Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 355 (2007) 23–27
BBRC
www.elsevier.com/locate/ybbrc

Transgenic insulin released from G cells preferentially signals in the liver ☆

Yu-Chun Lu [1], Enrique Rozengurt, Elena Zhukova *

Department of Medicine, Division of Digestive Diseases, David Geffen School of Medicine at UCLA and CURE: Digestive Diseases Research Center, Los Angeles, CA 90095, USA

Received 22 December 2006
Available online 22 January 2007

Abstract

We have previously produced transgenic *G-InsKi* mice, a model allowing regulated portal insulin delivery from gastric G cells without using β cells. Here, we report that in *G-InsKi* mice portal levels of transgenic human insulin are 6-fold higher than in peripheral circulation. Peptone-induced release of transgenic human insulin from G cells preferentially stimulated signaling cascades in the liver rather than in peripheral insulin-sensitive tissues, as judged by tyrosine phosphorylation of insulin receptor β subunit and phosphorylation of protein kinase Akt/PKB at Thr-308. *G-InsKi* mice provide a novel animal model for elucidating direct effects of insulin on liver functions.

Keywords: Transgenic *G-InsKi* mice; Insulin; G cell; Diabetes; Liver; Hepatic insulin receptor phosphorylation; Hepatic Akt phosphorylation; Hepatic glucose production; Senescence; Akita mice

Insulin exerts its potent effects on liver function, including hepatic glucose production (HGP), via complex mechanisms counting direct and indirect effects (reviewed in [1]). The indirect actions of insulin are exerted via regulation of glucagon secretion from the α cell, release of non-esterified fatty acid from the adipocyte, reduction in supply of gluconeogenic precursors by fat and muscle, as well as via neural inputs to the liver [2–7]. In mice, the direct effects of insulin on the regulation of HGP were demonstrated in liver-specific insulin receptor knock-out (LIRKO) mice, which showed near complete loss of insulin action on glucose production [8,9]. However, in wild-type mice acute decrease in the expression of the hepatic IR (up to 95%) by administration of antisense oligodeoxynucleotide did not modify hepatic insulin action [10]. Therefore, despite multiple studies including those in humans and dogs (reviewed in [11,12]), it remains unclear whether under physiological conditions a major component of insulin action on liver function is direct, i.e. via insulin receptor, or indirect.

We have previously produced transgenic *G-InsKi* mice in which human insulin is released portally by gastrin-producing stomach G cells in response to protein-rich meals, but not glucose [13]. Specifically, transgenic *G-InsKi* mice were generated by knocking-in the coding sequence of human insulin into the mouse gastrin gene. This genetic design positions *G-InsKi* mice as a unique model allowing regulated portal insulin delivery without using β cells. When crossed to diabetic *Akita* mice ($Ins2^{Akita}$), a genetic model of β cell loss characterized by depletion of mouse pancreatic insulin and early mortality [14–16], transgenic human insulin exerted dramatic effect on survival of diabetic *Akita* mice ($Ins2^{Akita/+}$: 50% survival at 10.3 months vs. $Ins2^{Akita/+}$/*G-InsKi*$^{+/-}$: no deaths by 13 months) despite modest levels of human insulin in peripheral circulation (10.7 pM fasted and 20.7 pM fed). In order to explain the dramatic effect

☆ This work was supported by the National Institute of Health Grant DK 63607 (E.Z.) and CURE Core Center Grant (DK 41301).
* Corresponding author. Fax: +1 310 794 5332.
E-mail address: ezhukova@ucla.edu (E. Zhukova).
[1] Present address: Department of Food Science, Central Taiwan University of Science and Technology, Taichung City 40601, Taiwan (R.O.C.).

0006-291X/$ - see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.bbrc.2007.01.059

that low levels of transgenic insulin exert on survival in *Akita* mice, we hypothesized that insulin released by G cells reaches significantly higher levels in portal circulation and therefore preferentially initiates signaling cascades in the liver rather than in peripheral insulin-sensitive tissues. The experiments presented here were designed to test this hypothesis. Our results demonstrate that peptone-induced release of transgenic human insulin preferentially stimulates insulin receptor tyrosine phosphorylation and Akt activation in the liver, but not in the skeletal muscle in *G-InsKi* mice. We propose that *G-InsKi* mice provide a novel model that should be useful to separate direct from indirect effects of insulin on liver cells.

