractions and flow rate were used to calculate energy expenditure (EE) as follows: 

$$EE = ((4.33*VO_2)+(0.67*VCO_2))*BW(\text{in kg})*60,$$

where gas fractions are expressed in ml/kg/min (16).

**Biotelemetry**
The C50-PT probe implanted rats had their core body temperature, heart rate and blood pressure values recorded every minute using Data Sciences system and averaged over 30 or 60 min for clarity. This was a cross-over study with all animals receiving pioglitazone and placebo in an alternating order, with a 10-day washout period in between. For each experimental period, data was collected for a total of 68 h from 19:00 with food being removed after 36 h, pioglitazone or vehicle being given by gavage after 38 h, and food being returned after 46 h.

**Cell culture**

INS832/13 cells (17) were cultured as described (9). Experiments were conducted in Krebs-Ringer bicarbonate buffer containing 10 mmol/l HEPES (pH 7.4) (KRBH) except during measurement of the oxygen consumption rate, where bicarbonate was omitted.

**Islet isolation and ex vivo insulin secretion measurement**

Islets were isolated from Wistar rats as previously described (9; 18). For ex vivo insulin secretion measurement, the islet isolation procedure was started 90 min after gavage. Batches of 10 islets were used for static insulin secretion measurements in KRBH as previously described (9).

**Oxygen consumption and insulin secretion in vitro**

Oxygen consumption rate (OCR) in vitro was measured using a XF24 respirometer (Seahorse Bioscience, Billerica, MA). INS832/13 cells were seeded a day before the experiments at 7x10^4 cells/well in XF24 microplates. Media were changed to RPMI 1640 supplemented as in (9) containing 3 mmol/l glucose 2 h prior to the experiments. Isolated islets were hand-picked into XF24 islet capture microplates, 3 h prior to the experiments, 75 islets/well in RPMI 1640 supplemented as in (9) containing 3 mmol/l glucose. Cells or
islets were then washed and incubated for 1 h (cells) or 25 min (islets) in KRBH containing no bicarbonate, 2 (cells) or 3 mmol/l (islets) glucose and 0.07% BSA (KRBH-XF) at 37°C under atmospheric CO\textsubscript{2} concentrations. After basal measurement for 20 min, pioglitazone or DMSO was added by 3 successive automatic injections of KRBH-XF to reach 25 (cells) or 50 µmol/l (islets) pioglitazone or equivalent DMSO volume. After a period of pre-incubation (20 min for cells and 1 h for islets), glucose was added by a fourth injection of medium to reach 8 or 25 mmol/l. OCR was measured for another 45 min after which media were collected to measure accumulated insulin secretion over the time of the total incubation (~150 min).

Testing of PPARγ agonists on insulin secretion in INS832/13 cells and isolated rat islets were performed as previously described for pioglitazone (9).

Statistical analysis

Statistical analysis was performed using Prism version 5.01 and InStat version 3.06 (GraphPad Software, San Diego, CA) with two-tailed unpaired Student’s test or, for multiple comparisons, Kruskal-Wallis test followed by Dunn’s post-hoc test, one-way ANOVA followed by Tukey post-hoc test, two-way repeated measures ANOVA or two-way ANOVA followed by Bonferroni post-hoc tests. Differences between groups were considered significant at \( P<0.05 \).
RESULTS

Pioglitazone acutely inhibits glucose-induced insulin secretion

We assessed the acute effect of pioglitazone on *in vivo* insulin secretion using HGC in Wistar rats (Fig. 1A). Pioglitazone-treated animals showed a lower insulinemia starting from the 75th min and throughout the second step of the clamp (Fig. 1C). During the latter, insulin levels in pioglitazone-treated rats were only about 50% of those of the vehicle-treated rats. The effect of the arginine bolus on insulinemia was not significantly changed by pioglitazone treatment (Fig. 1E). Similarly, plasma C-peptide was markedly reduced during the HGC (Fig. 1D) but not in response to arginine (Fig. 1E). This indicates an acute *in vivo* inhibitory effect of pioglitazone on GIIS. The glucose infusion rate during the HGC was the same in both groups (Fig. 1B).

The C-peptide/insulin molar ratio, an index of insulin clearance (19), was slightly elevated in pioglitazone-treated animals in comparison to control rats in the second step of the clamp (Fig. 1F), suggesting that increased insulin clearance could contribute to the lowered insulinemia observed in the pioglitazone-treated animals.

