Rat insulin promoter 2-Cre recombinase mice bred onto a pure C57BL/6J background exhibit unaltered glucose tolerance

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Abstract

β-Cell-specific gene targeting is a widely used tool when studying genes involved in β-cell function. For this purpose, several conditional β-cell knockouts have been generated using the rat insulin promoter 2-Cre recombinase (RIP2-Cre) mouse. However, it was recently observed that expression of Cre alone in β-cells may affect whole body glucose homeostasis. Therefore, we investigated glucose homeostasis, insulin secretion, and β-cell mass in our line of RIP2-Cre mice bred onto the C57BL/6J genetic background. We used 12- and 28-week-old female RIP2-Cre mice for analyses of insulin secretion *in vitro*, glucose homeostasis *in vivo* and β-cell mass. Our mouse line has been backcrossed for 14 generations to yield a near 100% pure C57BL/6J background. We found that fasting plasma glucose and insulin levels were similar in both genotypes. An i.v. glucose tolerance test revealed no differences in glucose clearance and insulin secretion between 12-week-old RIP2-Cre and WT mice. Moreover, insulin secretion *in vitro* in islets isolated from 28-week-old RIP2-Cre mice and controls was similar. In addition, β-cell mass was not different between the two genotypes at 28 weeks of age. In our experiments, we observed no differences in glucose tolerance, insulin secretion *in vivo* and *in vitro*, or in β-cell mass between the genotypes. As our RIP2-Cre mice are on a near 100% pure genetic background (C57BL/6J), we suggest that the perturbations in glucose homeostasis previously reported in RIP2-Cre mouse lines can be accounted for by differences in genetic background.

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Introduction

Gene inactivation, using the Cre/LoxP system, is an essential technique in tissue-specific gene targeting. The rat insulin promoter 2-Cre recombinase (RIP2-Cre) mouse (also called RIP-Cre or B6.Cg-Tg(Ins2-cre)25Mgn/J, originally based on a C57BL/6xDBA/2 background) is a transgenic mouse expressing the bacterial Cre driven by a 706 bp fragment of the RIP2 (Lee *et al.* 2006). While this mouse line exhibits high expression of the Cre restricted to the β-cells within pancreatic islets (Gannon *et al.* 2000), the RIP2-Cre mouse also displays a low level of Cre expression in the hypothalamus. Since then, several β-cell-specific knockout (KO) mice have been created using the RIP2-Cre mouse (Kulkarni *et al.* 1999, Postic *et al.* 1999, Silva *et al.* 2000, Ristow *et al.* 2003). When the target gene in β-cells is ubiquitously expressed, Cre expression in the brain may be problematic resulting in a double KO of the gene of interest. Further concerns were raised when it was reported that the RIP2-Cre mouse itself may be glucose intolerant due to perturbed insulin secretion (Lee *et al.* 2006). This could contribute to some of the phenotypical characteristics observed in the different β-cell-specific KO lines. The publication by Lee *et al.* (2006) has highlighted the importance of appropriate control experiments, when using the RIP2-Cre mouse for β-cell-specific gene targeting. Here, we show that the previously reported phenotypic changes in RIP2-Cre mice are not obligatory upon Cre expression in β-cells, but may rather be controlled by a rigorous backcrossing strategy.

Material and Methods

Animals

Male RIP2-Cre heterozygote mice were obtained from Michael Ristow (Potsdam, Germany) in 2002. The mice originated from Mark A Magnuson (Vanderbilt, Nashville, TN, USA). The generation of the RIP2-Cre transgenic mouse is described elsewhere (Postic *et al.* 1999). Our mice have been consistently bred (14 generations) onto a C57BL/6J background to >99·9% purity. C57/BL/6J mice for backcrossing were purchased from Taconic, Skensved, Denmark. This particular line of C57BL/6J mice originates from Jackson Laboratory (Bar Harbor, ME, USA) and was transferred to the NIH Animal Genetic Resource in 1951. Forty years later (1991), they were imported to the Taconic facility from the NIH via Cesarian, and have been inbred since (http://www.taconic.com/anmodels/B6.htm). Genotype of the

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RIP2-Cre transgene was determined by PCR on genomic DNA as previously described (Kulkarni *et al.* 1999, Postic *et al.* 1999, Silva *et al.* 2000, Ristow *et al.* 2003). In all experiments, female litter mates were used (12 and 28 weeks of age). The study was approved by the Regional Animal Ethics Committee in Lund.

