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Hyperinsulinemia and insulin resistance in Wrn null mice fed a diabetogenic diet

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Abstract

Werner syndrome (WS) is an autosomal recessive progeroid syndrome caused by mutations in the Werner gene (Wrn). WS patients have increased incidence of a number of chronic conditions including insulin resistance and type 2 diabetes. Since ingestion of foods that are high in fat and sugar is associated with increased incidence of diabetes, we examined if Wrn mutations might affect metabolic response to a diabetogenic diet. Four month old mice with a null mutation for the Wrn gene were fed a diet consisting of 36% fat (lard), 33% table sugar, and 20% protein plus balanced vitamins and minerals. Wrn null mice had significantly increased body weights, increased serum insulin levels, impaired glucose tolerance, and insulin resistance during four months of eating the diabetogenic diet. Diffuse fatty infiltration of the liver and pancreatic islet hyperplasia were characteristic morphological features. These observations suggest that Wrn null mice have impaired glucose homeostasis and fat metabolism, and may be a useful model to investigate metabolic conditions associated with aging.

Keywords

progeriod syndrome; Werner's syndrome; high fat and sugar diet; type 2 diabetes; obesity

1. Introduction

Werner Syndrome (WS) is an autosomal recessive progeroid syndrome characterized by cataracts, skin atrophy and pigmentation, short stature, graying and thinning of scalp hair, cardiovascular complications, osteoporosis, abnormal fat distribution, neoplasms, and insulin resistant (type 2) diabetes. Patients can survive up to their fourth or fifth decade of life, but then die of cancer or atherosclerotic cardiovascular disease (or microvascular complications associated with diabetes) (Epstein et al., 1966; Goto, 1997; Huang et al., 2006; Uhrhammer et al., 2006). Diabetes is thought to develop in WS patients due to decreased insulin signaling in tissues that respond to insulin, e.g. muscle, liver and adipose tissue (Beadle et al., 1978; Blohme and Smith, 1979). WS patients do not have mutations in the insulin receptor (Uotani et al.,

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1994), but do have evidence of impaired insulin secretion (Yamada et al.,1999). Thus understanding the development of type 2 diabetes in WS may be useful in understanding the development of type 2 diabetes in the aging population.

WS is caused by mutations in the Werner (Wrn) gene resulting in absence of functional gene product. The Wrn gene product is a member of the RecQ helicase family of genes and proteins that maintain DNA structure (Yu et al., 1996). The Wrn protein has 3′–5′ exonuclease, ATPase and 3′–5′ helicase activities and a nuclear localization signal (Opresko et al., 2003). Wrn is found and thought to function primarily in the nucleoplasm (Monnat and Saintigny, 2004). A number of genetic defects have been associated with Wrn mutations including genomic instability, telomere dysfunction, defective recombination and perhaps several other aspects of DNA replication, repair and gene expression (Bachrati and Hickson, 2003; Monnat and Saintigny, 2004; Opresko et al., 2003). Fibroblasts isolated from WS patients are hypersensitive to DNA damaging agents, such as DNA cross-linking drugs, 4-nitroquinoline and camptothecin (Okada et al., 1998; Poot et al., 1999; Poot et al., 2002). The Wrn protein is capable of resolving complex DNA structures, such as Holliday junctions and DNA bubbles/loops (Opresko et al., 2003), and interacts with several protein partners including p53, PARP, BLM (Opresko et al, 2003) and protein kinase A (Nguyen et al, 2002).

Several Wrn mutant mouse models have been developed. One mouse line, developed by Lombard et al. (2000) was described as having no phenotype, suggesting alternate functional pathways for Wrn in the mouse. Recently, Chang et al. (2004) bred these mice with telomere RNA component (Terc) knockout mice and were able to recapitulate signs of WS. Mice have telomeres that are 50–150kb compared to 15kb in humans (Greider 1996). In order to observe the WS phenotype, successive breeding to reduce telomere size in mice was necessary. Their findings suggest the genomic instability and defects seen in WS are partly because telomeric structure is not being maintained. However, it is not known whether the development of insulin resistance and diabetes in WS is dependent on telomere shortening. We therefore sought to examine whether a diabetogenic diet high in fat and sugar (HFS) would generate any features of a diabetic phenotype. Our data show an accelerated development of obesity, hyperinsulinemia, and insulin resistance in Wrn null mice compared with wild type control mice fed the same diet.

