

RIP2-Cre mice bred onto a pure C57BL/6J background exhibit unaltered glucose tolerance

Malin Fex^{1,2,4}, Nils Wierup¹, Marloes Dekker Nitert^{1,4}, Michael Ristow³ and Hindrik Mulder^{1,4}.

¹*Department of Experimental Medical Science, Lund University, Sweden*

²*Department of Clinical Science, Malmö University Hospital, Sweden*

³*Institute of Nutrition, University of Jena, Germany*

⁴*Lund University Diabetes Centre, Sweden.*

Short title: Glucose tolerance in RIP2-Cre mice

Key words: Insulin secretion, gene expression, glucose tolerance, transgenic mice

Corresponding Author:

Malin Fex, PhD. Department of Clinical Science, Unit for Diabetes and Celiac disease, Clinical Research Centre, Malmö University Hospital, SE-20502 Malmö, Sweden.

Phone: +4640391906 E-mail: malin.fex@med.lu.se

Keywords: RIP2-Cre, insulin secretion in vivo and in vitro, glucose tolerance,

Word count: Abstract 249

Main text: 2052

Abstract

Beta cell-specific gene targeting is a widely used tool when studying genes involved in beta cell function. For this purpose, several conditional beta cell knock outs have been generated using the RIP2-Cre mouse. However, it was recently observed that expression of Cre-recombinase alone in beta cells may affect whole body glucose homeostasis. Therefore, we investigated glucose homeostasis, insulin secretion and beta 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 beta 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 intravenous 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 were similar. In addition, beta 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 beta cell mass between the genotypes. Because our RIP2-Cre mice are on a near 100% pure genetic background (C57BL/6J), we suggest that the perturbations of glucose homeostasis previously reported in RIP2-Cre mouse lines can be accounted for by differences in genetic background.

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 recombinase (Cre) driven by a 706 bp fragment of the rat insulin promoter 2 (RIP2) (Lee, et al. 2006). While this mouse line exhibits high expression of the Cre-recombinase restricted to the beta cells within pancreatic islets (Gannon, et al. 2000), the RIP2-Cre mouse also displays a low level of Cre-recombinase expression in the hypothalamus. Since then, several beta cell-specific knock out (KO) mice have been created, using the RIP2-Cre mouse (Kulkarni, et al. 1999; Postic, et al. 1999; Ristow, et al. 2003; Silva, et al. 2000). When the target gene in beta cells is ubiquitously expressed, Cre-recombinase 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 beta cell-specific KO lines. The publication by Lee et al (Lee et al. 2006) has highlighted the importance of appropriate control experiments, when using the RIP2-Cre mouse for beta cell-specific gene targeting. Here, we show that previously reported phenotypic changes in RIP2-Cre mice are not obligatory upon Cre expression in beta 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. C57BL/6J mice for backcrossing were purchased from Taconic, Skensved, Denmark. This particular line of C57BL/6J mice originates 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 Caesarian, and have been inbred since (<http://www.taconic.com/anmodels/B6.htm>). Genotype of the RIP2-Cre transgene was determined by PCR on genomic DNA as previously described (Kulkarni et al. 1999; Postic et al. 1999; Ristow et al. 2003; Silva et al. 2000). 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/mouse; Dormicum®, Hoffman-La Roche) and a combination of fluanison (0.9 mg/mouse) and fentanyl (0.02 mg/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 radioimmuno assay (RIA; Linco Research, St. Charles, Missouri, USA).

Intravenous glucose tolerance test For the intravenous glucose tolerance test (IVGTT), D-glucose (1g/kg) was injected into the tail vein of 10 anesthetized mice of each genotype (see above for anaesthesia). 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 11.00 p.m. and retro-orbital blood was drawn from anesthetized mice at 7:00 a.m.

Insulin secretion in vitro Islets were isolated from 4 female 28 week old RIP2-Cre and 4 female WT mice by standard collagenase digestion, and handpicked as previously described (Ristow et al. 2003).

Immunocytochemistry and beta 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 analysed. All islets in every section were measured and the mean area of insulin-stained beta cells was calculated.

PCR analysis of regions corresponding to exon 8 and exon 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: CAGGGTCACAGGAGAACA) 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; Ristow et al. 2003; Silva et al. 2000) and total DNA was extracted from islets (Qiagen DNeasy blood and tissue kit, Hilden, Germany).

Results

Plasma glucose and insulin, and intravenous glucose tolerance test 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).

