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ORIGINAL PAPER

# Effects of lactogen resistance and GH deficiency on mouse metabolism: pancreatic hormones, adipocytokines, and expression of adiponectin and insulin receptors

## Lactogen resistance and GH deficiency in mice

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**Abstract** We recently described a novel mouse model that combines resistance to lactogenic hormones with GH deficiency (GHD). The GHD/lactogen-resistant males develop obesity and insulin resistance with age. We hypothesized that altered production of pancreatic hormones and dysregulation of adipocytokine secretion and action contribute to the pathogenesis of their insulin resistance. Double-mutant males (age 12–16 months) had fasting hyperinsulinemia, hyperamylinemia, hyperleptinemia, and a decreased ratio of adiponectin to leptin. Adiponectin receptor 1 and 2 (AdipoR1 and R2) mRNA levels in liver and skeletal muscle were normal but hepatic insulin receptor mRNA was increased. Relative to double-mutant males, GHD males had lower levels of insulin, amylin, and leptin, higher levels of adiponectin, and higher expression of hepatic AdipoR1 and insulin receptor mRNAs. Lactogen-resistant mice had reduced hepatic adipoR2 mRNA. In response to stress the plasma concentrations of MCP-1 and IL-6 increased in double-mutant males but not GHD or lactogen-resistant males. Our findings suggest that the insulin resistance of GHD/lactogen-resistant males is accompanied by dysregulation of pancreatic hormone and adipocytokine secretion and receptor expression. Phenotypic differences between double-mutant and GHD males suggest that lactogens and GH exert differential but overlapping effects on fat deposition and adipocytokine secretion and action.

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## Introduction

The lactogenic and somatogenic hormones of the pituitary gland and placenta (prolactin, placental lactogen, and growth hormone) constitute a family of polypeptides with similarities in structure and function. The physiological roles of the lactogens in mammary development and casein production and the role of GH in postnatal growth are well established. On the other hand, the roles of placental lactogen, prolactin (PRL), and growth hormone (GH) in fetal and postnatal metabolism have been more difficult to define, in part because the hormones have similar biological activities in a variety of experimental systems. For example, both the lactogens and somatogens induce beta cell proliferation and insulin production in pancreatic islets and insulinoma cells [1–4] and, depending on the experimental conditions, may exert lipogenic, lipolytic, and/or adipogenic effects in white adipose tissue and may antagonize or facilitate the action of insulin [5–16].

Clinical observations have not clarified the roles of the lactogens and somatogens in intermediary metabolism. In part, this is because no humans bearing mutations of PRL or the PRL receptor (PRLR), which binds both PL and PRL, have ever been identified. Moreover, in contrast to rodent and non-primate GHs, the primate GHs bind with high affinity to PRLR as well as GH receptors [17]. Thus human GH may exert "lactogenic" as well as "somatogenic" effects in human tissues.

To clarify the roles of lactogen and GH signaling in the control of carbohydrate metabolism, growth, and abdominal fat deposition, we generated [18] a novel mouse model that combines resistance to all lactogenic hormones with a severe deficiency of pituitary GH. The model was created by breeding PRLR "knockout" (KO) males, which are resistant to the actions of both placental lactogen (in utero) and PRL, with GH-deficient ("little") females, which harbor a mutation in the receptor for GH releasing hormone. Given the lack of binding of mouse GH to the mouse PRLR, the PRLR KO mice are responsive to GH; thus comparisons of double-mutant mice with PRLR KO or GH deficient (GHD) mice may distinguish defects in lactogen signaling from defects in somatogen (GH) signaling in vivo and may identify important lactogen/somatogen signaling interactions [18].

Lactogen-resistant/GHD double-mutant males are growth retarded, hypoglycemic, and hypersensitive to insulin during the first week of life but develop adiposity, insulin resistance, and impaired glucose tolerance with age; in contrast, males with isolated GHD or PRLR-deficiency remain insulin sensitive as adults. Female double mutants have lesser weight gain until after 6–9 months of age and, like GHD females, show normal or increased insulin sensitivity as adults [18].

We hypothesized that altered production of pancreatic hormones and dysregulation of adipocytokine secretion and action characterize the insulin resistance of double-mutant males and might contribute to its pathogenesis. To test that hypothesis, we measured the fasting plasma concentrations of pancreatic hormones (insulin, amylin, and glucagon) and adipocytokines (leptin, adiponectin, monocyte chemoattractant protein-1, interleukin-6, and resistin) in mutant mice and the expression of adiponectin and insulin receptors in liver and skeletal muscle. Because stress plays a role in the release of inflammatory cytokines and the development of insulin resistance [19], we also assessed the adipocytokine and insulin responses to the stress of separation and cooling. We hypothesized that secretion of adipocytokines during stress would be exaggerated in the insulin-resistant double-mutant males.

## Results

### Body weights of mutant and wild-type males and females

The weights (mean ± SE) of the animals in the four experimental groups are shown in Table 1. At 12–16 months of age the GHD and double-mutant mice weighed less than wild-type and PRLR-deficient mice ($P < 0.01$). However, double-mutant males weighed more ($P < 0.05$) than GHD males or double-mutant females. The weights of double-mutant females were slightly (but not significantly) greater than those of GHD females.

