

ARTICLE

Abnormal Glucose Metabolism in Heterozygous Mutant Mice for a Type I Receptor Required for BMP Signaling

Gregory J. Scott,¹ Manas K. Ray,^{1*} Toni Ward,¹ Kelly McCann,¹ Shyamal Peddada,² Fang-Xu Jiang,³ and Yuji Mishina^{1,4,5}

¹Knock Out Core, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

²Bio-statistical Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

³Beta Cell Development and Regeneration Program, Centre for Diabetes Research, Western Australian Institute for Medical Research, Perth, Western Australia, Australia

⁴Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

⁵School of Dentistry, University of Michigan, Ann Arbor, Michigan

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Summary: BMPRIA and its high-affinity ligand BMP4 have recently been shown to be expressed in the β -cells of the pancreas. Here, we report the abnormalities of heterozygous mice for *Bmpr1a* in glucose metabolism during the course of intraperitoneal glucose tolerance test. The heterozygous mice had increased blood glucose levels throughout the first 2.5 h after the administration of glucose. Analysis of glucose-stimulated insulin secretion (GSIS) indicates that insulin secretion in the heterozygous mice is compromised, and induction of secreted insulin by stimulation is substantially lower compared with the wild-type controls. No apparent abnormalities in pancreas, thyroid, and liver were seen upon histological examination. Real-time PCR results of selected genes showed an increase in the mRNA level of *Ins1* and *Ins2* in the heterozygous group. These results indicate that the glucose-sensing pathway in these heterozygous mice is altered because of the heterozygosity in *Bmpr1a*. Together, our data suggest that BMP signaling through BMPRIA plays an important role in glucose metabolism and possibly working through the GSIS pathway. *genesis* 47:385–391, 2009. © 2009 Wiley-Liss, Inc.

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other cells in the body to utilize sugar molecules in the blood stream. The cause of diabetes is largely unknown, but genetic and environmental factors such as obesity and lack of exercise seem to play a major role in the development of this chronic disease. Many factors are involved in regulating the blood glucose levels through modulation of insulin secretion and/or its proper utilization in glucose metabolism. In addition to glucose-stimulated insulin secretion (GSIS) from pancreatic β -cells, specific amino acids may acutely and chronically regulate insulin secretion from the β -cell (Newsholme *et al.*, 2007). Factors like free fatty acid and inflammatory adipocytokines play a critical role in the development and progression of diabetes (Pfeiffer, 2007). Decreased glucose tolerance in diabetes may lead to impaired renal function (Pessina, 2007). Increased blood glucose levels from elevated hepatic glucose production may happen because of diminished secretion of insulin, impaired hepatic sensitivity to insulin action, or a combination of both (Halter *et al.*, 1985). Chronic inflammation and developing insulin resistance are the two hallmarks of type 2 diabetes. It has been reported that cytokines play important roles in glucose metabolism besides their immunoregulatory roles, and infusion of interleukin-6 in healthy humans is shown to increase glucose disposal by working like a hormone and to help increase fatty acid oxidation (Pedersen, 2007).

INTRODUCTION

Diabetes is a chronic, debilitating, and costly disease that affects 20.8 million children and adults or 7% of the population in the United States. In type 2 diabetes, which accounts for most of the cases, either the pancreas does not produce enough insulin or the insulin is not properly utilized in maintaining the blood glucose level. Insulin is produced by the β -cells in the pancreas, allowing

*Correspondence to: Manas Ray, Knock Out Core, National Institute of Environmental Health Sciences, 111 T. W. Alexander Drive, Research Triangle Park, NC 27709.