1h 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 were incubated at 2.8 mmol/l and 16.7 mmol/l glucose for 1 h under both K_{ATP} -dependent and -independent conditions (35mmol/l KCl and 250 μ mol/l Diazoxide). In addition, various secretagogues were tested in the batch experiment: 1 mmol/l palmitate, 100 nmol/l glucagon like peptide-1 (GLP-1), 100 μ mol/l carbacholine, and 20 mmol/l α -ketoisocaproic acid. Using these secretagogues, we were unable to detect any significant differences in insulin secretion between the two genotypes (Fig. 2).

Immunocytochemistry and beta cell mass Next, we estimated beta 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 beta cells between RIP2-Cre and WT littermates (3488 ± 327 vs. 3277 ± 507 μ m²/islet; $p=0.8$; Student's t-test; Values are given as Means \pm S.E.M).

PCR analysis of regions corresponding to exon 8 and exon 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, 3 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. PCR reactions from all animals used, display perfect bands of exons 11 (**A**) and 8 (**B**).

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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 beta cells by high expression of an ectopic protein, in this case a bacterial enzyme, is not unlikely. Cre expression in beta 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 beta cell mass between RIP2-Cre and WT littermates.

The RIP2-Cre mouse originating from the laboratory of Mark A. Magnuson in Nashville, Tennessee (Postic et al. 1999) is now globally distributed; it is also available from the Jackson Laboratory (Cold Spring Harbour, MA, USA). 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 some of the different RIP2-Cre lines were found to exhibit perturbed glucose tolerance (Lee et al. 2006).

The expression of Cre-recombinase 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 beta cell may be the underlying cause of the phenotype observed by Lee et al., (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 were 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. Second, 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 harbour 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 intraperitoneal glucose tolerance test (IPGTT). In an IVGTT beta cells are much more rapidly exposed to elevated plasma glucose than in an IPGTT. Conceivably, the more or less instant response required from beta cells in this situation is more challenging, and will likely be more sensitive to reveal any beta 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 3 times since then (Lee et al. 2006). This mouse has also been investigated with

regard to beta cell mass (Pomplun, et al. 2007). Here, the authors provide convincing results of alterations in beta cell mass at four week and 36 week old RIP2-Cre mice. At four weeks, their RIP2-Cre mice exhibit a reduction in overall beta cell mass. Surprisingly, at 36 weeks RIP2-Cre beta cell mass is increased as compared to WT littermates. Whether these mice harbour the Nnt mutation or not is not known at this time. However, if they do, the observed changes in beta 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. 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 (Kulkarni, et al. 2003).

In conclusion, our experiments show that beta cell expression of Cre is not necessarily linked to beta cell dysfunction. Moreover, we believe that a possible negative influence of Cre can be controlled by rigorous back crossing 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.

Acknowledgement

We thank Ann-Helen Thorén Fischer and Doris Persson for technical assistance with IVGTTs and immunocytochemistry of sectioned pancreas. We also thank Professor Frank Sundler for critical proofreading of the manuscript.

Funding

These studies were supported by grant from the Swedish research council (14196-03A:H.M.), The Juvenile Diabetes Research Foundation, The Albert Pahlsson, Åke Wiberg, Ingrid and Fredrik Thuring foundation, The Swedish Diabetes Association, The Swedish Royal Physiographic Society, The Krapperup foundation (to M.F.) and the Faculty of Medicine, Lund University. N.W. is supported by The Novo Nordisk Foundation. European Foundation for the Study of Diabetes (EFSD) / Merck Sharp & Dohme (MSD) Award 'European Studies on Beta Cell Function and Survival' (to M.R.), and a Deutsche Forschungsgemeinschaft (DFG) grant (RI 1076/1-2, to M.R.). There is no conflict of interest by any of the authors included in this study.

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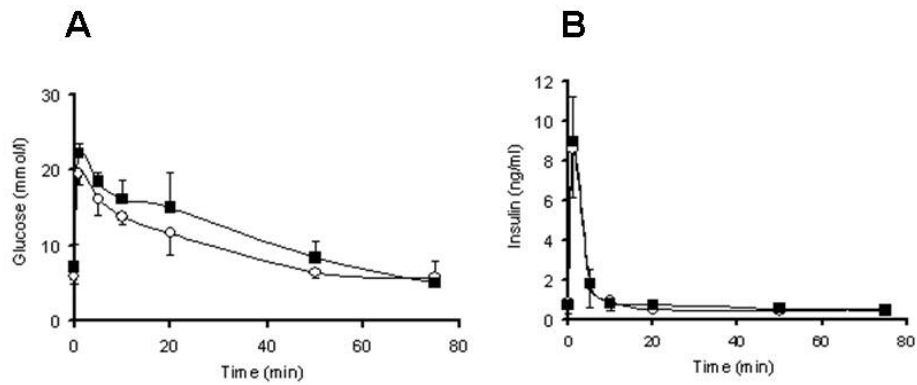


Figure 1. IVGTTs performed in 12 week old female RIP2-Cre and WT littermates (n=10) after an 8 hour fast. Plasma glucose (A) and insulin concentrations (B). White circles represent WT mice and black squares represent RIP2-Cre mice. Values are given as mean \pm S.E.M.