**Table 1** Body weights (mean ± SE) of wild-type and mutant mice at 12–16 months of age

| | Males | Females |
|---|---|---|
| Wild-type | 35.2 ± 1.6 | 31.8 ± 2.9 |
| PRLR-deficient | 33.9 ± 2.0 | 36.3 ± 1.5 |
| GH-deficient | 20.0 ± 2.0*** | 17.6 ± 0.9*** |
| Double-mutant | 27.8 ± 3.0**^$ | 20.3 ± 2.2** |

Values represent mean ± SE of 4–8 mice

** $P < 0.01$, *** $P < 0.001$ vs. wild-type, ^ $P < 0.05$ vs. GHD, $ $P < 0.05$ vs. females of same genotype

### Pancreatic hormone levels in mutant and wild-type male and female mice

Under fasting (6 h) conditions, plasma glucose, insulin, and amylin concentrations and the ratio of insulin to glucagon were higher in aging wild-type males than in wild-type females (Table 2).

Fasting glucose levels in GHD males (56.2 ± 4.8 mg%, $P < 0.01$) were lower than those in wild-type (154 ± 11.3 mg%), PRLR KO (129 ± 10.2 mg%), and double-mutant (104 ± 9.2 mg%) males. Relative to wild-type males, GHD males had lower fasting insulin and amylin levels (Fig. 1) but normal glucagon levels (wild-type 56.6 ± 6.0 pM, GHD 91.6 ± 26.1 pM, $P > 0.05$). In contrast, double-mutant males had higher fasting insulin and amylin concentrations and an increased ratio of insulin to glucose; glucagon levels were normal (87.4 ± 22.3 pM).

Fasting glucose levels in GHD (101.8 ± 5.0 mg%) and double-mutant females (115.0 ± 7.4 mg%) were comparable to those in wild-type (102.6 ± 5.5 mg%), PRLR KO (112.9 ± 6.0 mg%) females. Relative to wild-type females, GHD females had lower fasting insulin levels but normal

**Table 2** Fasting (6 h) pancreatic hormone levels in aging wild-type mice

| | Wild-type males | Wild-type females |
|---|---|---|
| Insulin (pM) | 183.7 ± 31.7* | 101.1 ± 27.4 |
| Glucose (mg%) | 148.3 ± 6.3* | 102.6 ± 5.5 |
| Insulin/Glucose | 1.2 ± 0.1 | 1.0 ± 0.2 |
| Amylin (pM) | 18.5 ± 3.6* | 8.7 ± 1.9 |
| Glucagon (pM) | 56.6 ± 6.0 | 81.3 ± 4.7 |
| Insulin/Glucagon | 3.2 ± 0.4* | 1.2 ± 0.5 |

Data are expressed as mean ± SE of 4 males and 3 virgin females. Similar results were obtained in three separate assays

* $P < 0.05$ vs. wild-type females

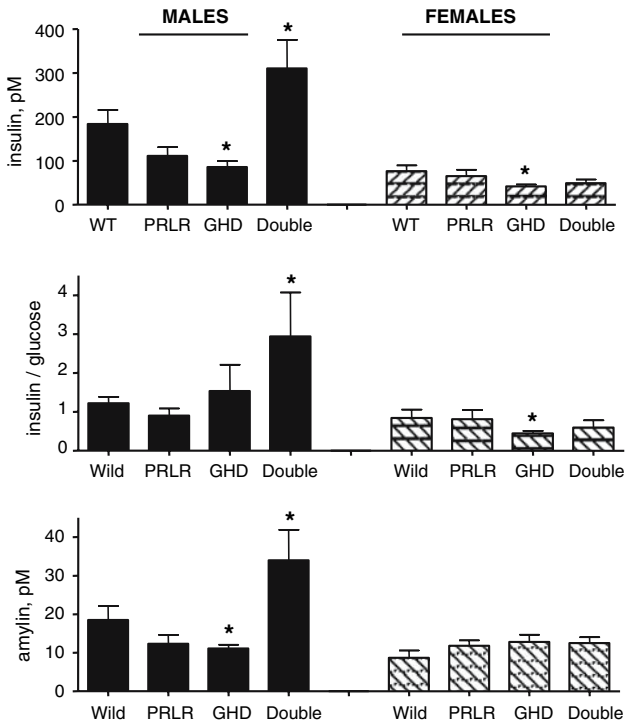


**Fig. 1** Pancreatic hormone levels in mutant and wild-type male and female mice. Blood was collected from mice under fasting (6 h) conditions. Values represent the mean ± SE of 4 wild-type, 5 PRLR KO, 5 GH deficient, and 6 double-mutant males and of 3 virgin wild-type, 5 PRLR KO, 7 GH deficient, and 7 double-mutant females. * $P < 0.05$ vs. wild-type controls