Materials and methods

Animals. Generation and genotyping of transgenic knock-in *G-InsKi* mice in which human insulin cDNA is inserted into the mouse gastrin gene is described in [13]. All mice were housed in specific pathogen-free barrier facilities, maintained on a 12-h light/dark cycle, and fed a standard autoclavable rodent diet (PMI Feeds, Inc., Richmond, IN). *G-InsKi* mice were maintained on C57Bl/6 background as heterozygotes to avoid creation of the gastrin-null phenotype. The studies were approved by the institutional committee on animal care.

In vivo experimental procedures and analytical procedures. All experiments were done with male 10–12 weeks old *G-InsKi* mice. For the measurements of circulating human insulin in peripheral and portal circulation mice in postabsorbtive state were anesthetized by i.p. injection of pentobarbital at a dose of 75 mg/kg of body weight. Blood for analyses was collected by retro-orbital bleeding from ophthalmic venous plexus and from canulated portal vein. For portal blood collection abdomen was opened with a large midline ventral abdominal incision. The intestine loops were moved away from the lower liver lobe and the pancreas to allow access to the portal vein. Portal blood was collected using 24G catheter and placed into heparinized tubes. Mice were euthanized immediately after blood collection by cervical dislocation. Human insulin in serum was determined using human insulin enzyme-linked immunosorbent assay kit, which has no cross-reactivity with mouse insulin (Linco Research, Inc., St. Charles, MO) as described in [13]. For the induction of transgenic insulin release from gastric G cells, 8% solution of peptone in water (Beckton–Dickinson, Sparks, MD) was instilled into anesthetized mice by intragastric gavage at the volume of 1% of body weight as described in [13].

Protein extract preparation, immunoprecipitation, and Western blot analysis. For immunoblotting, livers and skeletal muscles from posterior limbs were collected at various time-points (before and 5–30 min after gavage). Tissues were immediately frozen in liquid nitrogen and subsequently extracted in a buffer containing 20 mM Tris (pH 7.5), 1% NP40, 10 mM magnesium chloride, 10 mM EGTA, 1 mM DTT, 2 mM PMSF, and 1 mM sodium orthovanadate. Homogenates were clarified by centrifugation at 40,000*g* for 20 min.

After centrifugation, supernatants were transferred to fresh tubes and proteins were immunoprecipitated ON at 4 °C with protein A agarose and IR β-subunit antibody (SC-19; Santa Cruz Biotechnology, Inc.). Immunoprecipitates were washed 3× with lysis buffer and extracted in 2× SDS–PAGE sample buffer (200 mM Tris–HCl, pH6.8, 1 mM EDTA, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, and 10% glycerol). The resulting immunocomplexes were resolved on 8% SDS–PAGE gel, transferred to immobilon-P membranes and analyzed for total tyrosine phosphorylation using phosphotyrosine antibody 4G10 (Upstate Biotechnology). Membranes were blocked with 5% (w/v) non-fat skim milk in phosphate-buffered saline, pH 7.2 (PBS) for at least 30 min at room temperature, and then incubated ON at 4 °C with phosphotyrosine antibody 4G10 diluted 1:1000 in 3% (w/v) non-fat skim milk in PBS. Immunoreactive bands were visualized using horseradish peroxidase-conjugated antibodies against mouse IgG (GE Healthcare, NJ) and enhanced immunofluorescence methods. To normalize for protein content, membranes were stripped and re-probed with anti-IR antibody at a 1:200 dilution.

For detection of phosphorylated and total Akt, detergent-solubilized extracts were resolved on the 10% SDS–PAGE gel and transferred to immobilon-P membranes. Membranes were incubated ON at 4 °C with anti-phospho-Akt antibody (Cell Signaling Technology, Inc., MA) diluted 1:1000 in 3% (w/v) non-fat skim milk in PBS. Immunoreactive bands were visualized using anti-rabbit secondary antibody (GE Healthcare, NJ) and enhanced immunofluorescence methods as described above. To normalize for protein content, total Akt was detected by reprobing membranes with anti-Akt antibody (Cell Signaling Technology, Inc., MA) diluted 1:1000 in 3% (w/v) non-fat skim milk in PBS. The intensity of the bands corresponding to IR and Akt was measured using scanning densitometry of the autoradiograms.