2. Experimental procedures

2.1 Wrn null mutant mice

Wrn null mutant mice were a kind gift from Dr. Leonard Guarente (Lombard et al., 2000). They were backcrossed to C57BL/6J using mice obtained from Jackson Laboratories (Bar Harbor, ME). Mice used in this study were confirmed to lack other background strains based on microsatellite marker genotyping (Charles River Laboratories, Troy, NY). C57BL/6J wild type mice were used as controls because they develop a robust diabetic phenotype when fed a diabetogenic diet (Petro et al., 2004). Males were used exclusively in all experimental procedures.

The experimental diabetogenic diet (HFS) (No. F3282; Bio-Serv, Frenchtown, NJ) contained 35% (wt/wt) fat (lard) and 37% carbohydrate (primarily sucrose), plus 20% (wt/wt) proteins and essential vitamin and minerals. The control diet was standard rodent chow (CHOW) (LabDiet, St. Louis, MO) containing 4.5% (wt/wt) fat (soybean oil). For all experiments, mice were maintained in a 25 °C, specific pathogen-free, barrier facility with a strict 12-h light/dark cycle (6:00 am/6:00 pm) and were given free access to food and water. Mice were euthanized by CO_2 asphyxiation. This project was approved by the Institutional Animal Care and Use Committee of the University of Washington.

Wrn null and wildtype mice were maintained on CHOW until 4 months of age when the feeding trial was started. Four cohorts were established including wild type mice fed CHOW (n=8); Wrn null mice fed CHOW (n=8); wildtype mice fed HFS (n=8); Wrn null mice fed HFS (n=7). Body weights and blood glucose were monitored each week during the feeding trial. At 6, 8, and 10 months of age serum leptin and insulin levels were measured. Food intake was measured daily for 4 days and calculated as the total amount of food eaten per mouse. At 10 months of age, mice were euthanized, and individual fat pads were collected and weighed. Representative tissues were collected into 10% formalin for histological examination.

2.2 Serum glucose, leptin, insulin and triglyceride determinations

Serum glucose levels were determined with a standard glucometer using test strips and blood collected by retro-orbital sinus or tail vein puncture (Accu-chek, Advantage with Comfort Curve glucose strips, Roche, Newark, NJ). Serum insulin and leptin were measured using ELISA kits (No. EZRMI-13K and No. EZML-82K; Linco, St. Louis, MO) with rat insulin and mouse leptin as the respective standards. Serum triglycerides were measured using colometric kits (No. TR0100, Sigma, St. Louis, MO) with triolein (No. G7793, Sigma, St. Louis, MO) as the glycerol standard solution

2.3 Intraperitoneal Glucose Tolerance Test (IPGTT)

An intraperitoneal glucose tolerance test (IPGTT) was performed as described previously (Ladiges et al., 2005). Briefly, mice were fasted over night and injected intraperitoneally with 10% glucose in PBS at a dose of 2 g glucose/kg body weight. Serum glucose was monitored before glucose injection and at 30, 60, 120, and 240 minutes after injection.

2.4 Insulin sensitivity assay

The insulin sensitivity assay was performed as described previously (Ladiges et al., 2005). Briefly, mice were fasted overnight and injected subcutaneously in the sub-scapular region with human insulin (Humalin, Eli Lilly, Indianapolis, IN) at a dose of 1.0 unit insulin/kg body weight. Serum glucose was monitored before and 30 minutes after insulin injection. The percentage decrease in glucose between these time points was then calculated as follows: % glucose disposal = $[(\text{glucose}_{t=0} - \text{glucose}_{t=30})/\text{glucose}_{t=0}] \times 100$.

2.5 Immunohistochemistry

Insulin immunohistochemistry was done on pancreatic islets as previously described (Ladiges et al., 2005). Briefly, unstained slides were deparaffinized and antigens were retrieved with 0.1 M citrate, pH 6.0. Antigens were blocked with 5% Normal Goat Serum (GIBCO, Invitrogen, Carlsbad, CA). Rabbit anti-insulin primary antibody (Sigma, St. Louis, MO) and goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) were used to detect insulin. Cross-reaction was minimized with the ABC Vector Kit (Vector Laboratories, Burlingame, CA) and detection of insulin was done by DAB colorimetric detection kit (Sigma, St. Louis, MO).