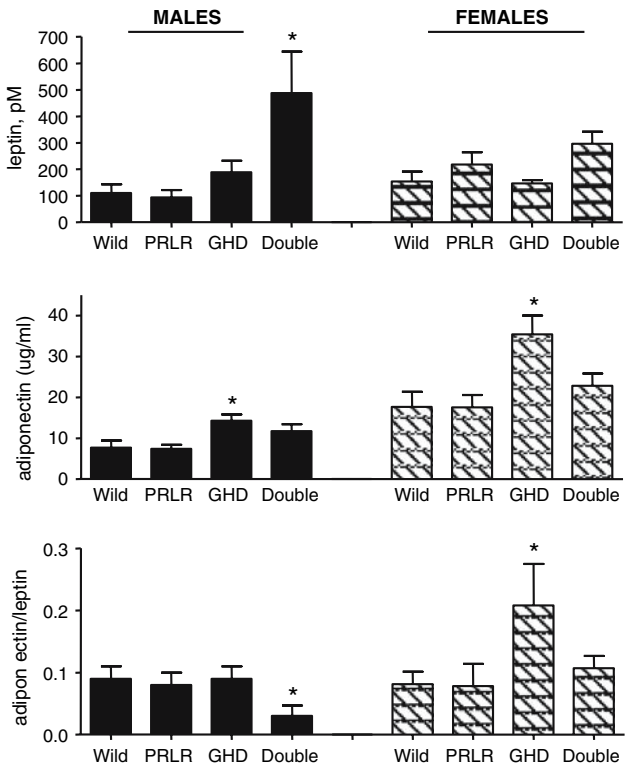


**Fig. 2** Fasting plasma concentrations of leptin and adiponectin and the ratio of adiponectin to leptin in mutant and wild-type males and females. Values represent the mean ± SE of 4–7 mice in each group. The distribution of mice in the various experimental groups is identical to that described in Fig. 1; however, one additional wild-type female was available for the studies shown in this figure. * $P < 0.05$ vs. respective wild-type controls. Adiponectin levels in wild-type females were significantly higher ($P < 0.05$) than those in wild-type males

amylin and glucagon levels; insulin, amylin, and glucagon levels and the ratios of insulin/glucose and insulin/glucagon were normal in double-mutant females (Fig. 1). Pancreatic hormone levels in PRLR-deficient mice did not differ significantly from those in wild-type mice.

## Plasma leptin and adiponectin levels

The adipocyte hormones leptin and adiponectin play central roles in food intake, energy expenditure, and peripheral insulin action [20, 21]. Fasting plasma concentrations of adiponectin were higher in wild-type females than in wild-type males, while plasma leptin levels were comparable (Fig. 2).

Relative to wild-type males, GHD males had increased adiponectin levels (Fig. 2). The ratio of adiponectin to leptin, a metric of insulin sensitivity, was normal (Fig. 2). Double-mutant males also had increased leptin levels but had lesser increases in plasma adiponectin; the ratio of adiponectin to leptin was significantly decreased (Fig. 2).

Relative to wild-type females, GHD females had increased plasma adiponectin and an increase in the ratio of adiponectin to leptin (Fig. 2). Leptin and adiponectin levels were not significantly higher in double-mutant females than in wild-type females and the ratio of adiponectin to leptin was normal (Fig. 2). Plasma concentrations of leptin and adiponectin were normal in PRLR-deficient males and females.

## Adiponectin and insulin receptor expression

Binding of adiponectin to target tissues is mediated by two distinct receptors, AdipoR1 and AdipoR2 [22, 23] AdipoR1 mRNA levels were 1.8-fold higher in wild-type gastrocnemius skeletal muscle than in liver. Conversely, AdipoR2 mRNA levels were 8.9-fold higher in liver than in skeletal muscle (Table 3). Hepatic AdipoR1 mRNA levels were increased (+41%, $P < 0.05$) in GHD mice but were normal in PRLR KO and double-mutant mice (Fig. 3). No significant differences in gastrocnemius AdipoR1 mRNA levels were detected among the experimental groups. Hepatic AdipoR2 mRNA levels were reduced 44% ($P < 0.05$) in PRLR KO mice but AdipoR2 expression was normal in liver and skeletal muscle of GHD and double-mutant mice (Fig. 3).

**Table 3** Insulin receptor, AdipoR1, and AdipoR2 mRNA levels in wild-type mice

| | Corrected CT values | Relative abundance |
|---|---|---|
| Insulin receptor | | |
| Liver | 21.6 ± 0.1 | 4.0 |
| Skeletal muscle | 23.6 ± 0.3 | 1.0 |
| AdipoR1 | | |
| Liver | 21.0 ± 0.1 | 1.0 |
| Skeletal muscle | 20.1 ± 0.1 | 1.8 |
| AdipoR2 | | |
| Liver | 18.9 ± 0.2 | 8.9 |
| Skeletal muscle | 22.0 ± 0.2 | 1.0 |

CT values (corrected for riboprotein mRNA) and relative abundance in liver and skeletal muscle. Values represent the mean ± SE

The actions of insulin are initiated through binding to the insulin receptor (IR) [24]. IR mRNA levels were 4-fold higher in wild-type liver than in skeletal muscle (Table 3). Hepatic IR mRNA levels were approximately 50% higher in GHD and double-mutant mice than in wild-type and PRLR KO mice ($P < 0.05$, Fig. 4). Skeletal muscle IR mRNA levels were comparable among the four groups (Fig. 4).

## Adipocytokine levels in male and female wild-type mice

Insulin action in peripheral tissues is modulated by adipocytokines in addition to leptin and adiponectin; these include interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and resistin. Fasting plasma concentrations of IL-6 were marginally (but not significantly) higher in wild-type females (15.7 ± 4.8 pg/ml, $P = 0.14$) than in wild-type males (5.7 ± 1.0 pg/ml); plasma MCP-1 (females 47.2 ± 9.7 pg/ml, males 73.3 ± 16.4 pg/ml) and resistin levels (females 3517 ± 553 pg/ml, males 2746 ± 251 pg/ml) were comparable in male and female mice.