Blood sampling and analysis

Blood was collected from anesthetized mice (midazolam (0·4 mg per mouse; Dormicum, Hoffman-La Roche) and a combination of fluanison (0·9 mg per mouse) and fentanyl (0·02 mg per mouse; Janssen, Beerse, Belgium)) by retro-orbital sampling. Glucose was determined in plasma by Infinity (Glucose Ox, TR 1521-125; Thermo Electron Corporation. Melbourne, Australia). Insulin *in vivo* and *in vitro* was measured by RIA (Linco Research, St Charles, Missouri, USA).

Intravenous glucose tolerance test

For the i.v. glucose tolerance test (IVGTT), D-glucose (1 g/kg) was injected into the tail vein of ten anesthetized mice of each genotype (see above for anesthesia). Plasma glucose and insulin levels were determined in retro-orbital blood samples collected at the time points indicated in Fig. 1. All animals were fasted at 2300 h, and retro-orbital blood was drawn from anesthetized mice at 0700 h.

Insulin secretion in vitro

Islets were isolated from four female 28-week-old RIP2-Cre mice and four female WT mice by standard collagenase digestion, and handpicked as previously described (Ristow *et al.* 2003).

Immunocytochemistry and β-cell mass

Indirect immunocytochemistry to detect insulin in pancreatic sections, and islet size measurements were performed as previously described (Harndahl *et al.* 2004). In brief, pancreatic sections from RIP2-Cre ($n=6$) mice and WT ($n=7$) were stained for insulin, and digitized images were collected. Three sections from each of three levels of the pancreatic tissue block from every mouse were analyzed. All islets in every section were measured and the mean area of insulin-stained β-cells was calculated.

*PCR analysis of regions corresponding to exons 8 and 11 in the nicotinamide nucleotide transhydrogenase (*Nnt*) gene*

PCR was performed on genomic DNA from either islets or tail tips from RIP2-Cre, WT littermates, C57BL/6J, and NMRI mice, using primers specific for exon 8 (Nnt exon 8 fwd primer: CCAGGCGAGCACTCTCTATT and rev primer: CAGGGTCACAGGAGAACACA) and exon 11 (Nnt exon 11 fwd primer: TCCTGCTATTCCTCCTCCTG and rev primer: GCTGCCTTGACTTTGGATATT) in the *nnt* gene as previously described (Freeman *et al.* 2006, Huang *et al.* 2006). Tail tips were digested with proteinase K (Ambion, Austin, TX, USA) as previously described (Kulkarni *et al.* 1999, Postic *et al.* 1999, Silva *et al.* 2000, Ristow *et al.* 2003) and total DNA was extracted from islets (Qiagens DNeasy blood and tissue kit).

Results

Plasma glucose and insulin, and IVGTT

Fasted plasma glucose (7·1±3 vs 5·9±1 mmol/l) and insulin levels (0·74±0·2 vs 0·85±0·5 pmol/l) were similar in 12-week-old RIP2-Cre and WT mice (Fig. 1). During the IVGTT, glucose clearance was not statistically different in the two genotypes (A). The curves of the insulin levels in the tolerance test were virtually superimposable (B).

One-hour static incubations in vitro *of isolated islets*

Insulin secretion *in vitro* was analyzed in isolated islets from 28-week-old RIP2-Cre and WT littermates ($n=4$ mice). Islets

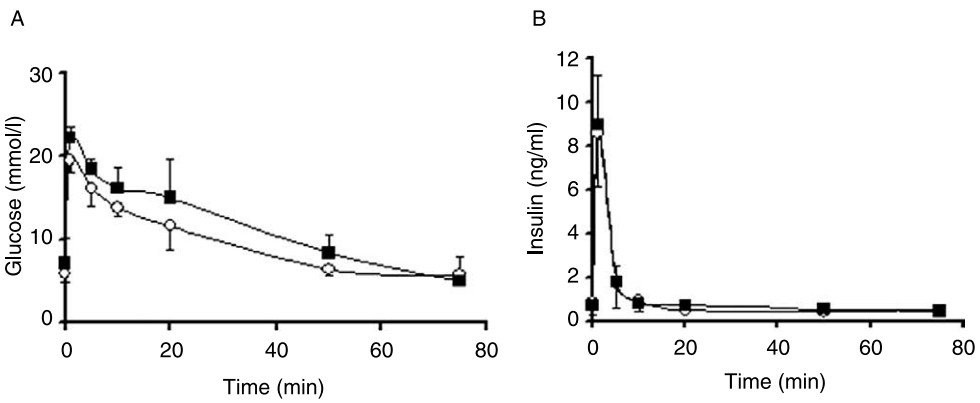

Figure 1 IVGTTs performed in 12-week-old female RIP2-Cre and WT littermates ($n=10$) after an 8-h fast. (A) Plasma glucose and (B) insulin concentrations. White circles represent WT mice and black squares represent RIP2-Cre mice. Values are given as mean±S.E.M.

were incubated at 2·8 and 16·7 mmol/l glucose for 1 h under both K_{ATP}-dependent and -independent conditions (35 mmol/l KCl and 250 μmol/l diazoxide). In addition, various secretagogues were tested in the batch experiment: 1 mmol/l palmitate, 100 nmol/l glucagons-like peptide-1, 100 μmol/l carbacholine, and 20 mmol/l α-ketoisocaproic acid. Using these secretagogues, we were unable to detect any significant difference in insulin secretion between the two genotypes (Fig. 2).