E-mail: ray6@niehs.nih.gov

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Other cytokines of interest include the TGF- β superfamily, and there is accumulating evidence that they play an important role in the development of endocrine pancreas as well as in insulin secretion (Gannon, 2007). TGF- β superfamily members, including TGF- β , activin, and bone morphogenetic proteins (BMPs), have been shown to be involved in islet morphogenesis and the establishment of β -cell mass in several gene ablation or overexpression studies in mice. Inhibition of TGF- β signaling resulted in reduced beta cell mass and poorly organized islets, which can be restored in overexpression studies using transgenic mice (Kim *et al.*, 2000; Sanvito *et al.*, 1994; Smart *et al.*, 2006; Yamaoka *et al.*, 1998). TGF- β signaling is involved in insulin production and secretion in adult mice, whereas inhibition of activin signaling resulted in significant decreases in insulin and glucagon expression. There is also a substantial decrease in the number and size of the islets in these mice (Gannon, 2007).

BMPs were originally identified by their ability to induce ectopic bone formation (Urist, 1965). These secreted signaling molecules are involved in various cellular processes including proliferation, differentiation, and the determination of cell fate (Kishigami *et al.*, 2004). BMP4 has been reported to promote the expansion of endocrine progenitor cells in the pancreas by blocking their differentiation, and hence, balancing between differentiation and expansion of pancreatic duct epithelial progenitors (Hua *et al.*, 2006). The role of BMP2 in converting the acinar-like AR42J cells into insulin-secreting β -cells in the presence of exendin-4 (glucagon-like peptide 1) through the Smad pathway has been reported recently, suggesting a role of BMP signaling in β -cell formation (Yew *et al.*, 2005).

BMPRIA (ALK3) is one of three type I receptors for BMPs and is expressed in many tissues during development as well as after birth (Kishigami and Mishina, 2005). The most studied ligands, BMP2 and BMP4, bind to BMPRIA with high affinity, whereas BMP7 binds only weakly to BMPRIA (Aoki *et al.*, 2001) (Miyazono *et al.*, 2005). Mice homozygous for *Bmpr1a* null allele fail to produce mesoderm and die at embryonic day 8.0 (E8.0) (Mishina *et al.*, 1995). Recently, it has been shown that BMPRIA and its high-affinity ligand BMP4 are expressed in differentiating and adult β -cells in the pancreas. Attenuated BMPRIA signaling in the β -cells reduces the expression of key genes that are involved in glucose metabolism leading to the eventual onset of diabetes (Goulley *et al.*, 2007). Here, we studied heterozygous knock out *Bmpr1a* to examine glucose metabolism. We found that the 6-month-old heterozygous mice showed increased blood glucose levels after intraperitoneal injection of glucose, despite their elevated levels of fasting plasma insulin. Glucose stimulation to these mice indicated that in fact, the mutant mice had impaired GSIS as observed during intraperitoneal glucose tolerance test (IPGTT). These results show that the heterozygous *Bmpr1a* mice are morphologically indistinguishable from their wild-type littermates, but manifested abnor-

malities in the glucose signaling pathway resulting in impaired insulin secretion upon glucose stimulation.

RESULTS

Abnormal Glucose Metabolism During IPGTT

In multiple independent experiments, using different groups of 6-month-old heterozygous mutant male mice and their wild-type littermates, blood glucose values rose substantially higher in the heterozygous *Bmpr1a* mice (Fig. 1a). The increased blood glucose levels in the heterozygous mice were maintained for the first 2.5 h postglucose injection and gradually became insignificant at 4 h postinjection. Beyond the increase in initial glucose readings, 13 of the 33 heterozygous mutant mice (39%) showed some form of glucose rebounding and ineffective glucose control over a period of up to 2.5 h. This abnormality presented in different manners, sometimes as oscillations, other times it might increase gradually over the 2.5 h period. Only one control mouse in 22 (4.5%) showed even a mild form of abnormal glucose control. The same experiment performed on 3-month-old mice did not show any overt changes between heterozygous mice and control mice (data not shown). These results suggest that the loss of one copy of the gene causes the delay or reduction in the initial event involved in the metabolism of sugar molecules including the release of insulin in response to the demand.

