Antidiabetic Actions of an Estrogen Receptor β Selective Agonist

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The estrogen receptor β (ERβ) is emerging as an important player in the physiology of the endocrine pancreas. We evaluated the role and antidiabetic actions of the ERβ selective agonist WAY200070 as an insulinotropic molecule. We demonstrate that WAY200070 enhances glucose-stimulated insulin secretion both in mouse and human islets. In vivo experiments showed that a single administration of WAY200070 leads to an increase in plasma insulin levels with a concomitant improved response to a glucose load. Two-week treatment administration increased glucose-induced insulin release and pancreatic β-cell mass and improved glucose and insulin sensitivity. In addition, streptozotocin-nicotinamide–induced diabetic mice treated with WAY200070 exhibited a significant improvement in plasma insulin levels and glucose tolerance as well as a regeneration of pancreatic β-cell mass. Studies performed in db/db mice demonstrated that this compound restored first-phase insulin secretion and enhanced pancreatic β-cell mass. We conclude that ERβ agonists should be considered as new targets for the treatment of diabetes.


Diabetes has become one of the most challenging health problems on a global scale, with an estimated 285 million people affected by this disease in 2010 (1,2). The most common form of diabetes is type 2 (T2D), which results from the interaction of a subject’s genetic background with the environment. Both insulin resistance and pancreatic β-cell dysfunction contribute importantly to the pathogenesis of this disease; however, T2D develops only when insulin secretion cannot meet the insulin demand (3–5). Therefore, the most effective therapy for T2D should control not only β-cell failure, but also the loss of β-cell mass. Today, although there is an extensive range of oral antidiabetic drugs, a single administration of WAY200070 leads to an increase in pancreatic β-cell mass and improved glucose-induced insulin release and pancreatic β-cell mass. Studies performed in db/db mice demonstrated that this compound restored first-phase insulin secretion and enhanced pancreatic β-cell mass. We conclude that ERβ agonists should be considered as new targets for the treatment of diabetes. Diabetologija 62:2015–2025, 2013

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were injected intraperitoneally with 0.75 IU/kg soluble insulin. The glucose, and blood samples were taken at the indicated intervals. In STZ-NA and tests (IGTTs), animals were fasted overnight for 12 h, and blood samples were cultured for 48 h in the presence of 10 mmol/L (Fig. 1a). The effect of WAY200070 on GSIS of human islets was also analyzed. Insulin release was enhanced in a dose-dependent manner, with a maximal effect at a dose of 100 nmol/L (Fig. 1E).

We next studied the in vivo effect of WAY200070. For this purpose, we used male C57BL/6 mice aged 3 months. Fasted animals were injected intraperitoneally with 2 g/kg glucose followed by the intraperitoneal administration of a vehicle or WAY200070 at different concentrations (3, 10, or 30 mg/kg). As shown in Figure 2A, glucose sensitivity was improved in those animals that received the agonist WAY200070, especially at a concentration of 10 mg/kg (W10) and 30 mg/kg (W30), although the effects were not statistically significant at the latter dose. When plasma insulin levels were measured at 30 min, a higher glucose-stimulated insulin release was observed at W10 compared with the vehicle-treated group (Fig. 2B). Under fasting conditions, no changes of glycemia were observed in animals treated with the ERβ agonist at any concentration assayed (Fig. 2C).

To unequivocally demonstrate that the in vivo insulinotropic effect of WAY200070 is an ERβ-mediated effect, we compared its action in wild type (WT) and ERβ knockout (BERKO) mice. A single administration of W10 in WT mice resulted in a lower rise of glycemia levels in response to a glucose challenge, indicating that these mice tolerated glucose better than WT vehicle-treated mice (Fig. 2D). However, when the same experiment was performed in BERKO mice, no differences were observed between vehicle and ERβ agonist-treated animals (Fig. 2E). When we measured plasma insulin levels 30 min after a glucose load, we observed a higher glucose-stimulated insulin release in the WT mice that had received a single dose of W10 (Fig. 2F) but no changes in the BERKO mice (Fig. 2F).

