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ORIGINAL PAPER

Loss of angiotensin-converting enzyme 2 leads to impaired glucose homeostasis in mice

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Abstract This study aimed to investigate the role of angiotensin-converting enzyme 2 (ACE2) in regulating glucose homeostasis. Immunohistochemistry was used to investigate ACE2 expression in the pancreas. Glucose tolerance test, insulin secretion test, and insulin tolerance test were performed in age-matched male ACE2 knockout (KO) and wild-type (WT) mice. We found that ACE2 was positively expressed in the pancreas. Male ACE2 KO mice displayed a selective decrease in first-phase insulin secretion in response to glucose and a progressive impairment of glucose tolerance compared with age- and sex-matched WT mice. On the other hand, insulin sensitivity of the peripheral tissue in age-matched ACE2 KO and WT mice showed no difference. These findings suggest that ACE2 might play an important role in glucose homeostasis as well as type 2 diabetes.

Keywords Angiotensin-converting enzyme 2 · Pancreas · Glucose homeostasis · Insulin sensitivity

Introduction

The type 2 diabetes epidemic is evident in the Western world and also in Asia. One of the most intriguing and clinically relevant findings related to renin-angiotensin system (RAS) inhibitors [1, 2] is their ability to delay the onset of type 2 diabetes in high-risk patients with cardiovascular disease. Angiotensin II is both pro-inflammatory [3, 4] and pro-oxidant [5, 6], resulting in cellular toxicity and apoptosis. The protective effects achieved by blocking RAS in clinical studies remain ambiguous. However, it is now apparent that blocking RAS has important and direct effects on the development of islet cell dysfunction associated with type 2 diabetes. Chu et al. [7] reported that oral losartan treatment delayed the onset of diabetes, and reduced hyperglycemia and glucose intolerance in db/db mice, but did not affect the insulin sensitivity of peripheral tissues. Chu et al. [7] also confirmed that Losartan selectively improved glucose-induced insulin release and (pro)insulin biosynthesis in db/db islets. RAS inhibition also seems to benefit the islets in other animal models of type 2 diabetes, such as the Zucker diabetic fatty rat [8] and the Otsuka Long-Evans Tokushima fatty rat [9]. These benefits seemed to be due to preservation of the islet architecture and β-cell mass.

Angiotensin-converting enzyme 2 (ACE2), a membrane-bound carboxymonopeptidase, negatively regulates the RAS. ACE2 acts as an endogenous ACE inhibitor by altering the relative generation of angiotensin II and angiotensin-(1–7). ACE2 is the first known homologue of ACE [10]. Although ACE2 cleaves a single residue from angiotensin I, generating angiotensin-(1–9), and a single residue from angiotensin II to generate angiotensin-(1–7) [10], the catalysis efficiency of angiotensin II to angiotensin-(1–7) by ACE2 is much higher than that of angiotensin I to angiotensin-(1–9) [11]. Thus, it appears that ACE2 negatively regulates the RAS and counterbalances ACE function.

Targeted disruption of ACE2 in mice results in increased plasma and tissue angiotensin II levels [12, 13]. Male ACE2 knockout (KO) mice exhibit progressive impairment of heart contractility at advanced ages, a