2.6 Statistics

Data are presented as means or median ± SEM. Differences between mouse cohorts were determined by using the Student's *t* test. Two-tailed P values ≤ 0.05 were accepted as statistically significant.

3. Results

3.1 Changes in body weight, adiposity and serum leptin

Wrn null mice fed HFS had significantly increased body weights (8%–21%; $P < 0.0001$) by the third week after starting the feeding trial (Figure 1), when compared to wildtype mice on HFS. This differential weight increase continued through the end of the study at 10 months of age (Figure 1). Fat pads (reproductive, inguinal, retroperitoneal/renal and intrascapular brown adipose tissue) collected at 10 months of age were significantly increased (>44%; $P < 0.001$) in Wrn mice fed HFS compared to mice fed CHOW, indicating the increased body weight was due to increased adiposity.

Fasting serum leptin levels were similar in both genotypes fed HFS at 6 and 8 months of age, but by 10 months of age Wrn null mice had significantly increased serum leptin levels relative to wildtype control mice (37 ± 0.8 ng/ml vs. 30 ± 7 ng/ml, $P < 0.03$; respectively) (Figure 2). However, Wrn null mice fed HFS had significantly reduced food intake (42% reduction; $P < 0.0004$) compared to mice fed CHOW. Therefore serum leptin levels may be influencing food intake in Wrn null mice after prolonged ingestion of HFS, but not be causally related to increased adiposity.

3.2 Hyperglycemia, hyperinsulinemia and hypertriglyceridemia

Median blood glucose levels were significantly increased at 6 months of age in Wrn null mice fed HFS compared to wildtype mice fed HFS (231 ± 14 mg/dl vs. 167 ± 5 mg/dl ($P < 0.022$) but by 10 months of age this difference was no longer significant (Figure 3). Therefore Wrn null mice develop hyperglycemia at an earlier age than wildtype mice, but this difference becomes less significant when fed the HFS diet for longer than several months. Fasting serum insulin levels in Wrn null mice fed HFS were also significantly increased relative to wild type control mice at 6 months of age (20.4 ± 1.1 ng/ml vs. 6.5 ± 1.4 ng/ml, $P < 0.0003$; respectively) and at 8 months of age (26.4 ± 0.9 ng/ml and 13.6 ± 1.9 ng/ml, $p < 0.001$; respectively) (Figure 4). By 10 months of age, serum insulin levels dropped significantly in both genotypes of mice fed HFS compared to levels similar to mice fed CHOW (2.8 ± 0.6 vs.1.6 ± 0.2 ng/ml, ($P = 0.142$); 0.9 ± 0.4 vs. 0.6 ± 0.2 ng/ml, ($P = 0.520$); respectively). The serum insulin levels between both mouse genotypes fed the CHOW diet remained unchanged after 6, 8 and 10 months of age. There were no detectable histological abnormalities or differences in insulin abundance between genotypes fed either diet at 4 months of age. By 10 months of age, both genotypes of mice fed HFS appeared to have increased islet size and islet cell hyperplasia compared to mice fed CHOW. Insulin staining by 10 months of age appeared less intense by visual observation and after correcting for increasing mass in Wrn mice fed HFS.

Fasting serum triglyceride levels in Wrn null mice fed HFS were significantly increased relative to wild type mice at 6 months of age (164 + 8.7 mg/dL vs. 135 ± 6.3 mg/dL, $P < 0.0175$; respectively). Serum triglyceride levels were not significantly different in mice at 8 and 10 months of age. The serum triglyceride levels between both mouse genotypes fed the CHOW diet remained unchanged after 6, 8 and 10 months of age. Diffuse fatty infiltration of the liver was also apparent histologically in HFS fed mice compared to CHOW fed mice.