## Adipocytokine responses to separation and cooling

Emerging evidence suggests a role for stress in the release of adipocytokines and the development of insulin resistance [19]. To determine if the insulin resistance of double-mutant males is accompanied by hypersecretion of adipocytokines during stress, we measured plasma MCP-1, IL-6, and resistin as well as plasma insulin and amylin before, during, and 14 days after a 48-h period of separation and mild cooling (18.5°C). That this procedure represented a form of stress was demonstrated by an increase in plasma corticosterone (expressed as ng/ml) in all groups of mice (wild-type: pre 42.8 ± 6.1; post 173.6 ± 46.4; PRLR-deficient: pre 40.7 ± 11.6, post 132.6 ± 43.0; GHD: pre 64.7 ± 15.1, post 116.2 ± 51.3; double-mutant: pre 40.9 ± 12.9, post 111.3 ± 48.3).

During the cooling period the wild-type, GHD, PRLR-deficient, and double-mutant mice lost weight (−2.5 ± 0.2%, −4.6 ± 0.9%, −5.7 ± 1.2%, and −7.2 ± 1.6%, respectively; all $P < 0.05$, Fig. 5); in each case, weight was restored during the 14-day re-warming (recovery) period.

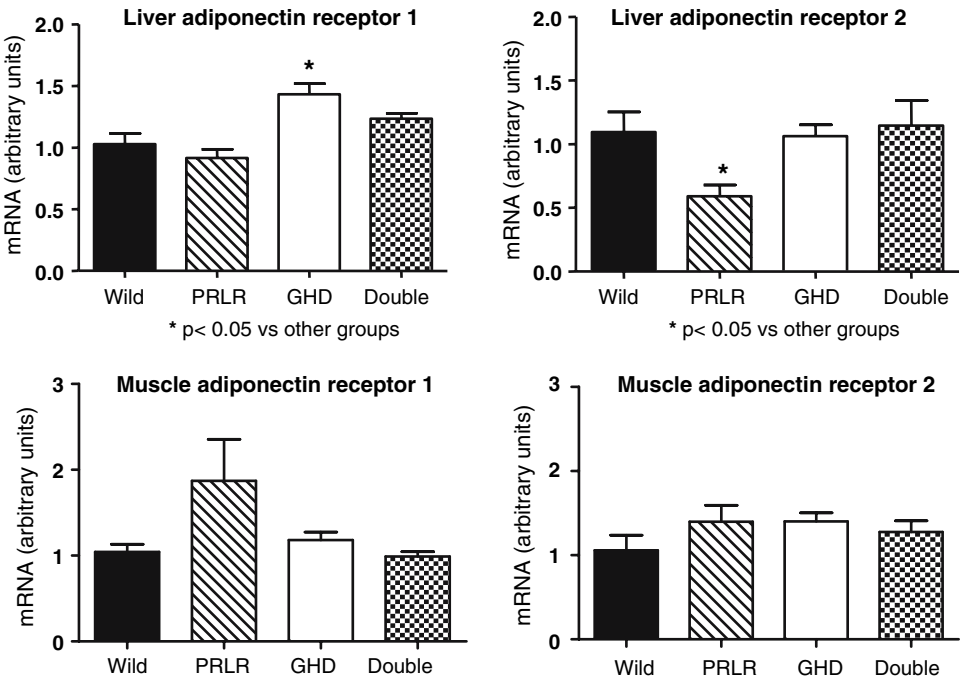


**Fig. 3** Hepatic and skeletal muscle adiponectin receptor 1 and 2 mRNA levels in mutant and wild-type male mice. The mRNA levels were assessed by real-time PCR. Control values, normalized to riboprotein mRNA levels, were arbitrarily set at 1.0. Values represent mean ± SE of 3 wild-type, 5 PRLR KO, 5 GH deficient, and 3 double-mutant males; similar results were obtained in three separate experiments.
* $P < 0.05$ vs. other groups