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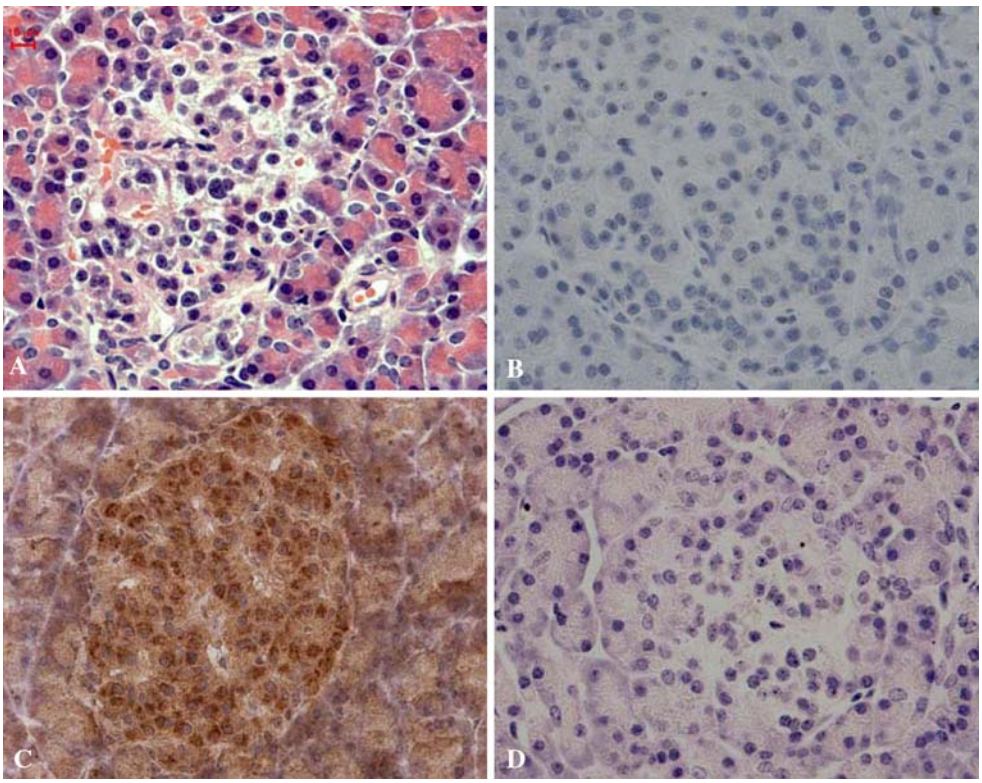

Fig. 1 Immunohistochemistry of mice pancreas (×400). **a** HE staining shows the exocrine tissue of pancreas and pancreatic islet (in the middle). **b** Negative control of immunohistochemistry to exclude non-specific staining. **c** Expression of ACE2 in the WT mice pancreas as assessed by immunohistochemistry shows ACE2 protein was localized to acini and islets. **d** No expression of ACE2 in the ACE2 KO mice pancreas ($n = 5$ per group)

phenotype that can be reverted by loss of ACE [12]. Male ACE2 KO mice develop late-onset glomerulonephritis resembling diabetic nephropathy [13]. Nevertheless, the in vivo role of ACE2 in the islet function and the RAS is unknown.

The aim of this study was twofold. First, to investigate ACE2 expression in the pancreas, and second to elucidate its role in regulating glucose homeostasis. In order to explore the physiological role of ACE2 in regulating blood glucose, in this study we performed glucose tolerance test, insulin secretion test, and insulin tolerance test in age-matched male ACE2 KO and wild-type (WT) mice. Our studies indicate that ACE2 is expressed in the pancreas and help regulate glucose homeostasis.

Results

Immunohistochemistry

We examined ACE2 expression in the pancreas. In the mouse pancreas, ACE2 protein was localized to acini and islets (Fig. 1).

Glucose tolerance test

ACE2 KO mice were born at the expected frequencies, and were fertile, and did not differ in weight from WT mice (Table 1). To assess the effect of the ACE2 KO on glucose homeostasis, glucose levels were measured in the fasted

Table 1 Characteristics of 3-, 6-, and 12-month-old male ACE2 KO and WT mice. ($n = 8$, each group)

	3-month-old ACE2 KO	3-month-old WT	6-month-old ACE2 KO	6-month-old WT	12-month-old ACE2 KO	12-month-old WT
Body weight (g)	27.03 ± 0.26	27.63 ± 0.33	32.09 ± 0.35	31.99 ± 0.30	34.45 ± 0.37	34.96 ± 0.40
Fasting glucose (mg/dl)	95.18 ± 4.09	95.85 ± 3.61	102.83 ± 4.92	98.10 ± 3.94	101.25 ± 4.48	104.85 ± 5.02
Fasting insulin (ng/ml)	0.78 ± 0.04	0.82 ± 0.04	0.79 ± 0.04	0.74 ± 0.03	0.81 ± 0.05	0.80 ± 0.03

Data are expressed as mean ± SEM ($n = 8$)