3.3 Glucose intolerance and insulin resistance

The effect of Wrn deficiency on total body glucose disposal was examined using an intraperitoneal glucose tolerance test on HFS-fed mice. Results show that loss of Wrn weakens the clearance of serum glucose in mice fed HFS (Figure 5A). Compared with wild type mice, Wrn null mice displayed higher glucose levels at 60 minutes (600 ± 12 mg/dl vs. 516 ±19 mg/dl; $P < 0.01$) and at 120 minutes (398 ± 25 mg/dl vs. 249 ± 25 mg/dl; $P < 0.008$) after glucose injection. These findings suggest that the HFS-fed Wrn null mice have decreased glucose

tolerance relative to WT mice. CHOW-fed mice from both genotypes had similar glucose tolerances (after 30 minutes: 291 ± 27 mg/dl vs. 279 + 22 mg/dl; P = 0.680).

Using an *in vivo* insulin-mediated glucose disposal assay, Wrn null mice on the HFS diet for six months (at 10 months of age) showed a significant reduction in the percent glucose cleared after insulin injection compared to WT mice, 43 ± 1% versus 52 ± 1% (P < 0.004) (Figure 5B). In addition, glucose clearance was calculated on a per gram body weight basis to determine if weight was a factor in insulin resistance. The HFS-fed Wrn null mice still had a significant decrease in glucose disposal compared to HFS-fed WT mice (P < 0.0006) (data not shown). This suggests that Wrn null mice are highly susceptible to the development of diet-induced insulin resistance after several months on a HFS diet. Therefore, we conclude that a diabetogenic diet increases resistance to insulin-mediated glucose disposal in C57BL/6 mice, and that loss of Wrn exacerbates this effect.

4. Discussion

Our studies demonstrate that Wrn null mice become obese and develop hyperglycemia, hypertriglyceridemia, hyperinsulinemia, insulin resistance and type 2 diabetes when fed a diabetogenic diet. Wild type mice on the same diet show similar but less severe metabolic signs, thus suggesting a role for the Wrn gene in glucose and/or adipose homeostasis. It is of interest that Wrn null mice on a standard rodent chow diet do not develop obesity or hyperglycemia. In fact, our data suggest they are quite efficient in glucose metabolism, and maintain consistent body weights as adults compared to wild type littermates. However, they are unable to maintain homeostasis with a change in diet high in fat and sugar. The molecular events that trigger this regulatory failure in the absence of Wrn gene product are not known. An understanding of how the loss of Wrn, a genetic stability protein, affects glucose and fat metabolism has broad implications for aging and diabetes, as well as other chronic age-associated diseases. Since the ingestion of high amounts of fat and sugar mimics a lifestyle factor common in Western societies, the Wrn null mutant mouse would seem to be an intriguing model system to investigate gene-nutrient interaction and aging.

As an organism ages, it is thought that genomic DNA incurs damage from numerous exogenous and endogenous sources. Although the exact function of Wrn is still not known, mutations have been associated with defects in the repair of specific types of DNA damage. However, in the presence of normal telomere function in the mouse, there may be redundant systems that maintain the integrity of DNA repair pathways. Recent evidence suggests that Wrn interacts with a number of genes directly involved in base excision repair (BER) (Bohr et al). BER is responsive to the repair of bases damaged by oxidative stress and other endogenous products, and the loss of Wrn may compromise efficient BER. It is possible that the increased adipose load increases oxidative stress and oxidative DNA damage in specific genes that control important metabolic cellular functions.

The cells or tissue most sensitive to a loss of Wrn function might be those that retain cell division potential, lack stringent cell editing during and after development, and are tolerant of and thus able to accumulate at least some type of genetic instability (Shimamoto et al., 2004; Salk, 1982). Mesenchymal or mesodermally derived cell lineages are preferentially affected by the loss of Wrn (Epstein et al., 1966) and mature adipocytes are derived from a defined mesenchymal pool of pre-adipocytes (Gregoire et al., 1998). We speculate that the metabolic phenotype observed in Wrn null mice is driven by a defect in adipocytes. Thus adipocytes and genes associated with adipocyte function are potential targets for compromise when Wrn protein is lacking. The exaggerated increase in adiposity seen in HFS-fed Wrn null mice suggests that adipocytes may be able to proliferate as well as take in more fat.