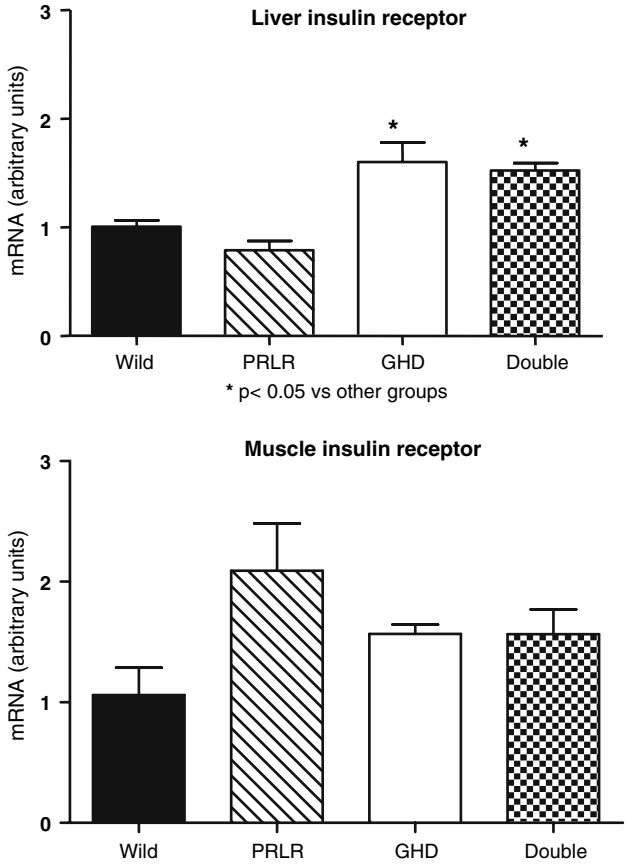


**Fig. 4** Hepatic and skeletal muscle insulin receptor mRNA levels in mutant and wild-type male mice. The mRNA levels were assessed by real-time PCR. Control values, normalized to riboprotein mRNA levels, were arbitrarily set at 1.0. Values represent mean ± SE of 3 wild-type, 5 PRLR KO, 5 GH deficient, and 3 double-mutant males; similar results were obtained in three separate experiments. * $P < 0.05$ vs. other groups

Food intake, expressed as a function of body weight, increased slightly in the PRLR KO, GHD, and double-mutant mice during the period of separation and cooling; however, food intake during stress was significantly lower ($P < 0.05$) in the double-mutant mice than in the GHD mice (Fig. 5). That the animals lost weight despite increases in food intake may reflect an increase in energy expenditure in response to cooling. The mild cooling had no significant effect on body temperature; however, prior to cold exposure, at an ambient temperature of 25°C, the rectal temperatures of double-mutant males (35.4 ± 0.5°C, $P < 0.05$) were lower than those of GHD (36.1 ± 0.3°C), PRLR-deficient (36.3 ± 0.4°C), or wild-type males (36.6 ± 0.2°C).

Under basal conditions, the circulating levels of IL-6, MCP-1, and resistin levels did not differ significantly among PRLR KO, GHD, double-mutant, and wild-type males (Fig. 6, resistin levels not shown). In response to the stress of separation and cooling, the levels of MCP-1 and IL-6 increased in double-mutant males while plasma insulin and amylin declined (Fig. 6). MCP-1 and IL-6 levels did not rise in PRLR KO or GHD mice. Adiponectin levels did not change significantly in any of the groups during cooling (not shown). The changes in insulin, amylin, and MCP-1 in double-mutant mice reversed after a 14-day recovery period, but plasma IL-6 levels remained elevated. Plasma insulin and amylin levels were lower ($P < 0.05$, Fig. 6) in GHD mice than in wild-type mice at the end of the recovery period. There were no significant differences in plasma resistin among the groups throughout the stress or recovery periods (not shown).

## Discussion

Like mice with isolated GHD [25], the double-mutant GHD/lactogen resistant mice develop male-predominant adiposity and sarcopenia with age [18]. However, relative to GHD mice, the double-mutant mice have higher body weights, higher percent fat mass, and lower percent lean body mass. As shown in this study, plasma leptin levels in the double-mutants (but not the GHD mice) are elevated, reflecting their increase in body fat mass [18]. Given the lipolytic effects of GH [26] and (in chronic excess) PRL [13, 27], and the anabolic effects of GH in skeletal muscle [28], it is possible that decreased hydrolysis of white adipose tissue depots and impaired muscle protein synthesis contribute to the adiposity and sarcopenia of aged double-mutant males. A severe deficiency of IGF-1 in double-mutant mice [18] contributes to the reduction in lean body mass. Sarcopenia may facilitate weight gain or maintenance of the obese phenotype because lean body mass is the major determinant of resting energy expenditure. A defect in energy expenditure in the double mutants is suggested by their reduction in basal body temperature and absence of hyperphagia; food intake, expressed as a function of body weight, was lower in double-mutant males than in GHD males. However, comprehensive studies of oxygen consumption, heat production, and the adaptation to cold will be required to fully assess energy balance.

In a previous paper, we showed that double-mutant males develop insulin resistance with age [18]; the ratio of insulin to glucose is elevated and the fall in glucose following insulin administration is blunted. Here we demonstrate that fasting amylin as well as insulin levels are high and the ratio of adiponectin to leptin is low. Amylin and insulin are stored in pancreatic beta cells and co-secreted in response to nutrients; their plasma concentrations are elevated in obesity and other states associated with insulin resistance [29]. In contrast, adiponectin levels and the ratio of adiponectin to leptin are low [20, 21]. Amylin suppresses insulin-mediated glucose uptake and