We studied the extent to which an ERβ agonist can be a positive modulator of energy balance and glucose homeostasis under healthy conditions. For this purpose, we analyzed glycemia and insulin sensitivity in C57BL/6 male mice treated with W10 for 14 days. The treatment with the agonist was found to improve glucose tolerance (Fig. 3A) and increase the glucose-stimulated in vivo insulin release 30 min after the glucose load (1.41 ± 0.19-fold increase over basal insulin levels in vehicle-treated mice versus 2.01 ± 0.16-fold increase in W10-treated mice) (Fig. 3B), suggesting that the pancreatic β-cells were working more vigorously. A slightly better insulin sensitivity was also observed in these animals compared with the controls (Supplementary Fig. 1A). In addition to this, β-cell mass and islet size were higher in W10-treated mice compared with the vehicle-treated mice (Fig. 3C and D). We studied whether the augmented β-cell mass was a result of an increased β-cell proliferation. We performed coimmunostaining for insulin and BrdU and found that animals treated with the ERβ agonist exhibited a larger number of proliferating β-cells (Fig. 3E). Dispersed islet cells were treated with a range of doses of this compound for 2 days, and β-cell proliferation was quantified by using BrdU incorporation over the same period. We observed a significant increase in pancreatic β-cell proliferation in the ERβ agonist–treated cultures, which suggests that the activation of ERβ signaling directly affects pancreatic β-cell replication (Fig. 3F).

RESULTS

In vitro experiments performed with isolated pancreatic β-cells from mice showed that WAY200070 (100 pmol/L) decreased K_ATP channel activity (Fig. 1A). In whole mouse islets of Langerhans, the agonist promoted both a greater global intracellular Ca2+ entry (Fig. 1B) and an increase of insulin secretion in response to glucose stimulation (8 mmol/L glucose) (Fig. 1C). WAY200070 at 100 pmol/L enhanced GSIS in the presence of 8 and 16 mmol/L glucose but, of interest, it did not have any effect when applied in the presence of low glucose concentrations (3 mmol/L) (Fig. 1D). The effect of WAY200070 on GSIS of human islets was also analyzed. Insulin release was enhanced in a dose-dependent manner, with a maximal effect at a dose of 100 nmol/L (Fig. 1E).
FIG. 1. In vitro exposure to an ERβ agonist (WAY200070) induced reduction of KATP channel activity and enhanced glucose-induced intracellular calcium concentration signals and insulin secretion. A: WAY200070 100 pmol/L decreased KATP channel activity in isolated pancreatic β-cells from mice (n = 6). B: This was accompanied by a greater increase in global intracellular Ca²⁺ entry after glucose stimulation (n = 6), as indicated by a greater AUC (inset). C: Insulin release in response to 8 mmol/L glucose, 8 mmol/L glucose and DPN (1 nmol/L), and 8 mmol/L glucose and WAY200070 (100 pmol/L, 1 nmol/L, and 10 nmol/L) was measured in intact mouse islets of Langerhans. Insulin levels were significantly higher in the presence of WAY200070 (n = 8). D: WAY200070-induced insulin secretion from mouse islets exposed to 3, 8, and 16 mmol/L glucose for 1 h (n = 8). WAY200070 was applied at 100 pmol/L. Note that WAY200070 action was significant only when stimulatory glucose concentrations were used. E: Insulin release in response to 8 mmol/L glucose, 8 mmol/L glucose and DPN (1 nmol/L), and 8 mmol/L glucose and WAY200070 (100 pmol/L, 1 nmol/L, and 100 nmol/L) was measured in intact human islets of Langerhans. Data are mean ± SE. *P < 0.05, **P < 0.001 versus control (Student t test). Statistical analyses between groups in C, D, and E were evaluated by one-way ANOVA, with P < 0.05 considered significant. G, glucose; W, WAY200070.
We also observed clear hyperinsulinemia in the fed state (Fig. 4A) but no differences in leptin (Fig. 4B) or triglyceride (Fig. 4C) levels, although glycerol levels were decreased in the ERβ agonist-treated group (Fig. 4D). Mild diabetes was induced by administering a single dose of STZ 150 mg/kg and NA 1,000 mg/kg. As shown in Figure 5A, 10 days after the STZ-NA treatment, these animals exhibited moderate fasting hyperglycemia (vehicle-treated control mice 90 ± 4.31 mg/dL, STZ-NA diabetic mice 126 ± 5.3 mg/dL, P < 0.05). An IGTT showed that STZ-NA mice presented consistent glucose intolerance as indicated by the bigger area under the curve (AUC) (Fig. 5A). At this point, we divided STZ-NA mice into two groups: One received an intraperitoneal injection of W10 for 7 days, and the other received a vehicle injection for the same period. The 1-week treatment provoked a decrease in fasting glycemia levels in the diabetic mice treated with the ERβ agonist (control mice 108 ± 2.36 mg/dL, diabetic mice treated with WAY200070 109 ± 4.03 mg/dL, diabetic mice treated with W10 140 ± 9.16 mg/dL, P < 0.05). We also observed from the IGTT that glucose tolerance was significantly improved in the diabetic group treated with WAY200070, with similar levels of glycemia as the control group (Fig. 5B). This improvement in glucose sensitivity was accompanied by an increase of β-cell replication (Fig. 5F). No differences in the plasma leptin or triglyceride levels were found between groups (Supplementary Fig. 1B and C). A marked tendency for glycerol levels to decrease was observed in the diabetic group treated with the ERβ agonist, although it was not statistically significant (Supplementary Fig. 1D).