A possible explanation that adipocytes from Wrn null mice have enhanced deposition of fat under conditions of increased energy intake may be a disruption in intracellular signaling. It has been shown that the p38MAPK gene product is activated in fibroblasts from Werner syndrome patients (Davis et al, 2005). However, it is unclear what role p38MAPK may play in adipogenesis. It is known that p38MAPK is highly expressed in adipocytes from patients with type 2 diabetes (Carlson et al., 2003). It has also been demonstrated that 3T3-L1 preadipocyte cell cultures treated with a p38MAPK inhibitor (SB203580) do not differentiate, suggesting that p38MAPK may be necessary for the maturation of preadipocytes. Since p38MAPK is activated by cellular stress and various cytokines, it is possible that cytokines released from adipocytes due to DNA damage or excessive fat deposition may increase the rate of fat deposition in the absence of Wrn. The implication is that Wrn is involved in modulating p38MAPK activity. This concept is of interest since a recent study by Davis et al., (2005) showed that treatment of human WS cells with a p38MAPK inhibitor could reverse this senescent phenotype.

There may be other molecular mechanisms associated with the metabolic phenotype in Wrn null mice. Several cytokine genes associated with adipocyte function may be affected by dietary factors in the absence of Wrn, including leptin. Leptin is an adipocyte-specific gene that functions physiologically as a signal to initiate food intake and accelerate energy expenditure. Leptin deficiency in rodents and humans is associated with increased body fat and insulin resistance (Jones et al., 2005). Leptin levels were increased in our Wrn null mice fed HFS, consistent with elevated levels associated with obesity (Pelleymounter et al., 1995). The increase in leptin in this case is most likely due to leptin resistance, and therefore probably not causally related to Wrn deficiency and insulin resistance.

It is possible that a defect in the insulin signaling pathway may play a role in dysfunctional regulation of glucose seen in the absence of Wrn gene product. Insulin resistance does not mean all insulin-regulated processes and tissues become equally resistant to insulin. For example, hyperinsulinemia acting on the liver leads to hypertrigylceridemia, which we see in our Wrn null mice. It is of interest that liver-specific insulin receptor knock out mice develop marked hyperinsulinemia, but have decreased levels of circulating free fatty acids and trigycerides (Biddinger and Kahn, 2006). In contrast, muscle-specific insulin receptor knock out mice have a three-fold increase in insulin-stimulated glucose transport in adipose tissue, with an increase in fat deposition and serum triglycerides and free fatty acids but not serum insulin (Biddinger and Kahn, 2006). The metabolic phenotype of Wrn null mice may represent a combination of the liver and muscle effects.

Our study supports an independent role for Wrn in the modulation of weight gain and insulin resistance associated with obesity, and suggests that alleles at loci coding for Wrn should be included in the list of candidate genes determining susceptibility to diabetes. Heterozygous carriers of single mutant Wrn alleles appear to be present in the United States at a frequency range of 1:250 (Goto et al., 1997; Matsumoto et al., 1997). In addition, single nucleotide polymorphisms occurring in the Wrn gene product have been reported to be associated with increased incidence of diabetes (Hirai et al., 2005). It is estimated that by 2030, at least a fifth of America’s population will be over the age of 65 years, and the majority will be at risk for developing insulin-resistant diabetes and the complications of cardiovascular and cerebrovascular disease associated with this metabolic epidemic. Our findings indicate that Wrn mutant mice with a complete null deletion may be useful in investigating homeostasis of fat and glucose metabolism associated with aging and Wrn gene function.