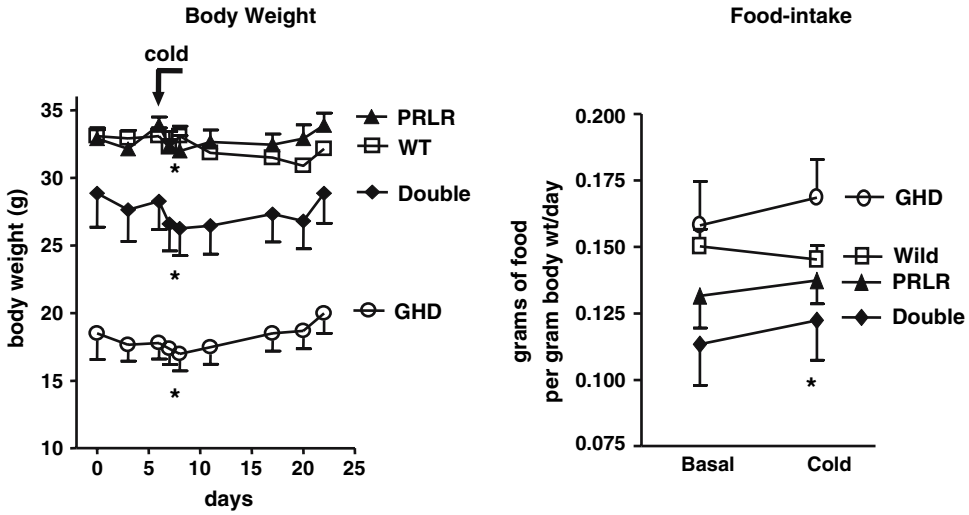


**Fig. 5** Effects of separation and cooling on body weights and food intake of male mice. Mice were maintained in groups of 3–4 at an ambient temperature of 25°C. The mice were then separated into individual cages and housed at an ambient temperature of 18.5°C. After 48 h of cooling, the mice were re-grouped and returned to an ambient temperature of 25°C. Data are expressed as mean ± SE of 4–6 mice in each group. * $P < 0.05$ vs. body weight at 25°C (figure on left) and * $P < 0.05$ double-mutant vs. GHD mice (figure on right)

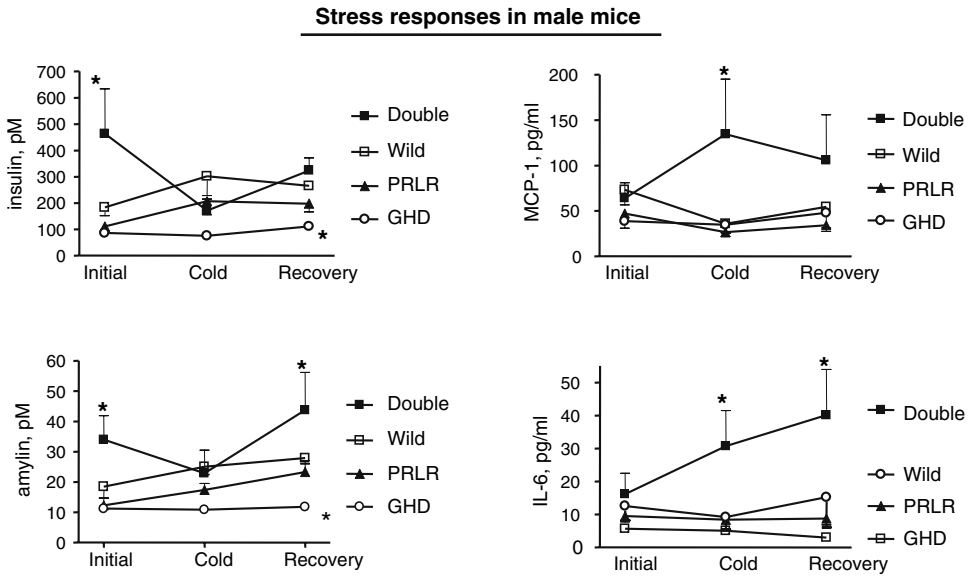


**Fig. 6** Changes in plasma insulin, amylin, MCP-1, and IL-6 in response to separation and cold stress in male mice. Mice were maintained in groups of 3–4 at an ambient temperature of 25°C. The mice were then separated into individual cages and housed at an ambient temperature of 18.5°C. After 48 h of cooling, the mice were re-grouped and returned to an ambient temperature of 25°C. Blood was obtained under fasting (6 h) conditions prior to, 48 h after initiation of, and 14 days after termination of the separation/cold stress. Data are expressed as mean ± SE of 4–6 mice in each group. Asterisks (*) represent significant differences (* $P < 0.05$) from wild-type mice

glycogen synthesis in skeletal muscle [30], while adiponectin has direct insulin sensitizing effects in skeletal muscle and liver [20, 21]. Thus, the hyperamylinemia and relative hypoadiponectinemia of double-mutant male mice likely contribute to insulin resistance during aging.

In comparison with double-mutant males, double-mutant females have lower insulin, amylin, and leptin levels and higher adiponectin levels; this correlates with, and may explain in part, their lack of insulin resistance with aging. We do not yet understand why plasma hormones and adipocytokines in female double-mutant mice differ from those in double-mutant males. However, other investigators have also noted gender differences in growth and metabolism in wild-type and mutant mice; for example, plasma adiponectin levels are higher in adult wild-type females than in wild-type males [31], and weight gain and fat deposition are greater in adult GH deficient "little" males than in GH deficient "little" females [25].

The studies presented herein show that GHD males have lower levels of insulin, amylin, and leptin than lactogen-resistant/GHD double-mutant males and higher levels of adiponectin. These findings suggest a role for lactogen signaling in the regulation of adipocytokine release. Moreover, liver AdipoR1 mRNA levels are higher in GHD

males than in double-mutant males; on the other hand, hepatic IR mRNA levels in both GHD and double-mutant mice are higher than those in wild-type mice. The hypoamylinemia, hyperadiponectinemia, and increased hepatic AdipoR1 mRNA, in combination with heightened hepatic IR expression, may explain, in part, why GHD mice are more sensitive to insulin than double-mutant, PRLR KO, or wild-type mice. Our findings concur with previous studies that reported increases in hepatic IR mRNA levels in GH receptor KO mice and GHD Ames dwarf mice [32, 33]. Interestingly, GH receptor KO and Ames dwarf mice have normal skeletal muscle IR mRNA levels [34], an observation consistent with our findings.