Results and discussion

Levels of gastric insulin in portal circulation in postabsorbtive state

Previous studies in *G-InsKi* mice demonstrated that levels of transgenic human insulin measured in peripheral circulation in postabsorbtive state were 20.7 ± 0.7 pM (means ± SEM, $n = 19$) [13]. Since ~80% of insulin released from the pancreas is extracted during the first liver passage [17], it is conceivable that in *G-InsKi* mice levels of transgenic human insulin released by G cells into the portal vein could be significantly higher than peripheral levels. To test this possibility, we measured human insulin levels in blood collected from ophthalmic venous plexus and from canulated portal vein of *G-InsKi* mice in postabsorbtive state. We found that the levels of transgenic human insulin in the portal circulation were 6.6 ± 2.1 (SEM, $n = 11$, $P < 0.03$) fold higher than in the peripheral circulation. To substantiate these findings, levels of transgenic human insulin in portal circulation following peptone gavage were determined as a function of time. As shown in Fig. 1, the increase in human insulin released from G cells in response

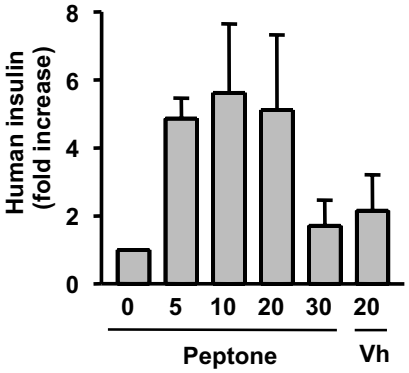

Fig. 1. Portal levels of transgenic human insulin in *G-InsKi* mice as a function of time. Transgenic human insulin was released by intragastric administration of 8% peptone. Levels are expressed as fold increase over basal (0 min) from at least five mice per time-point, means ± SEM. Increases at 5, 10, and 20 min were statistically significant ($P < 0.02$).

to peptone administration reached a maximum at 5–10 min and thereafter gradually declined.

Gastric insulin released by peptone meal activates hepatic insulin receptors in G-InsKi mice

Having characterized the human insulin levels in portal circulation at early time-points after peptone gavage, our next step was to determine whether transgenic human insulin initiates signaling in the liver as compared to peripheral tissues in *G-InsKi* mice. Specifically, we measured tyrosine phosphorylation of insulin receptor β-subunit in *G-InsKi* mice and non-transgenic controls. Release of gastric insulin was induced by intragastric instillation of 8% peptone, protein hydrolyzate that stimulates secretion from G cells, as previously described [13]. Livers were collected from *G-InsKi* mice and non-transgenic controls at various times (before and 5, 10, 20, and 30 min after gavage). The insulin receptor β-subunit (IRβ) was immunoprecipitated (IP) from liver homogenates and the resulting immunocomplexes were analyzed by Western blot (WB) for total tyrosine phosphorylation using phosphotyrosine antibody 4G10. As shown in Fig. 2A, in *G-InsKi* mice Western blot analysis of the resulting immunocomplexes revealed bands of 95 kDa corresponding to the apparent molecular mass of mature insulin receptor β chain. Intragastric administration of peptone caused a rapid (5 min) and statistically significant 8- to 9-fold increase in the IRβ phosphorylation that declined to near base-line levels by 30 min after treatment (Fig. 2B, gray bars). Intragastric administration of vehicle (water) did not cause a significant change in the phosphorylation of IRβ. Administration of peptone to non-transgenic mice did not induce any detectable change in the tyrosine phosphorylation of IRβ at early time-points (5–30 min), (Fig. 2B, open bars) indicating that peptone-induced activation of hepatic IR is specific to *G-InsKi* mice.

Insulin receptor phosphorylation in skeletal muscle of G-InsKi mice: absence of response to gastric insulin released by peptone

In order to assess if release of gastric insulin is able to elicit biological responses in skeletal muscle, skeletal muscle was collected from posterior limbs of *G-InsKi* mice