Islets from pioglitazone-treated and control animals were isolated 90 min after gavage and GIIS was assessed *ex vivo* after isolation. GIIS was unaltered by prior *in vivo* treatment with pioglitazone (data not shown). This lack of pioglitazone effect on *ex vivo* GIIS could be due to efflux of the drug from islets during their isolation, pre-incubation and incubation periods (approximately 5 h altogether).

**Acute pioglitazone administration has minimal effect on *in vivo* insulin sensitivity**

TZDs enhance insulin sensitivity under chronic treatment (20). We therefore performed HIEC to measure insulin sensitivity *in vivo* in Wistar rats. Both control and pioglitazone-
treated groups required similar rates of glucose infusion (Fig. 2B) to maintain euglycemia (Fig. 2A). However, the achieved insulinemia during the 60 min clamp period was slightly lower in the acute pioglitazone treated rats (Fig. 2C) such that the M/I measure of insulin sensitivity trended higher in the pioglitazone group, although the difference was not significant (Fig. 2D). The mildly reduced insulinemia achieved in pioglitazone-treated rats (at times 90 and 120 min) (Fig. 2C) occurred despite equivalent rates of insulin infusion. This could be due to increased insulin clearance in the pioglitazone treated Wistar rats (Fig. 1D).

**Pioglitazone acutely inhibits glucose-induced insulin secretion in ZDF rats**

With the objective to determine if pioglitazone can also reduce GIIS in an animal model of type 2 diabetes, we performed HGC in young obese ZDF rats at their transition phase to diabetes (Fig. 3A). At 10 weeks of age, ZDF rats show mild hyperglycemia, insulin resistance and hyperinsulinemia. The glucose infusion rate was the same in both the pioglitazone and control groups (Fig. 3B), suggestive of similar insulin sensitivity. Acute pioglitazone treatment in ZDF rats reduced glucose-induced increase in insulinemia and almost abolished glucose-induced C-peptide secretion (Fig. 3C, D). The increase in plasma insulin and C-peptide levels following arginine bolus were not significantly changed by pioglitazone (Fig. 3E). Insulin clearance was not changed in pioglitazone-treated ZDF rats (Fig. 3F).

**Pioglitazone administration acutely reduces whole body energy expenditure**

In our previous study employing isolated islets and INS832/13 β-cells, we hypothesized that pioglitazone-induced inhibition of GIIS could be due to metabolic deceleration of the
β-cell (9). We therefore assessed if acute reduction in whole body energy metabolism occurs in vivo in response to acute pioglitazone administration.

A first approach was to use indirect calorimetry. Energy expenditure (Fig. 4C), calculated from VO$_2$ (Fig. 4A) and VCO$_2$ (Fig. 4B) values, was elevated after restraining and gavaging the animal, and slowly decreased and became stable within 2 h. Starting from 1 h after gavage to 5 h post gavage, energy expenditure was significantly reduced in pioglitazone-treated animals by approximately 10%. During this time period, there was no difference in the respiratory exchange ratio calculated as VCO$_2$/VO$_2$ (Fig. 4D) indicating a lack of acute impact by pioglitazone administration on fuel selection. Reduced energy expenditure could not be attributed to reduced ambulatory activity (Fig. 4E).

Biotelemetry determinations showed that pioglitazone does not acutely change body temperature (Fig. 5A, D), heart rate (Fig. 5B, E) and blood pressure (Fig. 5C, F) in the 4 hours immediately following gavage.

On a longer time scale, a single administration of pioglitazone caused a small increase in heart rate (Fig. 5B), an effect that becomes statistically significant 4 h post gavage and is sustained for at least 24 h (Fig. S1B). Blood pressure was also significantly altered on this time scale, with a reduction 24 h post gavage (Fig. S1C). However, core body temperature was not changed (Fig. S1A).

**Pioglitazone reduces oxygen consumption rate in vitro**

Energy metabolism cannot currently be measured in β-cells in vivo. To complement the in vivo experiments on whole body energy metabolism with in vitro work in β-cells, oxygen consumption was measured in INS832/13 cells and isolated rat islets. In control
conditions, a rise in glucose from basal to 8 mmol/l and to 25 mmol/l elicited a similar rapid increase in oxygen consumption rate (OCR) (Fig. 6A, C). When pioglitazone was added, the increase in OCR induced by 8 mmol/l glucose was reduced by approximately 60% both in cells and isolated rat islets. The inhibitory effect of pioglitazone on OCR was partially alleviated at 25 mmol/l glucose, in accordance with the concept of metabolic deceleration. Insulin secreted during these measurements followed the same pattern (Fig. 6B, D) with the highest glucose concentration relieving in part the pioglitazone inhibitory effect.