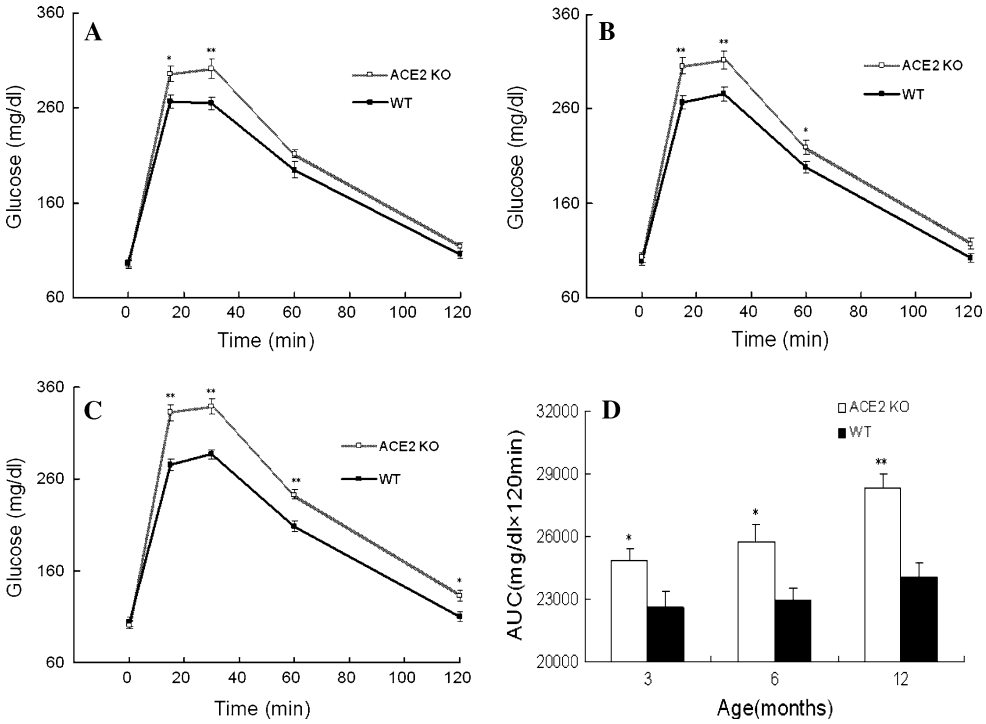

Fig. 2 Male ACE2 KO mice demonstrate a progressively impaired glucose tolerance. The ability to handle a glucose load was assessed by carrying out a glucose tolerance test (2 g/kg body weight IP) at 3 (**a**), 6 (**b**), and 12 (**c**) months of age in male WT (*filled squares*) and ACE2 KO (*open squares*) mice. **d** The areas under the curves (AUC) were expressed and compared. An age-dependent glucose intolerance was observed in male ACE2 KO mice ($P < 0.01$ by ANOVA). Results are expressed as mean ± SEM ($n = 8$, each group). *$P < 0.05$ vs. WT; **$P < 0.01$ vs. WT

states. No significant differences were observed in fasting blood glucose levels in male ACE2 KO mice as compared to those in male WT mice at either 3 months or 12 months of age. However, on glucose tolerance test, male ACE2 KO mice showed a progressively impaired glucose tolerance over 12 months (Fig. 2a–d).

Insulin secretion test and insulin tolerance test

Male ACE2 KO mice had fasting insulin levels similar to those of WT mice. For insulin release, glucose (3 g/kg body weight) was injected IP, in male WT mice, a three to fourfold increase in insulin secretion was observed 2 min after IP glucose injection, and the levels remained higher than baseline values for up to 30 min, indicating a second-phase response. In male ACE2 KO mice, the first-phase insulin secretory response to glucose was reduced. However, by 30 min insulin levels were not significantly different between the groups, suggesting some retention of second-phase insulin secretion in male ACE2 KO mice (Fig. 3a).

Insulin sensitivity of the peripheral tissue in age-matched male ACE2 KO and WT mice showed no difference (Fig. 3b). This was evidenced by the insulin tolerance test,