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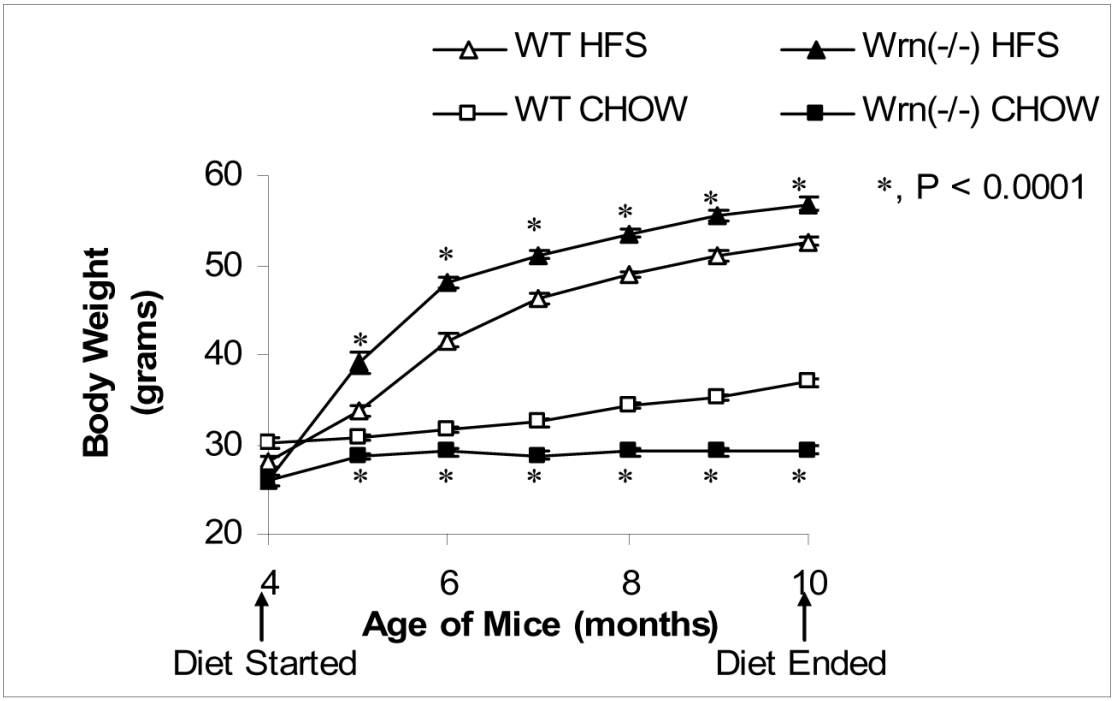

Fig. 1.

Body weights of mice fed a diabetogenic diet high in fat and sugar (HFS) or a standard rodent chow (CHOW) from 4 through 10 months of age. Values are presented as means ± SEM for 5 to 8 mice per cohort. Significance for each time point is shown by P values less than 0.0001*. $Wrn^{(-/-)}$ mice are represented as closed triangles and squares. Wildtype (WT) mice are represented as open triangles and squares.