Statistical Analysis of the IPGTT Data

For further comparison of glucose response over time relative to the baseline values, we divided the observed response at each time point by the response at the initial time point. Because we expected an exponential decay in response over time, we converted the data into log-scale. In doing so, we generated a linear response over time, and more importantly, the variability in response at each time point was approximately constant and was normally distributed (Fig. 1b). The two genotypes differed significantly in their intercepts ($P < 0.0001$), confirming that the heterozygous group had higher initial response to glucose, relative to the baseline, than the control group of animals. There was, however, no significant difference in the slopes between the two genotypes ($P = 0.9337$) (Table 1). Thus, the rate of decay in glucose levels over time, relative to the baseline values, did not differ significantly between the heterozygous and control group. These analyses highlight again that the heterozygous mice showed insufficient response during the first phase.

Abnormalities in Insulin Secretion in the Mutant Mice

There was a slightly elevated plasma insulin level in the heterozygous mutant mice after an overnight fast compared with the wild-type littermates (see Fig. 2). This data indicate that the available insulin may not be utilized properly in mutant mice, reflecting the scenario of type 2 diabetes. Another likely possibility would be a

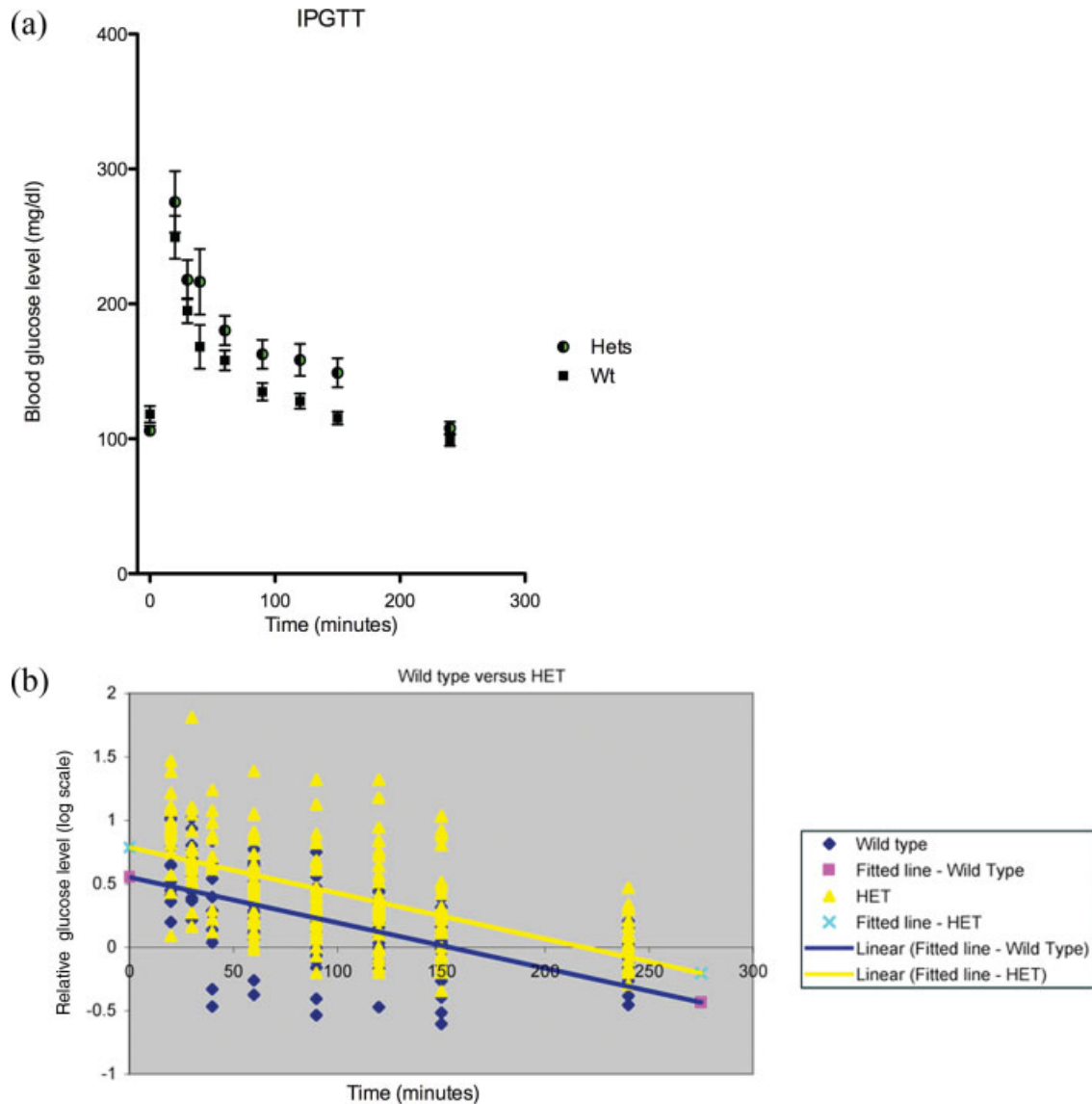