**Pioglitazone effect on insulin secretion is PPARγ-independent**

Because the effects of pioglitazone *in vivo* and *in vitro* in this series of experiments are acute (less than 4 h post drug) it is unlikely that they are mediated via PPARγ and gene expression changes. In an attempt to further substantiate that the acute effects of pioglitazone on insulin secretion are PPARγ-independent, two structurally-unrelated non-TZD PPARγ agonists were tested. Treatment of INS832/13 cells and isolated rat islets with GW1929 and CAY10599, at a concentration equal to 600 times their EC50 for PPARγ transactivation (21; 22), did not alter GIIS or KCl-induced insulin release (Fig. 7).
DISCUSSION

Long term treatment with pioglitazone lowers fasting insulinemia (20). Whether this effect is entirely indirect due to a reduction in insulin resistance or, in part, a consequence of a direct effect on the β-cell is unknown. Here, we report the surprising ability of pioglitazone to acutely inhibit GIIS \textit{in vivo} prior to any significant change in insulin sensitivity. Besides inhibiting insulin secretion, pioglitazone also caused a small rise in insulin clearance in healthy Wistar rats but not in ZDF rats. The results provide evidence for a novel acute TZD effect to lower insulinemia, not due to changes in insulin sensitivity or PPARγ activation (see below). The development of new drugs for treating type 2 diabetes focuses on potential agents to either enhance insulin secretion or reduce insulin resistance. However, here we show an effective anti-diabetic agent reducing insulin secretion, a finding of particular interest considering the view that hyperinsulinemia may in some instances drive obesity, insulin resistance and type 2 diabetes (1; 3). Thus, the possibility of a contribution to the beneficial effect of TZDs (23) and metformin (24) on glucose homeostasis via a direct action on the β-cell to reduce insulinemia might have been overlooked.

What is the evidence that pioglitazone acutely reduces GIIS \textit{in vivo}? During the HGC, the glucose-induced rise in insulinemia was reduced by 50% in Wistar rats by pioglitazone and by 65% in the hyperinsulinemic ZDF rats. This was quite remarkable considering that pioglitazone was given as a single oral dose only 135 min earlier. The fact that both insulin and C-peptide levels were reduced is consistent with this effect being a consequence of reduced insulin secretion.
During HGC in both Wistar and ZDF rats, the glucose infusion rate needed to reach and maintain hyperglycemia trended slightly lower in pioglitazone-treated animals, an observation compatible with lower insulin secretion but not with improved insulin sensitivity. Indeed, after acute oral pioglitazone dosing, insulin sensitivity was not significantly altered in Wistar rats, as directly assessed by HIEC, and unchanged in ZDF rats as indirectly shown by similar glucose infusion rate during the HGC in the face of unaltered insulin clearance. In Wistar rats undergoing HIEC, there was however a trend for a slightly lower insulinemia in the pioglitazone-treated animals, which could have been the result of increased insulin clearance or reduced endogenous insulin secretion. An effect on clearance is supported by the fact that human insulin (measured with a specific antibody) infused at identical rates in both groups, also tended to be lower with pioglitazone treatment (not shown). Considering the lower insulin levels in Wistar rats, insulin sensitivity according to the M/I measure was slightly higher in the pioglitazone group, although this difference was not significant. This should not acutely affect the response of pancreatic islets to hyperglycemia and, therefore, the interpretation that pioglitazone has an acute direct effect to reduce in vivo insulin secretion.

Chronic and acute TZD treatments increase insulin clearance in humans (25-27). A rapid TZD effect on insulin clearance has been reported by Farret et al. in healthy volunteers undergoing a HGC following single-dose rosiglitazone administration (26). However, the authors could not measure significant changes in C-peptide level in their subjects. A careful look at the C-peptide figure does show a slightly lower (15%) C-peptide level in the rosiglitazone vs the control group, suggesting a modest effect of acute rosiglitazone treatment on GIIS in humans. It is also interesting to note that intraperitoneal
administration of troglitazone increased AMPK activity in rat liver, muscle and adipose tissue within 15 min (28), showing an acute effect of a TZD in vivo.