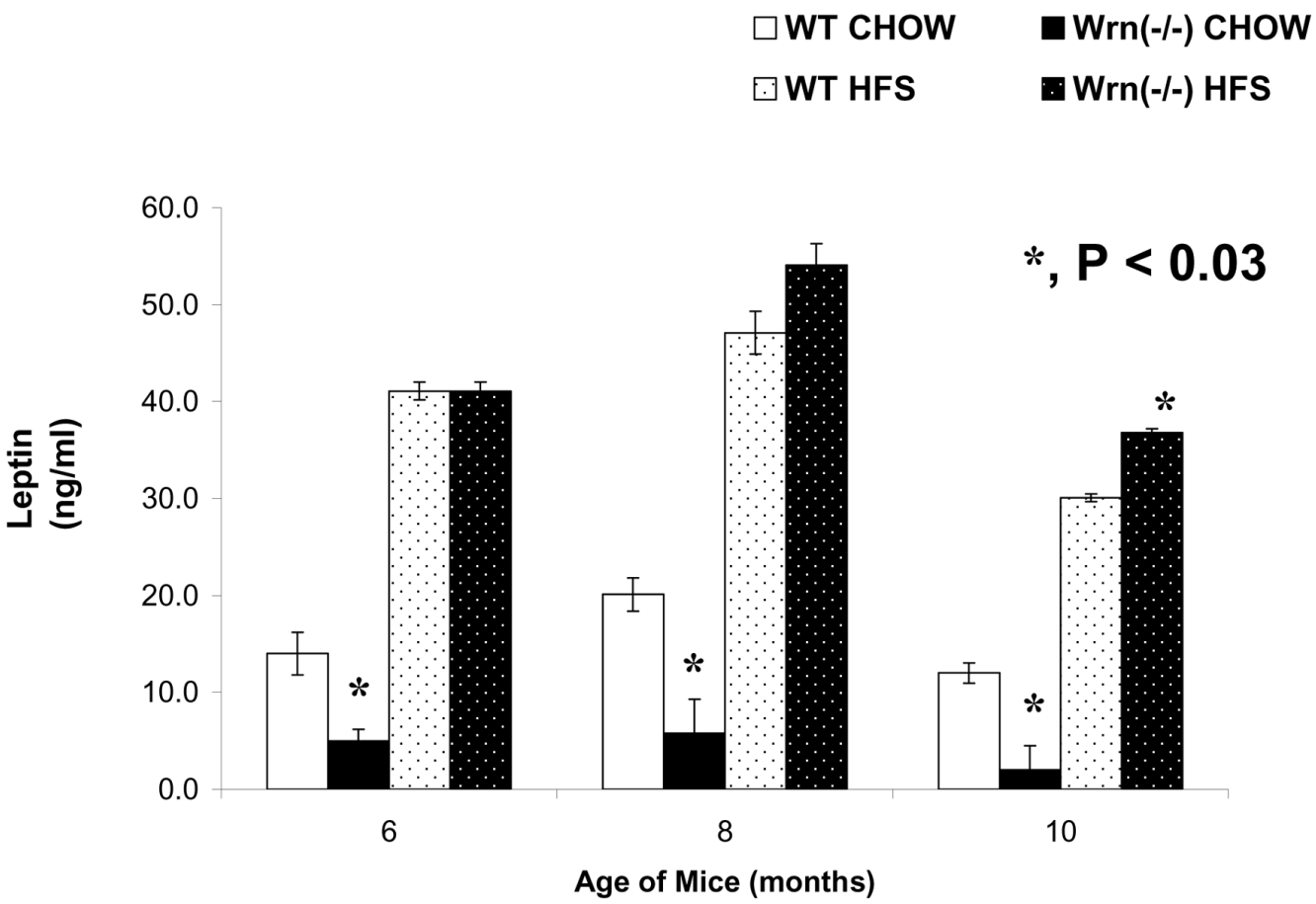

Fig. 2.

Fasting serum leptin levels were determined at 6, 8 and 10 months of age in mice fed HFS or CHOW. Values are presented as means ± SEM for 5 to 8 mice per cohort (HFS WT, open stipled bars; HFS $Wrn^{(-/-)}$, closed stipled bars; CHOW WT, open bars; CHOW $Wrn^{(-/-)}$, closed bars). P value denotes significant difference (< 0.03)* between WT and $Wrn^{(-/-)}$ mice fed HFS or CHOW for each time point.