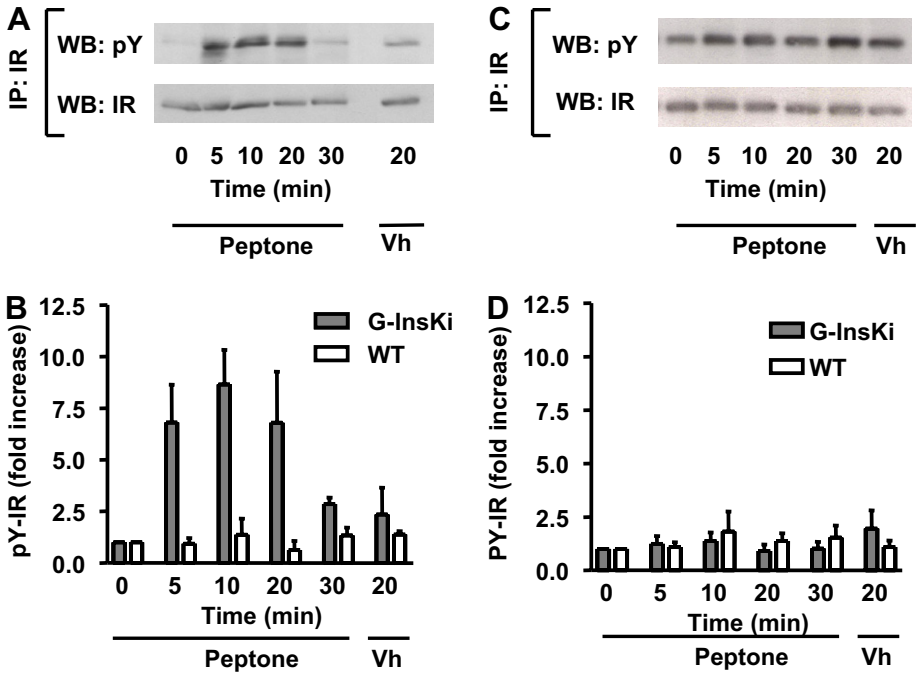

Fig. 2. Tyrosine phosphorylation of insulin receptor by transgenic human insulin in *G-InsKi* mice. (A) *G-InsKi* mice received 8% peptone or vehicle (Vh) by intragastric gavage. Liver tissue was collected before and at indicated time-points after gavage. The β-subunit of the insulin receptor (IRβ) was immunoprecipitated (IP) from liver homogenates with the specific antibody. The resulting immunocomplexes were analyzed by Western blot (WB) for total tyrosine phosphorylation using phosphotyrosine antibody 4G10 (pY, upper panel) or total IR antibody (IR, lower panel). Representative immunoblots are shown. (B) Quantification of hepatic IR phosphorylation kinetics by scanning densitometry normalized to the total amount of IR protein. Scanning data obtained from at least five independent experiments are expressed as fold increase over basal (untreated) phosphorylation. Results are presented as means ± SEM. For *G-InsKi* mice (gray bars), values obtained at 5, 10, and 20 min were statistically different from basal ($P < 0.02$). (C) *G-InsKi* mice received 8% peptone or vehicle (Vh) by intragastric gavage. Skeletal muscle tissue was collected from posterior limbs before and at indicated time-points after gavage. IRβ was immunoprecipitated (IP) from homogenates of skeletal muscle with the specific antibody. The resulting immunocomplexes were analyzed by Western blot (WB) for total tyrosine phosphorylation using phosphotyrosine antibody 4G10 (pY, upper panel) or total IR antibody (IR, lower panel). Representative immunoblots are shown. (D) Quantification of muscle IR phosphorylation kinetics by scanning densitometry normalized to the total amount of IR protein. Scanning data obtained from at least three independent experiments are expressed as fold increase over basal (untreated) phosphorylation. Results are presented as means ± SEM, $P > 0.05$.

and non-transgenic controls and tyrosine phosphorylation of insulin receptor β-subunit was characterized by Western blot. Release of gastric insulin was induced by intragastric administration of peptone as described above. Skeletal muscle was collected at various times (before and 5–30 min after gavage). The insulin receptor β-subunit was immunoprecipitated from homogenates of skeletal muscle and the resulting immunocomplexes were analyzed by Western blot for total tyrosine phosphorylation using phosphotyrosine antibody 4G10. As shown in Fig. 2C, Western blot analysis of the resulting immunocomplexes revealed bands of 95 kDa corresponding to the size of mature insulin receptor β chain. However, intragastric administration of peptone did not cause any significant change in IRβ phosphorylation neither in *G-InsKi* nor in non-transgenic control mice (Fig. 2D, black and open bars, respectively) at the times tested. In contrast with the differential stimulation of IRβ tyrosine phosphorylation in the liver and in skeletal muscle induced by peptone stimulation of transgenic insulin release, i.p. injection of insulin at 2.5 U/kg b.w. induced the expected increase in the IRβ tyrosine phosphorylation in both liver and skeletal muscle (results not shown). Taken together, these results indicate that transgenic human insulin triggers signaling in the liver within 5 min of peptone administration, but does not reach a concentration in the peripheral circulation sufficient to stimulate insulin signaling cascades in muscle.