The current study was carried out with a reasonable dose of pioglitazone for rodents (30 mg/kg body weight). Experiments in rodents with chronic daily administration have been carried out by others with pioglitazone doses between 2.3 (29) and 35 mg/kg (30), with most studies using 10 mg/kg. It represents roughly 50 times what a patient of 70 kg would receive with a 45 mg pill. Although it seems disproportionate, TZDs are cleared approximately ten times faster by rats when compared to human due to higher CYP2C expression (31). In addition, our experiments were performed with a single administration of pioglitazone.

What is the biochemical basis of pioglitazone action to reduce GIIS in vivo? Inasmuch as the in vitro results obtained earlier (9) indicated that the reduced GIIS by pioglitazone was related to reduced metabolic activation and ATP production in the β-cell, we examined the possibility that this holds true in vivo. Thus we assessed the effect of pioglitazone on whole body energy expenditure. It was found that pioglitazone acutely reduces whole body energy metabolism, as assessed by oxygen consumption and CO₂ production, without changing fuel selection, as indicated by determination of the respiratory exchange ratio. Pioglitazone did not change rat body temperature or ambulatory activity. The fact that body temperature did not fall does not preclude an effect of pioglitazone to reduce body heat production, as homeostatic mechanisms could result in improved conservation of body heat.

The demonstration of reduced whole body energy metabolism fits with the view that pioglitazone may reduce β-cell energy metabolism as well, and cause its metabolic
deceleration *in vivo*. In accordance with this possibility, results obtained with INS832/13 β-cells and isolated rat islets showed that β-cells oxygen consumption is rapidly reduced by pioglitazone, most efficiently at intermediate glucose concentrations. Similar reduction in oxygen consumption is likely to occur *in vivo* in the main organs.

What is the significance of this study to our understanding of the mode of action of the main insulin action sensitizers, TZDs and biguanides? First, these compounds may in addition be direct “insulin secretion decelerators”. Second, they may act by being mild restrictors of energy production. Thus, in our earlier work (9) we suggested metabolic deceleration as a possible mode of action of pioglitazone characterized by: 1) lack of effect at low fuel levels; 2) metabolic inhibition at sub-maximal substrate concentrations, and 3) the relief of inhibition in the presence of elevated fuel levels.

Interestingly, metformin appears to act on liver gluconeogenesis via reducing the hepatic energy state independently of AMPK (32; 33). We also found that metformin, similarly to pioglitazone, acutely reduces insulin secretion (9), glucose oxidation, oxygen consumption and counteracts the hyperpolarization of the mitochondrial membrane, most efficiently at intermediate glucose concentrations (JL and MP, unpublished). Thus, our earlier results *in vitro* suggested the possibility that pioglitazone and metformin share a common mode of action by restricting energy production (9). Moreover, certain aspects of this action resemble those of an acute exercise bout in muscle (reduction in ATP, increase in AMP and AMPK activation) (34). Therefore, interventions that cause a mild decrease in cellular energy status may be useful approaches to improve glucose homeostasis in type 2 diabetes by simultaneously and directly increasing insulin sensitivity and reducing hyperinsulinemia. We speculate that this dual action of these
drugs that tackle the two characteristic features of type 2 diabetes is the reason why they are so efficient. Reduction of energy status at both cellular and whole-body level and the pharmacological agents that lead to such effects are of great interest, not only for type 2 diabetes, but also for cancer (35), myocardial infarction (36) and lifespan (37).

In conclusion, we propose that reduction of β-cell energy metabolism helps by protecting the cells from fuel surfeit that causes β-cell dysfunction and possibly death via several factors, including mitochondrial dysfunction, ROS production, endoplasmic reticulum stress and overstimulation of fuel-induced insulin secretion leading to the depletion of insulin stores (38). We report that pioglitazone exerts a direct action on the β-cell to reduce insulin secretion most likely by causing mild metabolic restriction/deceleration. Thus, pharmacologically-induced metabolic deceleration represents an attractive avenue to prevent β-cell loss due to their exhaustion, to reduce insulin resistance and treat type 2 diabetes.
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J.L. performed experiments, contributed to discussions, wrote the manuscript and reviewed/edited the manuscript. É.J.-A. performed experiments. E.P. performed experiments and reviewed/edited the manuscript. M.-L.P., N.B.R., C.J.N and E.J. contributed to discussion and edited the manuscript. S.R.M.M. contributed to discussion, wrote the manuscript and reviewed/edited the manuscript. V.P. contributed to discussion and reviewed/edited the manuscript. M.P. contributed to discussion, wrote the manuscript and reviewed/edited the manuscript. M.P. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. No potential conflicts of interest relevant to this article were reported.