FIG. 1. Analysis of IPGTT data using the glucose response and Scatter plot. (a) Graph of glucose response to IPGTT. The heterozygous animals showed a larger response to the glucose challenge but tended to return back to resting levels at a normal rate. It is important to note that the erratic nature of many of the heterozygous individuals is lost in the averaging. (b) Scatter plot of log ratios, relative to baseline, of control (in blue) and heterozygous (in yellow), along with their respective linear regression lines. All the data points relative to the initial fasting glucose level from the wild-type control mice (Wt) and heterozygous mice (Het) were averaged to determine the significance of the values for intercept (a pink square and a cross on Y-axis) and the slope (a blue line and a yellow line) between the two genotypes.

defect in the GSIS pathway where the mutant mice do not secrete enough insulin to handle the surge of glucose in the plasma. To test this possibility, insulin levels were measured after glucose stimulation. The mutant mice had a much lower insulin release compared with the control animals after glucose challenge (Fig. 2a). The average percent change in insulin level between time 0 and 30-min postglucose injection was substantial. In control animals, the average percent change was 74%, whereas the mutant animals had only an 11% change with a *P*-value of 0.03 (*P* = 0.03) (Fig. 2b). These results indicated that mutant mice not only had a poor response to the glucose surge but also impaired GSIS.

Table 1
Statistical Analysis of the Intercept and the Slope

	Intercept (SE)	Slope (SE)
Wt	0.5512 (0.05978)	-0.00359 (0.000236)
Het	0.7869 (0.05537)	-0.00361 (0.000247)
Difference	0.2357 (0.08148)	-0.00002 (0.000342)
<i>P</i> -value	<i>P</i> < 0.0001	<i>P</i> = 0.9337

Values for intercepts and slopes were obtained from Figure 1b. As shown in the table, there is no statistical significance between the Wt and Het slopes but the difference in the intercept is highly significant.