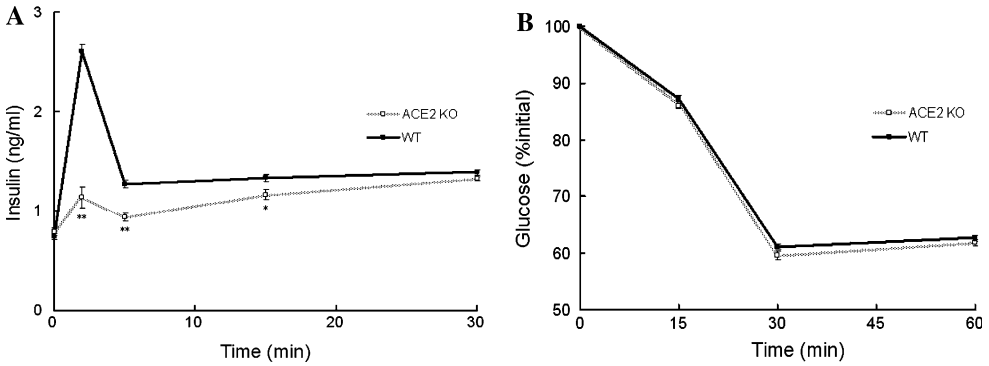

Fig. 3 Male ACE2 KO mice demonstrate a selective decrease in first-phase insulin secretion. Insulin sensitivity of the peripheral tissue in ACE2 KO and WT mice showed no difference. **a** Glucose (3 g/kg body weight) was injected intraperitonally in 6-month-old male WT (*filled squares*) and ACE2 KO (*open squares*) mice. Blood samples were collected at the indicated time intervals for insulin ELISA. A significantly decreased first-phase insulin secretion response was observed in male ACE2 KO mice as compared with WT mice. **b** Insulin tolerance test of 6-month-old male WT, ACE2 KO mice performed with 0.75 U/kg human regular insulin on fed animals. Results are expressed as mean ± SEM of percent of initial blood glucose value ($n = 8$, each group). *$P < 0.05$ vs. WT; **$P < 0.01$ vs. WT

as the change of percent of initial blood glucose level did not differ between ACE2 KO and WT mice after an intraperitoneal (IP) injection of insulin.

Discussion

It is well known that humans with type 2 diabetes exhibit defects in β-cell function, the most characteristic of which is decreased first-phase insulin secretion [14]. This defect is intrinsic, as demonstrated by recent studies using islets isolated from subjects with type 2 diabetes [15]. Our data show that the ACE2 was positively expressed in the mouse pancreas. In this study, male ACE2 KO mice exhibit a selective decrease in first-phase insulin secretion in response to glucose and a progressive impairment of glucose tolerance compared with age-matched male WT mice. On the other hand, insulin sensitivity of the peripheral tissue in age-matched male ACE2 KO and WT mice was not different. Interestingly, fasting glucose levels were preserved in the male ACE2 KO mice, which show that the β-cell responds appropriately for fasting. Results suggest that the loss of ACE2 is associated with impaired glucose homeostasis in male ACE2 KO mice.

ACE2 plays a key role in reducing levels of the vasoconstrictor angiotensin II and in increasing levels of the vasodilator angiotensin-(1–7) [16]. Hence, the major role of ACE2 in angiotensin metabolism seems to be production of angiotensin-(1–7), whose actions oppose those of angiotensin II. ACE2 enzyme plays a biological role in degrading angiotensin II, which provides a rationale for further exploration of its role in pathophysiological states, including myocardial ischemia, hypertension, heart failure, renal failure, atherosclerosis, and diabetic complications.

Recently, it has been reported that ACE2 levels are reduced in experimental diabetic nephropathy [17]. It is not yet known whether this reduction in ACE2 is of pathophysiological significance in diabetic nephropathy, but one could postulate that ACE2 deficiency leads to a local increase in tubular angiotensin II, with subsequent effects such as promotion of interstitial fibrosis. For instance, local increases in angiotensin II have been also reported in damaged tubules in various experimental models of progressive renal disease [18], such as passive Heymann nephritis [19] and glomerulosclerosis [20]. For instance, in glomerulosclerosis, it has been suggested that elevated angiotensin II levels might contribute to late development of glomerular injury and proteinuria [20]. These studies support the view that local unopposed action of the ACE enzyme is generally associated with enhanced angiotensin II formation, resulting in increased renal damage.