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FIGURE LEGENDS

Fig. 1 Pioglitazone administration acutely inhibits insulin secretion and increases insulin clearance in Wistar rats. Two-step hyperglycemic clamps were performed in animals 45 min after pioglitazone (Pio = black symbols or bars; 30 mg/kg) or vehicle (Ctl = white symbols or bars) administration by gavage. Glycemia was clamped consecutively at 8.3 mmol/l (1st step, from min 30 to 60) and 16.7 mmol/l (2nd step, from min 90 to 120) as represented by dotted areas, then an arginine bolus (174 mg/kg) was administered (min 120, after last sampling). A-D, Plasma glucose values (A), glucose infusion rate (GIR) (B), plasma insulin (C) and plasma C-peptide concentrations (D) during the procedures. Two-way repeated measures ANOVA post-hoc analyses: * P<0.05, ** P<0.01, *** P<0.001 vs vehicle-treated group; E, increment (Inc.) in plasma insulin and C-peptide concentrations 1 min after arginine (Arg) administration; F, insulin clearance estimated via C-peptide (C-pep) over insulin (Ins) molar ratio for first (30-60 min) and second (90-120 min) step of the clamps. Unpaired t-test: * P<0.05 vs vehicle-treated group. Data expressed as means +/- SEM, n = 7-8 animals. Ctl, control.

Fig. 2 Pioglitazone administration does not acutely change insulin sensitivity in Wistar rats. Hyperinsulinemic-euglycemic clamps were performed in animals 45 min after pioglitazone (Pio = black circles or bar; 30 mg/kg) or vehicle (Ctl = white circles or bar) administration by gavage. Glycemia was clamped at 6.5 mmol/l. A, Plasma glucose values during the procedures; B, Glucose infusion rate (GIR) during the procedures; C, Total (human plus endogenous) plasma insulin measured from samples collected at the indicated time points; D, M/I index of insulin sensitivity calculated from GIR and total plasma insulin. Unpaired t-test: not significant (n.s.) vs vehicle-treated group. Data expressed as means +/- SEM, n = 6-5. Ctl, control.

Fig. 3 Pioglitazone administration acutely inhibits glucose-induced insulin secretion in ZDF rats. One-step hyperglycemic clamps were performed in rats 45 min after pioglitazone (Pio = black symbols or bars; 30 mg/kg) or vehicle (Ctl = white symbols or bars) administration by gavage. Glycemia was clamped at 16.7 mmol/l and an arginine bolus (174 mg/kg) was administered at min 120, after last sampling. A-D, Plasma glucose values (A), glucose infusion rate (GIR) (B), increase in plasma insulin (C) and C-peptide (D) concentrations above baseline during the procedures. Basal insulin concentrations were 1.8 +/- 0.2 nmol/l and 2.1 +/- 0.5 nmol/l for Ctl and Pio groups, respectively. Basal C-peptide concentrations were 4.0 +/- 0.4 nmol/l and 4.4 +/- 0.9 nmol/l for Ctl and Pio groups, respectively. Two-way repeated measures ANOVA post-hoc analyses: * P<0.05 vs vehicle-treated group; E, increment (Inc.) in plasma insulin and C-peptide concentrations 1 min after arginine (Arg) administration; F, insulin clearance estimated via C-peptide (C-pep) over insulin (Ins) molar ratio for first (0-15 min) and second phase (60-120 min) of insulin secretion. Data expressed as means +/- SEM, n = 6 animals. Ctl, control.

Fig. 4 Pioglitazone administration acutely reduces whole body energy expenditure in Wistar rats. Pioglitazone (Pio = black circles or bar; 30 mg/kg) or vehicle (Ctl = white circles or bar) was administered 2 h after food withdrawal and data collected during the
following 6 h. A, Oxygen consumption (VO$_2$); B, Carbon dioxide production (VCO$_2$); C, Energy expenditure (EE) calculated from VO$_2$ and VCO$_2$; D, Respiratory exchange ratio (RER) measured as VCO$_2$/VO$_2$; E, Total horizontal and vertical distance traveled by the animals during the 6-h air sampling. Data expressed as means +/- SEM, n = 7-6 for calorimetry and n = 13 for locomotor activity. Two-way repeated measures ANOVA: * P<0.05 vs vehicle-treated group. Ctl, control.