At ~14 weeks of age, db/db mice presented hyperglycemia, glucose intolerance, and decreased in vivo insulin secretion in response to a glucose load (Supplementary Fig. 2A and B). At this moment, the animals were treated with W10 for 14 days or with the vehicle.
Glucose levels after overnight fasting were slightly reduced in W10 \textit{db/db} mice (241 \pm 18.5 mg/dL) compared with the vehicle \textit{db/db} mice (303 \pm 31.5 mg/dL), although differences were not significant (\(P = 0.1\)). To explore differences in glucose homeostasis, we performed an IGTT. At 15 and 30 min, blood glucose levels were lower in treated diabetic mice than in untreated mice. We also observed a smaller AUC, suggesting a better glucose tolerance at least after short periods (Fig. 6A). We measured the plasma insulin of mice in a fasted state and 30 min after a glucose load (Fig. 6B). Of interest, we found that in vivo GSIS was practically absent in vehicle \textit{db/db} mice, showing a 1.04 \pm 0.14-fold increase over basal insulin levels, whereas in \textit{db/db} animals treated with the ER\(\beta\) agonist, insulin secretion showed a 1.70 \pm 0.22-fold increase over basal insulin. No differences were observed in the significance of insulin sensitivity between groups (Supplementary Fig. 2C).

In addition to the ER\(\beta\)-agonist effect on pancreatic \(\beta\)-cell function, ER\(\beta\) activation also increased pancreatic \(\beta\)-cell mass (Fig. 6C). We observed an increase in the size of the islets, although differences between groups were not significant (\(P = 0.1\)) (Fig. 6D). We found a higher rate of \(\beta\)-cell proliferation in the treated \textit{db/db} mice (Fig. 6E).

In the fed state, no differences in plasma insulin or leptin levels were found (Fig. 7A and B); however, a clear decrease of triglyceride and glycerol plasma levels was detected in the W10 \textit{db/db} mice (Fig. 7C and D), which was in consonance with the weight loss observed in these animals throughout the treatment from mainly day 8 to the end (Fig. 7E). No changes in food intake were observed (Supplementary Fig. 2D).

**DISCUSSION**

In the current study, we demonstrate the potential use of the ER\(\beta\) ligand WAY200070 as an effective modulator of insulin release and pancreatic \(\beta\)-cell mass, with a significant improvement in glucose homeostasis. It is clear now that both ER\(\alpha\) and ER\(\beta\) play a pivotal role in the regulation of lipid and glucose metabolism and that a correct balance of the ER\(\alpha\)/ER\(\beta\) ratio is crucial for that (23,24). However, most studies have focused on the role of both receptors in the central nervous system, adipose tissue, liver, and skeletal muscle. It has been described that the anorectic effect of E2 seems to be mediated mainly by ER\(\alpha\) (25,26), although the involvement of ER\(\beta\) cannot be disregarded (27). In the liver, ER\(\alpha\) is the main regulator of hepatic insulin sensitivity (28,29). In skeletal muscle, ER\(\beta\) is suggested
to play a repressive role on GLUT4 expression because the ERβ agonist DPN severely downregulates its expression in ArKO (aromatase-deficient) mice (30). Finally, in adipose tissue, as is described later, ERβ could have antiobesity effects (31).