The effects of lactogen resistance on adiponectin receptor expression have not been examined previously. We found a reduction in liver AdipoR2 mRNA levels but no change in AdipoR1 mRNA levels in PRLR KO mice. Nilsson et al. [35] reported that PRL increased the levels of AdipoR1 mRNA levels in human adipose tissue explants, while GH reduced adipose AdipoR2 mRNA levels.

Emerging evidence suggests that insulin action in peripheral tissues is modulated by inflammatory cytokines produced in adipose tissue. Our studies suggest that differences in the secretion of adipocytokines during stress might contribute to differences in insulin sensitivity among the GHD, PRLR KO, double-mutant, and wild-type mice. Fasting levels of IL-6 and MCP-1, which decrease adiponectin expression and reduce insulin sensitivity in liver and skeletal muscle [35–38], were statistically indistinguishable in the four experimental groups under basal conditions but increased significantly in double-mutant males in response to the stress of separation and mild cooling. In contrast, GHD and PRLR KO mice showed no rise in MCP-1 or IL-6 during stress. Heightened release of IL-6 and MCP-1, in the setting of adiposity, hyperamylinemia, and relative hypoadiponectinemia, might facilitate the development of insulin resistance in the double mutants.

Despite their insulin resistance, the plasma levels of insulin and amylin in double-mutant mice declined acutely during stress. The decline in plasma insulin and amylin levels likely reflects stress-dependent increases in plasma corticosterone and catecholamines, which suppress insulin secretion [39].

Over-expression of resistin also impairs insulin action in skeletal muscle and liver; conversely, targeted deletion of resistin in mice increases insulin sensitivity [40]. Circulating levels of resistin are elevated in leptin-deficient ob/ob mice and in most (but not all) studies of diet-induced obesity [40–42]; on the other hand, resistin levels were reported to be low in obese db/db and KK-A(y) mice [42]. We found no significant differences in plasma resistin levels among our groups of wild-type and mutant mice. Differences among the groups might have been obscured because resistin levels normally decline markedly with age [31].

In previous investigations [18] we noted that the development of insulin resistance in double-mutant males during aging correlated with an increase in fat mass and a reduction in percent lean body mass. Developmental changes in adiposity and insulin sensitivity have also been observed in other models of GH and PRL signaling. For example, GH- and PRL-deficient Ames and Snell dwarf mice have fasting hypoglycemia, hypoinsulinemia, and increased insulin sensitivity before 3–4 months of age [18, 33, 43, 44]. However, Snell dwarf males, like GHD/lactogen-resistant double-mutant males, develop obesity and hyperleptinemia with aging (22–23 months old); in contrast, weight gain is blunted and leptin levels are normal in aging female Snell mice [43–45]. Similarly, leptin levels are low in Ames female dwarfs at 11 months of age but normal in adult male dwarfs [46]. Changes in body composition during development are accompanied by changes in insulin sensitivity; blood glucose and insulin levels in Snell dwarf males normalize with aging, in association with reductions in hepatic insulin receptor substrate (IRS)-1 tyrosine phosphorylation, IRS-2 content, and IRS-associated phosphatidyl inositol (PI)-3 kinase activity [43, 44]. Thus aging alters body composition and insulin sensitivity in GH- and PRL-deficient mice as well as GHD/lactogen-resistant mice, and the changes are gender-dependent.

We found no significant changes in pancreatic hormone levels, adipocytokine secretion, or expression of insulin receptors in aging mice with isolated PRLR-deficiency. AdipoR2 mRNA levels in liver, however, were reduced. Previous studies found no significant changes in insulin sensitivity in younger female mice (5–12 months of age) with lactogen resistance, despite diminished abdominal fat stores [47, 48]. In addition, PRLR-deficient males and females (3 weeks–8 months) had decreased beta cell mass, glucose-stimulated insulin secretion and mild glucose intolerance [49]. Differences between the current and previous investigations might be explained in part by differences in the age of the animals studied or in their genetic background (129/Bl6 vs. pure 129/Sv). It should also be noted that the number of animals studied here was low, reflecting the considerable difficulty in generating age-matched groups of female and male mutant mice.

In sum, our findings suggest that chronic defects in GH and/or lactogen signaling alter body composition and insulin action in an age- and gender-dependent manner. The combination of lactogen resistance and GHD in aging male mice is associated with hyperinsulinemia, hyperamylinemia, hyperleptinemia, and relative hypoadiponectinemia and heightened secretion of MCP-1 and IL-6 in response to stress. Relative to double-mutant males, the GHD males have lower body weights and percent fat mass, higher lean

body mass, lower levels of insulin, amylin, and leptin, higher levels of adiponectin and hepatic Adiponectin receptor 1 mRNA levels, and a blunted cytokine response to stress. The phenotypic differences between double-mutant and GHD males suggest that the lactogens and GH may have differential but overlapping effects on fat storage and adipocytokine secretion and action.

## Materials and methods

### Generation of mutant and wild-type mice

The generation of GHD and lactogen resistant mice has been described in a previous manuscript [18]. Homozygous PRLR KO males (129 background) were mated with homozygous GH-deficient "little" females (C57BL/6 background) to yield mice that were heterozygous at both loci on a hybrid 129/Bl6 background. Double-heterozygous males and females were bred to produce wild-type, PRLR-deficient, GH-deficient, and double-mutant mice. The various genotypes were identified through PCR analysis as described previously [48, 50, 51].