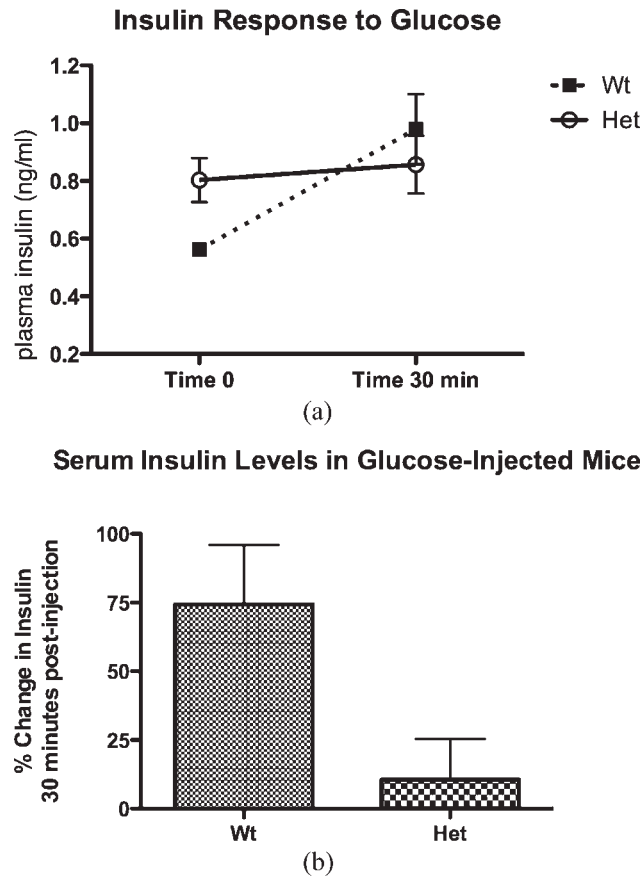


FIG. 2. The average percent change in insulin level after glucose stimulation. (a) The average change of insulin level between the heterozygous (Het) and the control (Wt) mice between 0- and 30-min time period after glucose stimulation. (b) Percent change in serum insulin levels. The percent change in control mice (Wt) is fivefold higher than the heterozygous mice (Het) in that time period.

Histological and Blood Chemistry Analysis of *Bmpr1a* Heterozygous and Wild-Type Mice

Histological analysis was conducted on primary organs involved in controlling blood glucose levels to determine whether there was any gross structural or organizational alteration. The pancreas showed islets that were well formed, of normal size and general morphology, and seemed to occur in a relatively normal quantity (Fig. 3a,b). Besides pancreas, we also performed the histological analyses on thyroid and liver. The oscillations in blood glucose values after glucose exposure prompted us to further analyze whether there is any difference in thyroid hormones. There were no significant differences in T3 (triiodothyronine) or T4 (thyroxine) values between the control and heterozygous mutant mice (1.00 vs. 1.11 ng/ml for T3 and 4.00 vs. 4.35 mg/ml for T4). T3 and T4 values in mice can vary substantially by strain and age, and many factors are known to interfere including autoantibodies with thyroid hormone assays. Thyroid also appeared unremarkable showing no overt phenotype (Fig. 3c,d). Liver was

selected for its role in overall glucose homeostasis and glucose metabolism. Histological examination of the liver revealed no gross abnormalities in the mutant heterozygous mice (data not shown). It has been reported that abnormally high fructosamine concentration in serum correlates with suboptimal glycemic control in humans (Yahaya *et al.*, 2006). The mice, we examined, however, showed no difference in fructosamine levels between the two genotypes (data not shown). Overall, no obvious differences between the heterozygous animals and the wild type littermates were observed in histological analysis of the major three organs involved in maintaining blood glucose levels or serum chemistry.

Gene Expression Analysis

Several genes involved in glucose regulation and metabolism on both the liver and pancreas were selected and tested whether their expression levels were altered in the heterozygous mice. In the liver, *Irs2* (insulin receptor substrate-2), *Srebf1* (Sterol regulatory element binding transcription factor 1), *Srebp-1a*, *Srebp-1c*, *Gck* (glucokinase), and *Gcg* (glucagon) were all tested and showed no significant changes in the heterozygous animals (data not shown). In the pancreas, we tested *Pdx1* (pancreatic and duodenal homeobox 1, *Ipf1*), *Ins1* (insulin 1), *Ins2* (insulin 2), *Slc2a2* [solute carrier family 2 (facilitated glucose transporter), member 2, *Glut2*], and *Gck* (Fig. 4a); although most showed an insignificant difference, the average *Ins1* was increased over 50-fold in the heterozygous mice, and *Ins2* increased over 100-fold. (Fig. 4b).

DISCUSSION

We report here an observed abnormality in glucose metabolism in *Bmpr1a* heterozygous mice. The elevated glucose response observed in 6-month-old heterozygous male mice was consistent among different independent experiments, which were performed using heterozygous and wild-type mice. The higher glucose response in the heterozygous mice was maintained for the first 2.5 h after glucose injection, and it became statistically insignificant thereafter in a 4-h experimental period. This indicates that the initial signaling mechanism required to initiate the glucose metabolism pathway may have been impaired in the heterozygous mice. If insulin resistance in these mice is a possibility, that would make it a mouse model similar to type 2 diabetes in humans.