Immunocytochemistry and β-cell mass

Next, we estimated β-cell mass in pancreatic sections from 28-week-old female RIP2-Cre and WT mice. Based on analysis of all islets in nine sections from three different portions of the pancreas, we were unable to detect any difference in area of stained β-cells between RIP2-Cre and WT littermates (3488 ± 327 vs 3277 ± 507 μm^2/islet; $P = 0{\cdot}8$; Student's *t*-test; values are given as means ± S.E.M.).

PCR analysis of regions corresponding to exons 8 and 11 in the nnt *gene*

It has recently been reported that C57BL/6J mice spontaneously become glucose intolerant due to impaired insulin secretion (Freeman *et al.* 2006). This was attributed to a mutation in the *nnt* gene where a deletion of exons 7–11 results in complete removal of Nnt protein. As shown in Fig. 3, our strains of mice (one NMRI mouse as a positive control, three female C57BL/6J mice, one RIP2-Cre, and one WT), were analyzed with PCR for exons 8 and 11 of the *nnt* gene. None of these animals carried the Nnt mutation. PCRs from all animals used display perfect bands of exons 11 (A) and 8 (B).

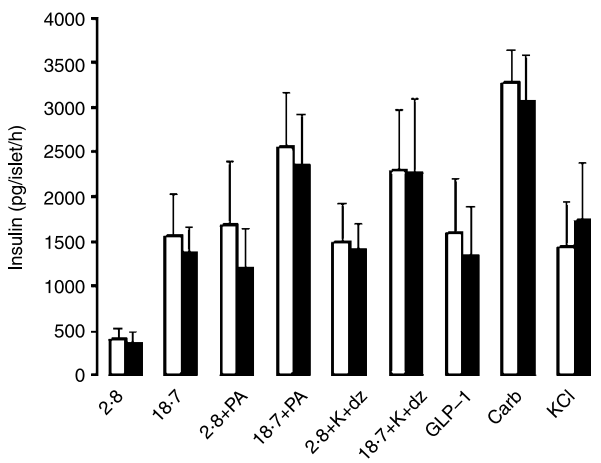

Figure 2 Insulin secretion in isolated islets from 12-week-old female mice ($n = 4$) in response to 2·8 and 16·7 mmol/l glucose, 1 mmol/l palmitate (PA), 35 mmol/l KCl, and 250 μmol/l diazoxide (K+dz), 100 nmol/l GLP-1, and 100 μmol/l carbacholine (Carb). White bars indicate insulin secretion from WT islets and black bars from RIP2-Cre islets. Values are given as mean ± S.E.M.

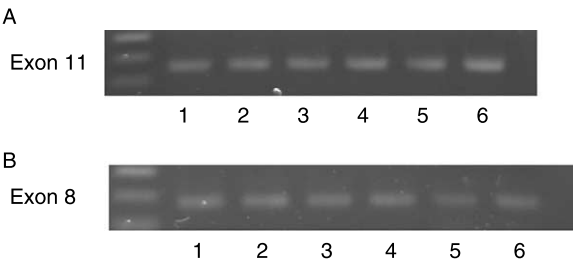

Figure 3 PCR analysis of exons 8 and 11 of the *nnt* gene from genomic DNA extracted from WT littermates, RIP2-Cre, C57/BL/6J, and NMRI mice. Lanes in order of appearance on the gel: 1. NMRI mouse positive control, 2. C57/BL/6J, 3. C57/BL/6J, 4.C57/BL/6J, 5. WT., and 6. C57/BL/6J. (A) Exon 11 displays a 250 bp band; (B) exon 8 displays a 182 bp band.

Discussion

The present study was prompted by a recent report (Lee *et al.* 2006), where investigators from three laboratories demonstrated retarded glucose elimination due to impaired insulin secretion in three different RIP2-Cre lines on different genetic backgrounds. While the cause of this disturbance is unknown, a toxic effect on β-cells by high expression of an ectopic protein, in this case a bacterial enzyme, is not unlikely. Cre expression in β-cells (and/or brain) may cause problems when interpreting the phenotype of mouse lines created with the aid of the RIP2-Cre mouse. However, we found no significant differences in glucose tolerance *in vivo*, *in vivo* and *in vitro* insulin secretion, and β-cell mass between RIP2-Cre and WT littermates.