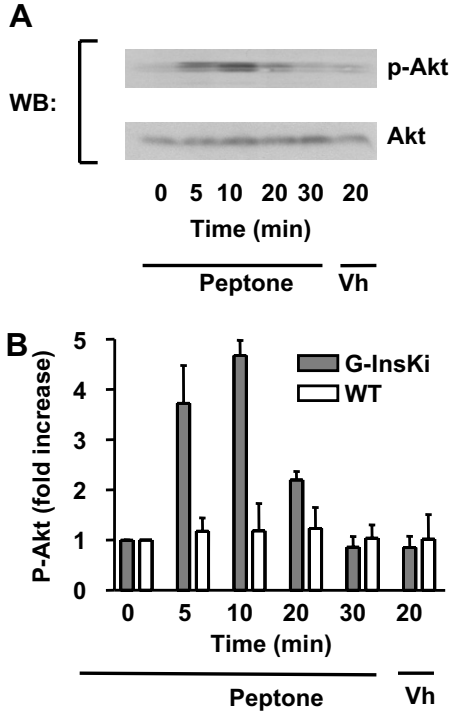

Fig. 3. Peptone-induced release of transgenic gastric insulin activates hepatic Akt in *G-InsKi* mice. (A) *G-InsKi* mice received 8% peptone or vehicle (Vh) by intragastric gavage. Liver tissue was collected before and at indicated time-points after gavage. Phosphorylation of Akt at T308 was analyzed by Western blot (WB, p-Akt, upper panel) and compared to the total amount of Akt protein (Akt, lower panel). Representative immunoblots are shown. (B) Quantification of Akt phosphorylation kinetics by scanning densitometry normalized to the total amount of Akt protein. Scanning data obtained from at least five independent experiments are expressed as fold increase over basal (untreated) phosphorylation. Results are presented as means ± SEM. For *G-InsKi* mice (gray bars), values obtained at 5 and 10 min were statistically different from basal ($P < 0.01$).

Gastric insulin released by peptone meal causes phosphorylation of Akt at T308 in liver of G-InsKi but not wild-type mice

The serine/threonine kinase Akt/PKB is activated by insulin through a pathway that requires PI3 K-dependent generation of PI(3,4,5)P3. To demonstrate that phosphorylation of hepatic insulin receptors by transgenic human insulin in *G-InsKi* mice stimulated signaling pathways, we determined phosphorylation of Akt/PKB kinase at Thr-308.

As described above, release of gastric insulin was induced by intragastric instillation of 8% peptone, and livers were collected at various time-points (before and 5–30 min after gavage). Phosphorylation of Akt at Thr-308 was analyzed by Western blot analysis of liver homogenates. As shown in Fig. 3A and B, intragastric administration of peptone caused a significant 3.5- to 4-fold increase in phosphorylation of Akt at Thr-308 in *G-InsKi* mice. This increase was time-dependent, reached maximum within 5 min and gradually decreased by 30 min after administration of peptone (gray bars). Similarly to IRβ tyrosine phosphorylation, no increase in phosphorylation of Akt at Thr-308 was observed in non-transgenic mice at any of the time-points tested (0–30 min, open bars), indicating that peptone-induced activation of hepatic IR is specific to *G-InsKi* mice.

Concluding remarks

Despite extensive investigations, the direct and indirect effects of insulin on liver response are difficult to dissect. As demonstrated in this paper, *G-InsKi* mice—a unique model allowing portal insulin delivery without using β cells—are characterized by the preferential and regulated insulin signaling in the liver. Specifically, in *G-InsKi* mice, peptone-induced release of transgenic human insulin into portal circulation causes stimulation of insulin signaling cascades in the liver at early time-points, including tyrosine phosphorylation of insulin receptor and phosphorylation of protein kinase Akt/PKB at Thr-308. In contrast, the levels of transgenic human insulin in the peripheral circulation are not sufficient to activate signaling in muscle. Taken together, these results indicate that *G-InsKi* mice offer a novel transgenic model that allows preferential regulated activation of insulin receptors in the liver.

The feasibility of this model is further increased by the fact that it can be studied on the background of diabetic Akita mice as has been demonstrated in our previous studies [13]. Akita mice are diabetic because of a dominant mutation in the Ins2 gene, and are characterized by

shortened life span and a wide range of senescence-associated phenotypic abnormalities [18]. A striking feature of $Ins2^{Akita/+}/G\text{-}InsKi^{+/-}$ mice is the restoration of the normal duration of the life span in the presence of the portally delivered transgenic human insulin. Therefore, an important implication of the results presented here is that the direct effect of insulin on liver may play a critical role in promoting the survival of diabetic Akita mice, a proposition that warrants further experimental work.