Another possible explanation for this abnormality may lie in GSIS where there may not be enough insulin available in the plasma for glucose disposal. Subsequent analysis of GSIS in these mice determined that the insulin secretion pathway is impaired in the heterozygous animals. The initial insulin level in the heterozygous animals was elevated after an overnight fast. The increase in insulin after the glucose challenge, however, was much lower in the heterozygous mice. Also of importance was the notable inability of some mice to control their glucose level after the initial response. Analysis of the indi-

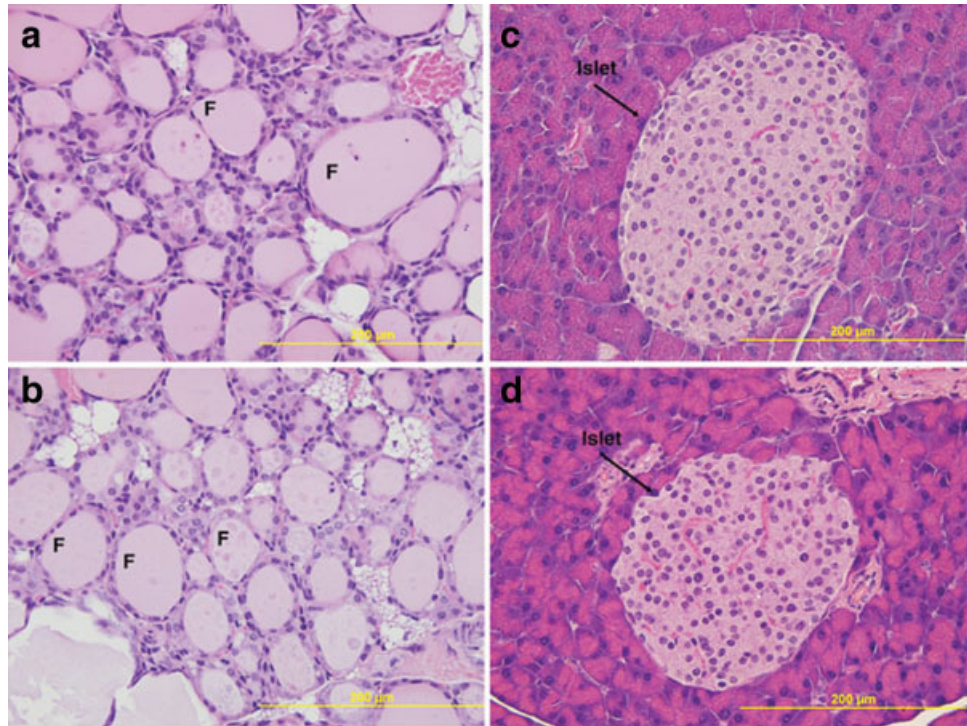


FIG. 3. Histological analysis of pancreas and thyroid using H&E staining. Histology of thyroid (**a, b**) and pancreas (**c, d**), where panels a, c and b, d correspond to the wild type and heterozygous mice, respectively. Paraffin sections were stained with hematoxylin and eosin. No gross abnormalities were observed in either tissue. F, denotes the follicle and the bar represents 200 μm ($\times 40$ original magnification).

vidual results showed that 39% of the heterozygous group had some form of glucose rebounding or instability, this would seem a considerable and significant portion. This could possibly relate back to disrupted timing in the secretions of the β -cells. Islets do secrete insulin in bursts, and can vary, and even be modified in their frequency (Ritzel *et al.*, 2003). It is therefore possible that the frequency of insulin secretion in heterozygous mice is altered and perhaps inconsistent. Much of the difference in the two groups of mice came from this phenotypic 39%. In the only significant gene expression results, *Ins1* and *Ins2* were increased over 50- and 100-fold, respectively, in the heterozygous mice. Closer examination of the data showed that of the 10 heterozygous mice tested for *Ins1* and *Ins2*, none was in the normal range for both, and four (40%) showed an enormous increase (>10 -fold) of *Ins1* and *Ins2*. However, the simple averages do not do justice to this data. The range of results was remarkable in their own right, ranging from 0.40 to 324 fold induction in *Ins1* and 0.34 to 958 fold induction in *Ins2*. This would seem to point to another compounding factor involved in this phenotype.