The RIP2-Cre mouse originating from the laboratory of Mark A Magnuson in Nashville, TN, USA (Postic *et al.* 1999) is now globally distributed; it is also available from the Jackson Laboratory. The RIP2-Cre mouse has been cross bred with a variety of different mouse strains, depending on the laboratory where it is housed, and the objective of the breeding. This further implies that the RIP2-Cre lines are genetically heterogeneous, and raises the possibility that genetic factors other than Cre, specifically for each individual line, may influence the phenotype of any mouse derived from a given RIP2-Cre mouse. For this reason, it is important that appropriate controls are employed in genetic experiments involving RIP2-Cre. This is further underscored by the fact that the some of the different RIP2-Cre lines were found to exhibit perturbed glucose tolerance (Lee *et al.* 2006).

The expression of Cre in the brain previously described in this mouse (Gannon *et al.* 2000) is consistent with reports of insulin expression in the brain (Havrankova *et al.* 1978) but may also be due to promiscuous RIP2 promoter activity. It is thus not unlikely that Cre expression in the brain combined with Cre expression in the β-cell may be the underlying cause of the phenotype observed by Lee *et al.* (2006). However, there may be differences in the penetrance of Cre expression in the brain of the different RIP2-Cre lines. Whether such

differences is determined by the genetic background of the RIP2-Cre lines remains to be shown.

While the RIP2-Cre line in Henninghausen's laboratory shares a similar genetic background with ours (Lee *et al.* 2006), the result from the glucose tolerance tests is still divergent. This may be due to several reasons. First, the identity of WT controls in Henninghausen's experiments is not clearly stated. Therefore, it is not known whether controls in that particular experiment were WT littermates or pure C57BL/6J control mice. Secondly, the C57BL/6J mice used to backcross their RIP2-Cre mouse may originate from the Jackson Laboratory. It was recently published that C57/BL/6J mice from Jackson Laboratory harbor the Nnt mutation (Freeman *et al.* 2006, Huang *et al.* 2006). This mutation has been implied to cause impaired insulin secretion due to mitochondrial uncoupling, which results in decreased ATP production, and hence decreased insulin secretion (Freeman *et al.* 2006). Finally, a difference in the route of glucose administration may explain the divergent results. We performed an IVGTT whereas Henninghausen and colleagues used an i.p. glucose tolerance test (IPGTT). In an IVGTT, β-cells are much more rapidly exposed to elevated plasma glucose than in an IPGTT. Conceivably, the more or less instant response required from β-cells in this situation is more challenging, and will likely be more sensitive to reveal any β-cell dysfunction. Nevertheless, the difference in glucose clearance between the two lines still remains, and needs to be further investigated.

The founder mouse from our line was established in the laboratory of Michael Ristow (Potsdam, Germany) and from the laboratory of Mark Magnuson originally on a (C57BL/6J/6xDBA2) background. This line was 66% C57BL/6J at the time we received it, and has been backcrossed thrice since then (Lee *et al.* 2006). This mouse has also been investigated with regard to β-cell mass (Pomplun *et al.* 2007). Here, the authors provide convincing results of alterations in β-cell mass at 4- and 36-week-old RIP2-Cre mice. At 4 weeks, their RIP2-Cre mice exhibit a reduction in overall β-cell mass. Surprisingly, at 36 weeks, RIP2-Cre β-cell mass is increased when compared with WT littermates. Whether these mice harbor the Nnt mutation or not is not known at this time. However, if they do, the observed changes in β-cell mass may very well be a result of this mutation. This issue further indicates that genetic background is crucial when analyzing genetically modified mice. Thus, genetic background may have a major impact on phenotypical characteristics, such as glucose homeostasis. For instance, Kulkarni *et al.* (2003) showed that mice heterozygous for a double inactivation of the insulin receptor and insulin receptor substrate-1 alleles in three different genetic backgrounds exhibited markedly different glucose tolerance.

In conclusion, our experiments show that β-cell expression of Cre is not necessarily linked to β-cell dysfunction. Moreover, we believe that a possible negative influence of Cre can be controlled by rigorous backcrossing of the mice onto a pure genetic background, preferably one that does not contain the Nnt mutation. Clearly, studies employing RIP2-Cre for conditional gene targeting should always include control experiments with RIP2-Cre mice to ensure that the mice used are not glucose intolerant.

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