Crackower et al. [12] reported that ACE2 KO mice show no gross abnormalities, and are fertile, and have normal blood pressure and renal function despite elevated angiotensin II levels. Closer examination, however, revealed male ACE2 KO mice exhibit progressive impairment of heart contractility at advanced ages. Significantly, ablation of the ACE gene against an ACE2 KO background rescues the cardiac phenotype, suggesting that ACE and ACE2 negatively regulate each other. In addition to the heart, ACE2 helps control kidney function. In another study, loss of ACE2 is associated with the development of gender-specific and age-dependent glomerular injury in male ACE2 KO mice. These structural and functional changes in the glomeruli of male ACE2 KO mice were prevented by treatment with the angiotensin II type-1 (AT1) receptor antagonist irbesartan [21]. Additionally, Ye et al. [22] propose that ACE2, by regulating the degradation of angiotensin II, prevents angiotensin II accumulation in the glomerulus. Moreover, because it has been shown that ACE2 acts not only on angiotensin I and angiotensin II, but also efficiently cleaves the C-terminal residues from several unrelated peptides such as apelin-13 or dynorphin A 1–13 [11], ACE2 functions may not be limited only to the RAS.

In the endocrine pancreas, islet RAS plays a novel role in regulating glucose homeostasis. In this context, pancreatic blood flow and preferentially islet blood flow is significantly impaired by locally generated angiotensin II, as demonstrated by perfused rat pancreas; this inhibitory effect was rescued by RAS antagonists [23]. Captopril and irbesartan selectively enhance pancreatic islet blood flow, insulin secretion, and glucose tolerance [24]. Interestingly, angiotensin II dose-dependently inhibits insulin release from isolated mouse islets in response to a high glucose concentration [25].

Several clinical trials have shown that RAS blockers can prevent type 2 diabetes development in high-risk individuals. In this respect, a recent meta-analysis of clinical trials of ACE inhibitors and AT1 receptor blockers has been conducted in order to evaluate these protective effects [26]. This meta-analysis concluded that the mean risk for developing type 2 diabetes was reduced by 27% with ACE inhibitor treatments, 23% with AT1 receptor antagonist treatments, and 25% overall in a pooled analysis of these two RAS blockers. AT1 receptor antagonist treatment reduces the incidence of type 2 diabetes in high-risk individuals in clinical trials despite no improvement in insulin sensitivity in most human studies using the hyperinsulinemic-euglycemic clamp technique (the gold standard method of assessing insulin sensitivity) [27]. Nevertheless, the exact mechanisms for the protective effect of RAS blockade in type 2 diabetes remain ambiguous. Of note, recent identification of an islet RAS in the pancreas and its emerging role in islet function might provide a novel and alternative explanation for the reduced incidence of type 2 diabetes observed in these clinical trials [25].

In order to delineate this issue, several animal models of type 2 diabetes have recently been developed for this topical research. For the long-term effect (24 weeks) of ACE inhibitor on pancreatic islets, ramipril is administered to Otsuka Long-Evans Tokushima fatty rats; ramipril treatment can prevent islet destruction by fibrosis in these diabetic rats, as shown by the expression profile of TGF-β and its downstream signaling molecules [9]. Using another rat model of type 2 diabetes, Zucker diabetic fatty rats, chronic treatment (10 weeks) of either an ACE inhibitor (perindopril) or AT1 receptor blocker (irbesartan) attenuates islet fibrosis as well as apoptosis and oxidative stress [8]. More recently, Chu et al. [7] reported that AT1 receptor antagonist improves β-cell function and glucose tolerance in db/db mice. These data suggest that islet RAS activation may be involved in oxidative stress-induced islet apoptosis and fibrosis, and thus islet dysfunction is observed in type 2 diabetes. In the rat pancreas, ACE2 protein was localized to acini and islets [8]. ACE2 activity may counterbalance the angiotensin II promoting effects of ACE by preventing angiotensin II accumulation in tissues [17, 28]. The finding that the pancreas contains a functional RAS may go a long way in accounting for the beneficial effects of ACE2 activator therapy on the onset of type 2 diabetes.

Glucose homeostasis in mammals is tightly controlled by a balanced interaction between peripheral tissue sensitivity to insulin and insulin secretion. Insulin sensitivity was evidenced by the insulin tolerance test. Insulin sensitivity was not significantly different between the two groups, suggesting that ACE2 KO mice do not experience any change in insulin sensitivity. This is somewhat surprising since an inverse relation between insulin secretion and insulin sensitivity is often seen [29]. On the other hand, the measure of insulin action used here might be too general. Thus, more detailed measures of insulin sensitivity (such as using the hyperinsulinemic-euglycemic clamp technique) and glucose effectiveness need to be performed in further studies. Because female ACE2 KO mice were not available, all experiments in this study were performed using male mice. Some future studies should be done with female mice.