The consequence of increased *Ins1* and *Ins2* found in insulinomas (Asfari *et al.*, 1992) generally results in a hypoglycemic state with increased insulin levels. In the case of *Bmpr1a* heterozygous mice, despite higher *Ins1* and *Ins2* expression and elevated resting levels of plasma insulin, they fail to respond to the increase in blood glucose. These facts reinforce the idea that the mutant mice cannot respond properly to a glucose challenge. There are significant examples linking the *Tgf- β*

family to *INS*, showing that *Tgf- β 1* increases insulin transcription and release (Sayo *et al.*, 2000). However, there are examples that some human insulinomas show a decrease of *Tgf- β 1* that results in a decrease of its target genes including *TGFBI* and *NMMT* (Nabokikh *et al.*, 2007), suggesting relations between growth factor signaling and insulin expression would be context dependent. Despite accumulating reports describing that BMPs stimulate differentiation of pancreatic cells (Jiang and Harrison, 2005; Jiang *et al.*, 2002), the only study directly assessing *Bmpr1a* signaling on *INS* is found in the pancreas-specific knockout study of *Bmpr1a* (Goulley *et al.*, 2007), which shows a reduction in resting insulin secretion. The increase in *Ins1* and *Ins2* and resting insulin after a reduction of *Bmpr1a* in heterozygous mutant mice seems counter to these previous reports. This would suggest that either the cause of the abnormal blood glucose response in the *Bmpr1a* heterozygous mutant mice is located away from the pancreas, or, more likely, we have a situation where the phenotype is not simply dose dependent, and in fact it is the reduced *Bmpr1a* expression in the pancreas that is responsible. It would seem very possible that an increase of such magnitude in *Ins-1* and *Ins-2* could cause a subsequent reduction of a similar magnitude in the insulin receptor substrates, or their downstream targets, resulting in the inability to respond to the increase in glucose. Further *in vitro* and *in vivo* work will elucidate the exact cause of this inconsistency in insulin secretion.

To determine whether the heterozygous mice suffer any form of hypothyroidism, we tested T3 and T4 levels