Glucose homeostasis requires a tight communication between peripheral tissues and the pancreas, but ultimately, hyperglycemia only develops when pancreatic β-cell function fails. In previous studies, we demonstrated that ERβ regulates stimulus-secretion coupling in pancreatic β-cells, as does E2 (11). In the present study, we confirm these results, showing that the selective ERβ agonist WAY200070 rapidly decreased K\textsubscript{ATP} channel activity and increased glucose-stimulated calcium signaling and GSIS. Remarkably, in human islets, WAY200070 also enhanced insulin release, with a maximal effect at a dose of 100 nmol/L. In mice, we observed that a single injection of WAY200070 in parallel with a glucose load rapidly enhances insulin secretion. Under fasting conditions, the selective ERβ agonist does not have an effect on glycemia, suggesting that the action of this compound depends on the actual glucose concentration and provides the possibility of glucose normalization without the risk of hypoglycemia.

This phenomenon is mediated at least partially by ERβ, as judged from experiments performed in WT and BERKO mice, in a process that mimics the effect of low physiological concentrations of E2 (100 pmol/L–1 nmol/L), a natural ligand of ERβ. However, we cannot rule out the possibility that other estrogen receptors can provoke similar effects. A recent report described that the activation of G protein-coupled receptor 30 enhanced GSIS and decreased glucagon and somatostatin release in female human and mouse islets, although this effect is only visible when E2 is applied at supraphysiological concentrations of \( \geq 100 \text{ nmol/L} \) (32–34). ERα has been proposed to play a significant role in the regulation of insulin synthesis by E2 but with no effects on pancreatic β-cell mass (10). Its role in cytoprotection has also been suggested (35).

**Potential clinical implications of the insulinotropic ERβ-mediated effect.** Insulin deficiency is the common denominator for both type I diabetes and T2D. In the current study, we evaluated the insulinotropic effect of an ERβ agonist by using STZ-NA diabetic mice. This model has been reported to be useful for the assay of insulin secretagogues (17,36,37). We observed that the ERβ agonist modulates insulin secretion capacity and restores

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**FIG. 4.** Plasma hormones and lipid metabolites in C57BL/6 mice after administration of WAY200070. Plasma was collected from C57BL/6 mice treated with the ERβ agonist W10 or vehicle (6–7 mice/group) during a 14-day period, and insulin (A), leptin (B), TG (C), and glycerol (D) levels were measured. Data are mean ± SE. *P < 0.05 versus vehicle (Student t test). TG, triglyceride.
FIG. 5. In vivo administration of the ERβ agonist (WAY200070) improved glucose tolerance in mildly diabetic mice and restored plasma insulin levels and pancreatic β-cell mass. A: Mild diabetes was induced by the administration of a single dose of STZ 150 mg/kg and NA 1,000 mg/kg. Ten days after the treatment, these animals exhibited moderate hyperglycemia and impaired glucose tolerance compared with controls (8–15 mice/group). The AUC in these mice was significantly increased (inset). *P < 0.05 versus control (Student t test). B: At this point, 1-week treatment with the ERβ agonist resulted in a decrease of fasting glycemia as well as a better sensitivity to a glucose challenge in the W10 diabetic group compared with the vehicle-treated diabetic group (7–8 mice/group). *P < 0.05 diabetic vehicle vs. diabetic W10; #P < 0.05 diabetic vehicle vs. control. C: Plasma insulin levels in diabetic mice treated with WAY200070 during 1 week compared with control and nontreated diabetic mice. We observed that levels of plasma insulin in treated diabetic mice were similar to controls; meanwhile, levels in diabetic mice were significantly reduced (8–10 mice/group). D, E, and F: Measurement of pancreatic β-cell mass and islet size in treated diabetic mice (5 mice/group) and β-cell replication measured as the percentage of BrdU-positive cells (5 mice/group). Data are mean ± SE. Statistical analysis between groups was evaluated by one-way ANOVA, with *P < 0.05 considered significant.
plasma insulin levels, but beyond this regulatory role of insulin secretion, we demonstrate that a selective ERβ agonist enhances β-cell proliferation in pancreatic islets, and thus, it could act as a regulator of β-cell growth. When pancreatic β-cell mass was quantified in STZ-NA diabetic mice, a clear decrease was observed compared with controls. However, in the diabetic animals that were treated with the agonist, there was a marked increase that resulted in the recovery of β-cell mass. Of interest, in vitro studies suggested that the ERβ agonist directly stimulates β-cell division, although the molecular basis mediating this phenomenon still needs further clarification. Mechanistically, E2 promotes islet regeneration in different animal models of diabetes through increased β-cell proliferation (38–41). Choi et al. (41) proposed that the E2 proliferative effect could be mediated by the activation of the cAMP-responsive element–binding protein, which in turn leads to an increased level of insulin receptor substrate 2 and pancreatic duodenal homeobox-1 levels and most probably leads to the activation of an IGF-I signaling cascade. In addition, genistein, another natural estrogenic compound, has been shown to improve islet β-cell proliferation, survival, and mass in diabetic mice in a process mediated by the activation of the protein kinase A and the subsequent phosphorylation of extracellular signal–related kinase 1/2 in β-cells (42). The authors speculated about the possibility that this signaling cascade is connected with an upregulation of cyclin D1 and, thus, with the regulation of the cell cycle machinery. Whether the ERβ agonist used in this study works through any of the presently proposed mechanisms remains unknown. In any case, we propose that ERβ selective agonists are considered as newcomers in the field of β-cell regeneration that potentially act alone or in synergy with other known factors, such as the hepatocyte growth factor, the glucose-dependent insulinotropic polypeptide, the transcription factors Pax 4, and the orphan nuclear liver receptor homolog-1 (43).