The animals were studied at 12–16 months of age. All animal protocols were approved by the Duke University Medical Center Institutional Animal Care and Use Committee and followed federal guidelines.

### Animal maintenance and response to stress

The mice were housed in groups of 3–4 under non-sterile conditions at an ambient temperature of 25°C under a 12-h light, 12-h dark cycle. Food and water were provided ad libitum. The mouse chow was a standard preparation (Laboratory Rodent diet 5001, Ralston Purina Co., St. Louis, MO) containing 12.1% of calories as fat, 28% as protein, and 59.8% as carbohydrate.

To assess the effects of stress on plasma hormone and adipocytokine levels, we placed mice in individual cages in an ambient temperature of 18.5°C. After 48 h of cooling the mice were re-grouped and returned to an ambient temperature of 25°C. Blood was obtained under fasting (6 h) conditions prior to, 48 h after initiation of, and 14 days after termination of the separation/cold stress. Body weight and food intake (expressed as grams of chow per day per gram body weight) were measured on three consecutive days prior to cooling and every 1–2 days thereafter. Basal body temperature was measured using a rectal probe (Thermalert TH-5-RET-3, Physitemp Instruments Inc., Clifton, NJ).

### Hormone and cytokine levels

Afternoon (3–5 pm) blood samples were obtained by retro-orbital puncture after 6 h of fasting. Plasma glucose concentrations were measured using a One-Touch Ultra glucometer (Lifescan, Milpitas, CA). Plasma insulin, glucagon, amylin, and leptin were assayed using the multi*plex* mouse endocrine kit from LINCO Research (St. Louis, MO). Plasma corticosterone levels were measured by EIA using a kit purchased from Assay Designs (Ann Arbor, MI). Plasma adiponectin was measured using a single*plex* kit from LINCO Research. Plasma IL-6, MCP-1, and resistin were assayed using a multi*plex* mouse serum adipokine kit from LINCO Research. Intra- and interassay variations of the assays were less than 20%. All assays were performed at least three times.

### Hepatic and skeletal muscle receptor expression

Total RNA was isolated with Trizol (Invitrogen, Frederick, MD), and cDNA was prepared using the High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's protocol. mRNA levels were quantified with a ABI 7300 Real time PCR system, as described previously [52, 53]. Oligonucleotide primers were designed using the Primer Express program from Applied Biosystems. The primer sequences used were as follows: mouse insulin receptor (IR) forward: 5′-GCA GTG TGG CAG CCT ACG T-3′ and reverse: 5′-CAG GGC CAA CGA TGT CAT CT-3′; mouse adiponectin receptor 1 (AdipoR1) forward: 5′-AGA AGG TCT CTC GGA CTT TTT CC-3′ and reverse: 5′-GAA CGA AGC TCC CCA TAA TCA G-3′; mouse adiponectin receptor 2 (AdipoR2) forward: 5′-CAC ACA GAG ACG GGC AAC AT-3′ and reverse: 5′-CCC CAG GCA CAG GAA GAA TA-3′; and acidic ribosomal phosphoprotein PO (riboprotein) forward: 5′-CCC TGA AGT GCT CGA CAT CA-3′ and reverse: 5′-GCG GAC ACC CTC CAG AAA GC-3′.

For measurements of mature mRNA, all primer pairs spanned introns; amplicon lengths ranged from 90 to 150 bp. Thermal cycling conditions were 10 min at 95°C followed by 35–40 cycles for 15 s at 95°C and 1 min at 57°C; SYBR green incorporation into a single peak was monitored using a dissociation curve. Expression levels were normalized against the levels of acidic riboprotein, a housekeeping gene that shows little change during cellular growth or differentiation [54]. The levels of mRNA were quantified using the comparative threshold cycle ($C_T$) method. $C_T$ was determined from a log-linear plot of the PCR signal vs. cycle number.

Data analysis

The breeding of double heterozygotes is predicted to yield only one homozygous wild-type, one PRLR-deficient, one GH-deficient, and one double-mutant mouse for every 16 pups. Because litters typically contained 5–11 pups, no single litter contained both males and females of the four genotypes of interest. Consequently, it was impossible to perform direct statistical comparisons within a single litter. We, therefore, used animals from multiple litters of comparable age as well as true littermates.

The necessity of breeding double heterozygotes limited the number of age-matched mice available for comparative analysis. The analysis of hormone and adipocytokine levels included data from 4 wild-type, 5 PRLR KO, 5 GH deficient, and 6 double-mutant males and from 3 to 4 virgin wildtype, 5 PRLR KO, 7 GH deficient, and 7 double-mutant females. To insure the reproducibility of the results, we repeated all hormone and cytokine assays at least three times. Hepatic and skeletal muscle mRNA levels were measured three times. All data were expressed as mean ± SE. Statistical differences among the various groups of mice were assessed by one-way (baseline values) or two-way (response to separation and cooling) ANOVA followed by the Bonferroni test of comparisons. $P < 0.05$ was considered statistically significant. Significant differences detected by one-way ANOVA were confirmed using the non-parametric Kruskal–Wallis test.

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