**Fig. 5** Short term assessment of the action of pioglitazone on body temperature, heart rate and blood pressure in Wistar rats. Core body temperature (A), heart rate (B) and blood pressure (C) were monitored by biotelemetry. Pioglitazone (Pio = black circles or bars; 30 mg/kg) or vehicle (Ctl = white circles or bars) was administered 2 h after food withdrawal. Horizontal bold lines correspond to clamp’s last hour (performed with other animals in Figures 1 and 2); D-F, Averaged values of data corresponding to clamp’s last hour. Data expressed as means +/- SEM, n = 6, where animals were all used as both treated and control rats alternatively, with 10 days washout in between experiments. BPM, beats per minute; Ctl, control.

**Fig. 6** Pioglitazone reduces oxygen consumption rate in INS832/13 cells and isolated rat islets. A and C, Oxygen consumption rate (OCR) presented as fold change over last measurement before glucose addition. Baseline OCR were, in cells, 174 +/- 6 pmol/min and 166 +/- 6 pmol/min for Ctl and Pio conditions, respectively and, in islets, 42 +/- 4 pmol/min and 40 +/- 5 pmol/min for Ctl and Pio conditions, respectively. Pioglitazone (Pio = black symbols or bars; 30 mg/kg) or vehicle (Ctl = white symbols or bars) was added after basal measurements at the time indicated by the bold vertical arrow and glucose (2 or 3 mmol/l (2G/3G) = circles; 8 mmol/l (8G) = squares; 25 mmol/l (25G) = triangles) was added after pre-incubation at the time indicated by the thin vertical arrow. Two-way repeated measures ANOVA post-hoc analyses: * P<0.05, ** P<0.01 vs vehicle-treated wells at same glucose concentration. B, Insulin secreted during OCR measurement in cells. Kruskal-Wallis post-hoc analyses: * P<0.05 vs vehicle-treated cells at same glucose concentration. D, Insulin secreted during OCR measurement in islets. One-way ANOVA post-hoc analyses: * P<0.05, ** P<0.01 vs vehicle-treated islets at same glucose concentration. Data expressed as means +/- SEM, n = 8 in A-B (8 different wells in 2 separate experiments, except for 2 mmol/l glucose conditions where n = 6) and n = 6-8 in C-D (6 to 8 different wells in 2 separate experiments, except for 3 mmol/l glucose conditions where n = 5). Ctl, control.

**Fig. 7** Lack of effect on insulin secretion of non thiazolidinedione PPARγ agonists in INS832/13 cells and isolated rat islets. Cells (A-B) or islets (C-D) were incubated in the presence of the indicated glucose concentrations (Glc) and either a non TZD PPARγ agonist (hatched bars; A and C, GW1929 (GW); B and D, CAY10599 (CAY)) at a concentration equal to 600 times its EC$_{50}$ for PPARγ transactivation, pioglitazone (Pio = black bars) or vehicle (Ctl = white bars). Data expressed as means +/- SEM, n = 9 in A-B (9 different wells in 3 separate experiments), n = 8 in C (8 different wells in 2 separate experiments) and n=12 in D (12 different wells in 3 separate experiments). Two-way ANOVA post-hoc analyses: * P<0.05, ** P<0.01 vs vehicle-treated wells at same glucose concentration. Ctl, control.
Figure 1
Figure 2
Figure 4
**Figure 5**

153x255mm (300 x 300 DPI)
Figure 6
Figure 7

81x36mm (300 x 300 DPI)
SUPPLEMENTAL DATA

**Fig. S1** A single pioglitazone administration increases heart rate and reduces blood pressure in Wistar rats. Core body temperature (A), heart rate (B) and blood pressure (C) were monitored by biotelemetry. Pioglitazone (Pio = black circles; 30 mg/kg) or vehicle (Ctl = white circles) was administered 2 h after food withdrawal (indicated as “gavage”). Data expressed as means +/- SEM, n = 6. Two-way repeated measures ANOVA: * P<0.05 vs vehicle-treated group. Ctl, control.