In addition to the STZ-NA model, we studied the possible benefits of ERβ-agonist treatment in a different model of diabetes, db/db mice. The administration of this drug led to an improvement of fasting glucose levels and glucose homeostasis, although the latter seemed to be a short-term effect. Importantly, while no insulin release in response to a rapid glucose stimulus was detected in the group of db/db vehicle-treated mice, the ERβ-agonist–treated group exhibited a clear release of insulin, indicating that this compound is able to restore the blunted first phase of insulin secretion. This phase is almost absent in individuals with T2D (44,45). Moreover, it has been shown that there is a delay of the first-phase insulin secretion in BERKO mice (46), reinforcing the importance of ERβ in the maintenance of postprandial glucose homeostasis.

When looking at the effects of WAY200070 in STZ-NA diabetic mice compared with db/db mice, we found that the recovery of pancreatic β-cell function and glucose homeostasis in the first model was much more effective
than in the second model. We hypothesized that this recovery is a result of the animals exhibiting a less severe degree of diabetes in the first case than in the second case. Similarly, the hypoglycemic effect of E2 depends on the severity of the diabetic condition, with no positive effects when hyperglycemia exceeds 500 mg/dL and islet damage is too severe (38). For these reasons, we believe that treatment at an earlier stage of the disease would be more effective for the db/db mice.

We also observed that the ERβ-agonist treatment has other extrapancreatic effects. It significantly decreased triglyceride and glycerol plasma levels in db/db mice, which were accompanied by a reduction of body weight. However, more information in terms of energy expenditure

FIG. 7. Plasma hormones, lipid metabolites, and increment of weight in db/db mice. Plasma was collected from W10 db/db or vehicle-treated db/db mice after 14 days of treatment, and insulin (A), leptin (B), TG (C), and glycerol (D) levels were measured. E: Increment of weight in db/db mice during the treatment with W10 or vehicle (6–7 mice/group). Data are mean ± SE. *P < 0.05 versus vehicle (Student t test). TG, triglyceride.
would largely contribute to the understanding of the role of WAY200070 from an energetic point of view. One report indicated that the administration of selective ERβ ligands prevented body weight gain in animals fed with a high-fat diet as well as in ovariectomized mice because of the reduction in fat mass and the increase in lean mass (31). In contrast, a diabeticogenic role for ERβ has been proposed because BERKO mice exposed to a high-fat diet had improved whole-body insulin sensitivity and glucose tolerance (47). In any case, the activation of ERβ demonstrates antilipogenic actions in pancreatic β-cells (40). Its role in cytoprotection has also been suggested (35).

In summary, we propose that an ERβ agonist could act as a regulator of pancreatic β-cell growth and that it promotes an increase of β-cell mass under pathological conditions. In addition, by using different experimental animal models of diabetes, we demonstrate the capacity of this agonist to normalize fasting glucose levels, to enhance endogenous insulin secretion, and to improve glucose homeostasis. Therefore, ERβ agonists may be useful new drugs for the treatment of T2D.

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P.A.-M. designed the study, researched data, and wrote the paper. A.B.R. contributed to discussion and researched data. M.G.A., S.S.S, S.J.M., and A.S. researched data. I.Q. and J.A.-G. contributed to discussion and reviewed and edited the manuscript. A.N. helped with the study design, contributed to discussion, and reviewed and edited the manuscript. P.A.-M. 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.

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