References

[1] J. Girard, The inhibitory effects of insulin on hepatic glucose production are both direct and indirect, Diabetes 55 (Suppl. 2) (2006) S65–S69.

[2] H. Ishihara, P. Maechler, A. Gjinovci, P.L. Herrera, C.B. Wollheim, Islet beta-cell secretion determines glucagon release from neighbouring alpha-cells, Nat. Cell Biol. 5 (2003) 330–335.

[3] J. Diao, Z. Asghar, C.B. Chan, M.B. Wheeler, Glucose-regulated glucagon secretion requires insulin receptor expression in pancreatic alpha-cells, J. Biol. Chem. 280 (2005) 33487–33496.

[4] G.F. Lewis, M. Vranic, P. Harley, A. Giacca, Fatty acids mediate the acute extrahepatic effects of insulin on hepatic glucose production in humans, Diabetes 46 (1997) 1111–1119.

[5] S.D. Mittelman, R.N. Bergman, Inhibition of lipolysis causes suppression of endogenous glucose production independent of changes in insulin, Am. J. Physiol. Endocrinol. Metab. 279 (2000) E630–E637.

[6] S. Obici, B.B. Zhang, G. Karkanias, L. Rossetti, Hypothalamic insulin signaling is required for inhibition of glucose production, Nat. Med. 8 (2002) 1376–1382.

[7] R.W. Gelling, G.J. Morton, C.D. Morrison, K.D. Niswender, M.G. Myers Jr., C.J. Rhodes, M.W. Schwartz, Insulin action in the brain contributes to glucose lowering during insulin treatment of diabetes, Cell Metab. 3 (2006) 67–73.

[8] M.D. Michael, R.N. Kulkarni, C. Postic, S.F. Previs, G.I. Shulman, M.A. Magnuson, C.R. Kahn, Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction, Mol. Cell 6 (2000) 87–97.

[9] S.J. Fisher, C.R. Kahn, Insulin signaling is required for insulin's direct and indirect action on hepatic glucose production, J. Clin. Invest. 111 (2003) 463–468.

[10] C. Buettner, R. Patel, E.D. Muse, S. Bhanot, B.P. Monia, R. McKay, S. Obici, L. Rossetti, Severe impairment in liver insulin signaling fails to alter hepatic insulin action in conscious mice, J. Clin. Invest. 115 (2005) 1306–1313.

[11] A.D. Cherrington, The role of hepatic insulin receptors in the regulation of glucose production, J. Clin. Invest. 115 (2005) 1136–1139.

[12] A.D. Cherrington, Banting Lecture 1997. Control of glucose uptake and release by the liver in vivo, Diabetes 48 (1999) 1198–1214.

[13] Y.C. Lu, C. Sternini, E. Rozengurt, E. Zhukova, Release of transgenic human insulin from gastric g cells: a novel approach for the amelioration of diabetes, Endocrinology 146 (2005) 2610–2619.

[14] M. Yoshioka, T. Kayo, T. Ikeda, A. Koizumi, A novel locus, Mody4, distal to D7Mit189 on chromosome 7 determines early-onset NIDDM in nonobese C57BL/6 (Akita) mutant mice, Diabetes 46 (1997) 887–894.

[15] J. Wang, T. Takeuchi, S. Tanaka, S.K. Kubo, T. Kayo, D. Lu, K. Takata, A. Koizumi, T. Izumi, A mutation in the insulin 2 gene induces diabetes with severe pancreatic beta-cell dysfunction in the Mody mouse, J. Clin. Invest. 103 (1999) 27–37.

[16] D. Ron, Proteotoxicity in the endoplasmic reticulum: lessons from the Akita diabetic mouse, J. Clin. Invest. 109 (2002) 443–445.

[17] J.J. Meier, J.D. Veldhuis, P.C. Butler, Pulsatile insulin secretion dictates systemic insulin delivery by regulating hepatic insulin extraction in humans, Diabetes 54 (2005) 1649–1656.

[18] M. Kakoki, C.M. Kizer, X. Yi, N. Takahashi, H.S. Kim, C.R. Bagnell, C.J. Edgell, N. Maeda, J.C. Jennette, O. Smithies, Senescence-associated phenotypes in Akita diabetic mice are enhanced by absence of bradykinin B2 receptors, J. Clin. Invest. 116 (2006) 1302–1309.