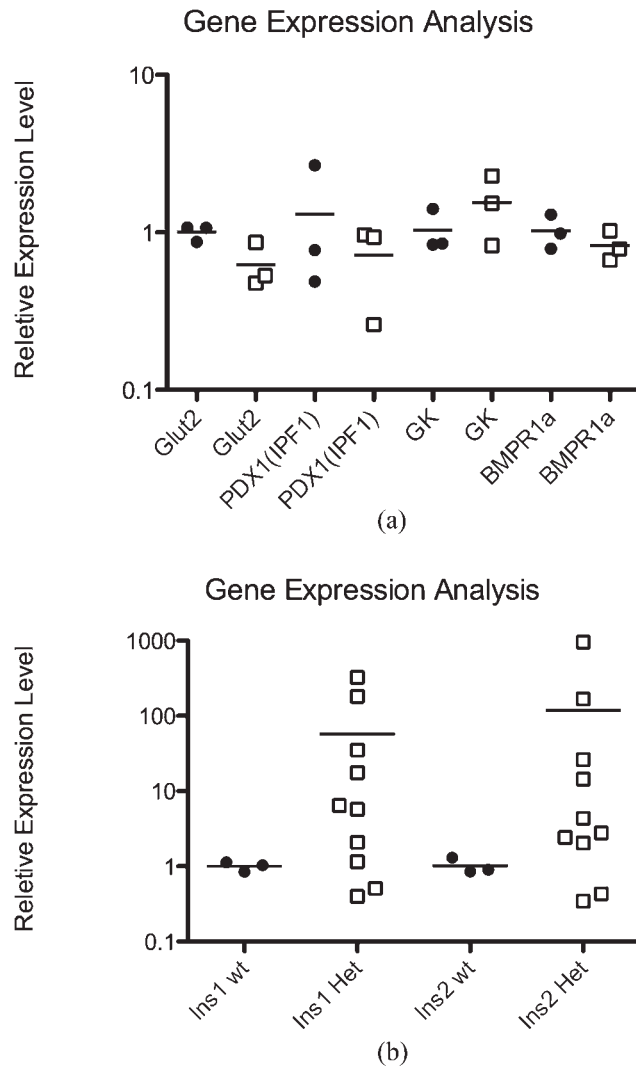


FIG. 4. Real-time RT PCR analysis. Real-time analysis of selected genes revealed that most of the tested genes were not modified in a substantial way (a). The only genes showing any significant changes were *Ins1* and *Ins2*, with average increases over 50- and 100-fold, respectively (b). Relative expression of genes shown in fold increase on a log₁₀ scale. λ = WT, \square = Hets.

in these animals. T3 and T4 values in the heterozygous animals were not different from those of the control animals and fell in the normal range. The heterozygous mice showed no difference in fructosamine level from that of the controls as well.

Overall, these heterozygous mice could well be used for further study to reveal the molecular mechanisms involved in this glucose intolerance, and would be interesting to explore how BMPRIA signaling is linked to normal glucose metabolism. Although the broad ranging and sometimes inconsistent phenotypes of heterozygous mice make them difficult to study, their potential application to human conditions seems to demand that we look further in this area.

METHODS

Generation of Mutant *Bmpr1a* Mutant Mice

Generation of the mutant *Bmpr1a* mouse line was described previously (Mishina *et al.*, 1995). Heterozygous mice were maintained in mixed 129SvEv and C57BL/6 background. Animal protocols were approved by the Institutional Animal Care and Use Committee at the National Institute of Environmental Health Sciences, NIH.

Intraperitoneal Glucose Tolerance Test

Mice were subjected to an overnight fast (greater than 6 h) with access to drinking water. Mice were weighed the next morning and a fasting glucose level was obtained. Blood was collected from the lateral marginal vein in the hind leg, and glucose levels were measured using a Glucometer (Accu-Chek, Roche, NJ). Glucose (2,000 mg/kg) was injected intraperitoneally according to the weight of the individual mouse, and blood glucose levels were measured at either 20, 40, 60, 90, 120, 150, and 240 min or 30, 60, 90, 120, 150, and 240 min postinjection.

Enzyme-Linked Immunosorbent Assay

Serum insulin levels were measured by ELISA (Linco Research, Charles, MO) following the protocol supplied by the manufacturer. Three wild type and five heterozygous mice each had ~ 20 μ l of blood drawn by submandibular puncture at time 0- and 30-min postglucose injection. Blood was allowed to clot and spun at 2,500g for 15 min at 4°C in hemostat tubes to separate serum.

Histology

Tissue samples were collected from animals after standard CO₂ euthanasia. Liver, pancreas, and thyroid glands were removed, fixed in 10% formalin, and embedded in paraffin for sectioning. Tissue was sectioned at 8 μ m and stained with standard H&E protocol.

Gene Expression

Three wild type and 3-10 heterozygous individuals were selected for RNA extraction and real-time analysis. RNA was collected from both the liver and pancreas using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was then made using superscript II (Invitrogen, Carlsbad, CA). Real-time PCR was done using Power SYBR Green from Applied Biosystems (Foster City, CA). All cycles were run with an annealing temperature of 58°C, and all samples were run in at least triplicate and compared using the 2^{DDt} method (Livak and Schmittgen, 2001).

Statistical Methods

Because repeated measurements are recorded on each animal, we expect the responses within animals to be correlated. To account for this correlation, we used

mixed effects models by treating animals as random effects. Using PROC MIXED (statistical software package SAS, version 9.1.3), we fitted a linear regression model with an intercept and a slope for each genotype.

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