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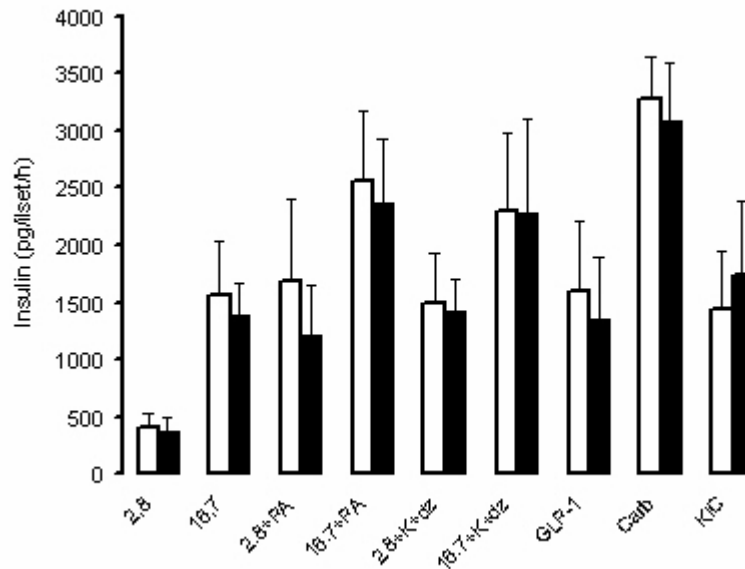


Figure 2. Insulin secretion in isolated islets from 12 week old female mice (n=4) in response to 2.8 and 16.7 mmol/l glucose, 1mmol/l palmitate (PA), 35 mmol/l KCl and 250 μ mol/l diazoxide (K+dz), 100 nmol/l GLP-1, 100 μ mol/l carbacholine (Carb). White bars indicate insulin secretion from WT islets and black bars from RIP2-Cre islets. Values are given as mean \pm S.E.M.

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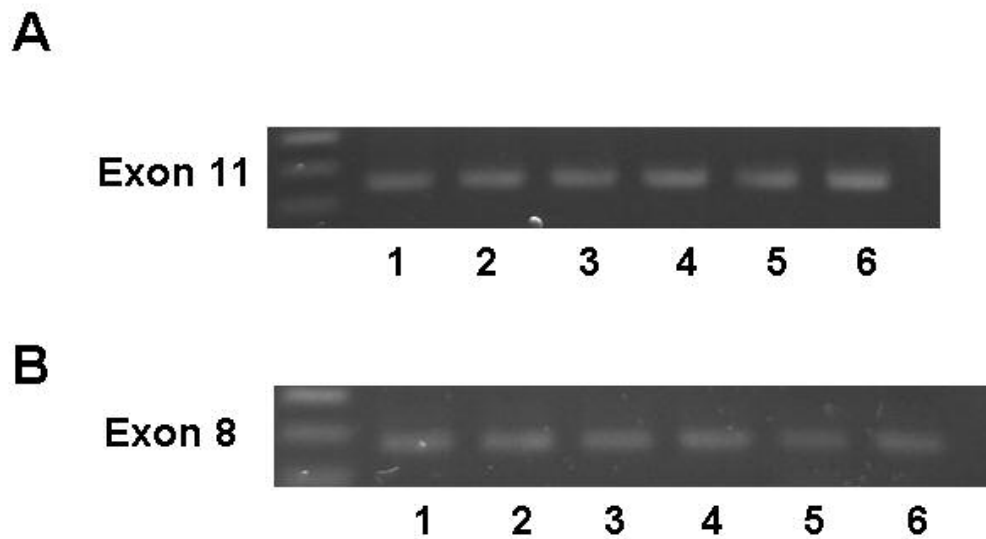


Figure 3 A and B. PCR analysis of exons 8 and 11 of the *nnt* gene from genomic DNA extracted from WT littermates, RIP2-Cre, C57/BL/6J and NMRI mice. Lanes in order of appearance on the gel: 1. NMRI mouse positive control, 2. C57/BL/6J, 3. C57/BL/6J, 4. C57/BL/6J, 5. WT. 6. C57/BL/6J. Exon 11 displays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

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