In summary, in this study, male ACE2 KO mice show impaired islet function. Results show that ACE2 plays a novel role in regulating glucose homeostasis. Notably, ACE2 functions as a protease on additional molecular targets that could contribute to the observed in vivo phenotypes of ACE2 KO mice. Thus, ACE2 seems to be a molecule that plays a protective role in the pancreas. The development of drugs that could activate ACE2 function would extend our treatment options in diabetes. These findings provide a novel and at least partial explanation for the reduced incidence of type 2 diabetes observed in a number of clinical trials, where ACE inhibitors and AT1 receptor blockers are applied to individuals at high risk for this disease. These findings have important implications for our understanding of ACE2, the RAS, and pancreatic disease, with ACE2 likely to be an important therapeutic target in pancreatic disease.

Materials and methods

Animals

ACE2 KO mice and their age- and sex-matched WT littermates were provided by the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences; the mice were maintained on the C57BL/6 background. ACE2 KO mice were identified by PCR on genomic DNA extracted from tail biopsies. All experiments followed the national ethical guidelines implemented by our institutional Animal Care and Use Committee and were approved by the local authorities. All mice were maintained on a 12 h dark/light cycle. Because female ACE2 KO mice were not available, all experiments in this study were performed using male mice.

Immunohistochemistry and ACE2 localization

Continuous paraffin section slides were made for ACE2 analyses and negative controls. The following procedure was then performed on each slice: in brief, 4 μm sections of Bruin's solution-fixed, paraffin-embedded tissues were deparaffinized with xylene and alcohol. Immunohistochemical staining for ACE2 was carried out, immersing the sections in 3% H_2O_2 to block endogenous peroxide. Antigen retrieval was performed by immersing the slides in 0.01 mM citrate buffer for 15 min, then allowing the slides and buffer to cool for half an hour. After soaking in PBS for 5–10 min, non-specific binding was blocked using normal goat serum for 30 min. The goat anti-ACE2 antibody (Santa Cruz Biotechnology, Santa Cruz) was used at 1:50 dilution with a 4°C overnight incubation. After excess antibody was removed, slides were treated with biotin-labeled rabbit anti-goat antibody at room temperature for 20 min. Streptavidin-Horseradish peroxidase was applied and the slides were incubated for 20 min at room temperature. After washing, slides were incubated with diaminobenzidine as a chromagent for 5–15 min or until the desired color intensity was obtained. The slides were then rinsed and counterstained lightly with Mayer hematoxylin, then examined under a conventional microscope after removing the excess substrate in ddH_2O. Negative controls were performed without the primary antibody, just applying dilution buffer of the primary antibody.

Glucose tolerance test and insulin secretion test

Mice were fasted overnight for 14 h, but allowed free access to water. Mice were fasted overnight for 14 h, followed by IP glucose injection (2 g/kg body weight) [30]. Blood samples were taken from the tail vein at 0, 15, 30, 60, and 120 min after glucose injection. Blood glucose was measured using an automatic glucometer (One Touch, Lifescan).

For insulin release, glucose (3 g/kg body weight) [30] was injected IP in 6-month-old male mice, and blood samples were collected at 0, 2, 5, 15, and 30 min in a centrifuge tube containing no anti-coagulant. Blood samples were left at room temperature for 30 min, and centrifuged for 15 min at 4°C. We transferred and stored serum samples at −70°C. Insulin levels were measured with the mouse insulin ELISA kit (Linco Research, USA).

Insulin tolerance test

Mice were fed freely and then fasted during the study. IP insulin tolerance test was performed on fed mice with 0.75 U human insulin (Novolin R; Novo Nordisk Pharma Ltd) per kg body weight as previously described [31] and blood samples were taken from the tail vein at 0, 15, 30, and 60 min after insulin injection. Blood glucose was measured using an automatic glucometer (One Touch, Lifescan).

Statistical analysis

All statistical analyses were performed using SPSS 11.5. Data are expressed as mean ± SEM. Statistical significant of differences was assessed using one-way analysis of variance, and unpaired Student's *t*-test, as appropriate. A *P*-value <0.05 was considered significant. Area under the curve for glucose levels during the GTT was determined using the trapezoidal rule.

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