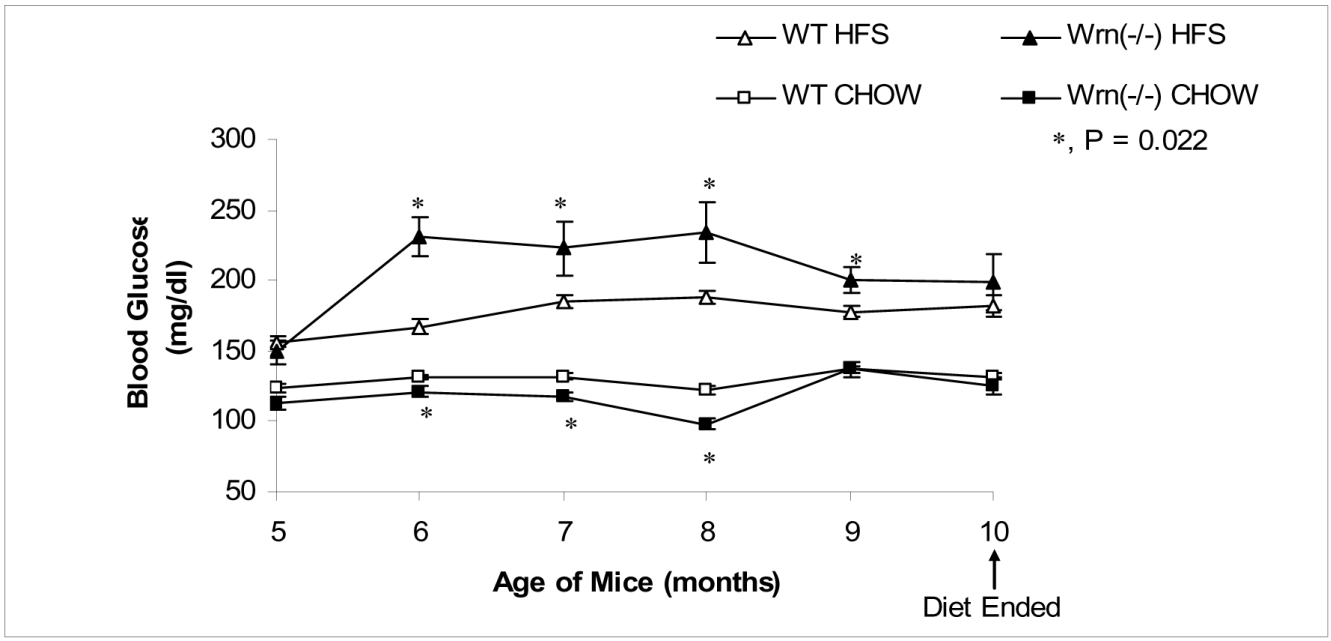

Fig. 3.

Blood glucose levels in mice fed HFS diet starting at 4 months of age and continuing to 10 months of age. Values are presented as medians ± SEM for 5 to 8 mice per set with significant difference (P = 0.022)* between the WT and $Wrn^{(-/-)}$ mice. $Wrn^{(-/-)}$ mice are represented as closed triangles and squares and WT mice are represented as open triangles and squares.

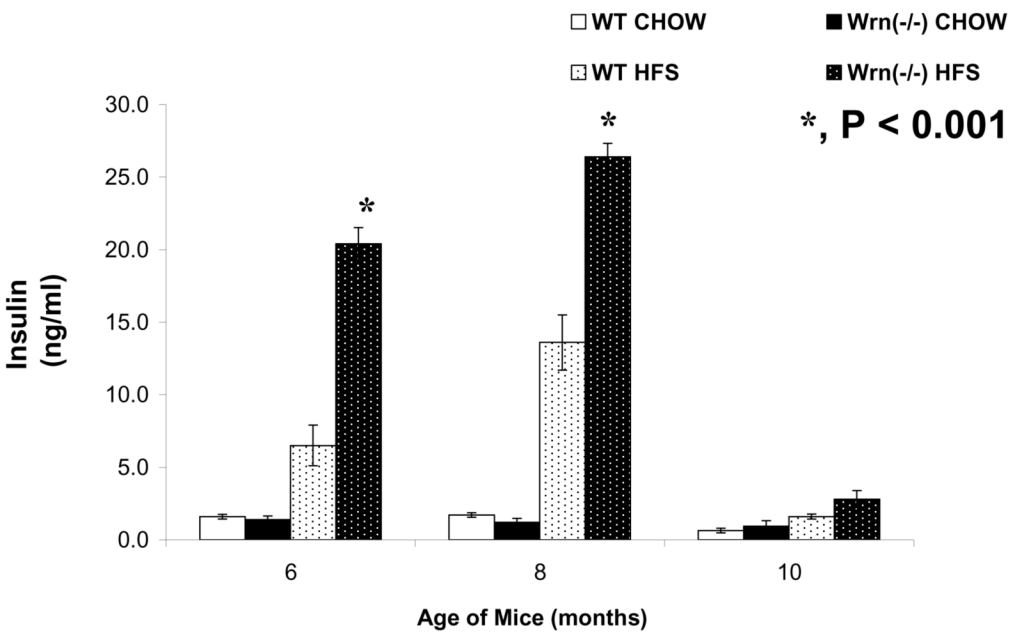

Fig. 4.

Fasting serum insulin levels in mice fed HFS at 6, 8 and 10 months of age. Mice were introduced to the diet at 4 months of age. Values are presented as means ± SEM for 5 to 8 mice per cohort (HFS WT, open stipled bars; HFS $Wrn^{(-/-)}$, closed stipled bars; CHOW WT, open bars; CHOW $Wrn^{(-/-)}$, closed bars). P values (< 0.0003** and < 0.001*) denote significant difference between the WT mice and the $Wrn^{(-/-)}$ fed HFS at 6 and 8 months of age, respectively. Serum insulin levels at 10 months of age were similar to that observed in mice fed CHOW.

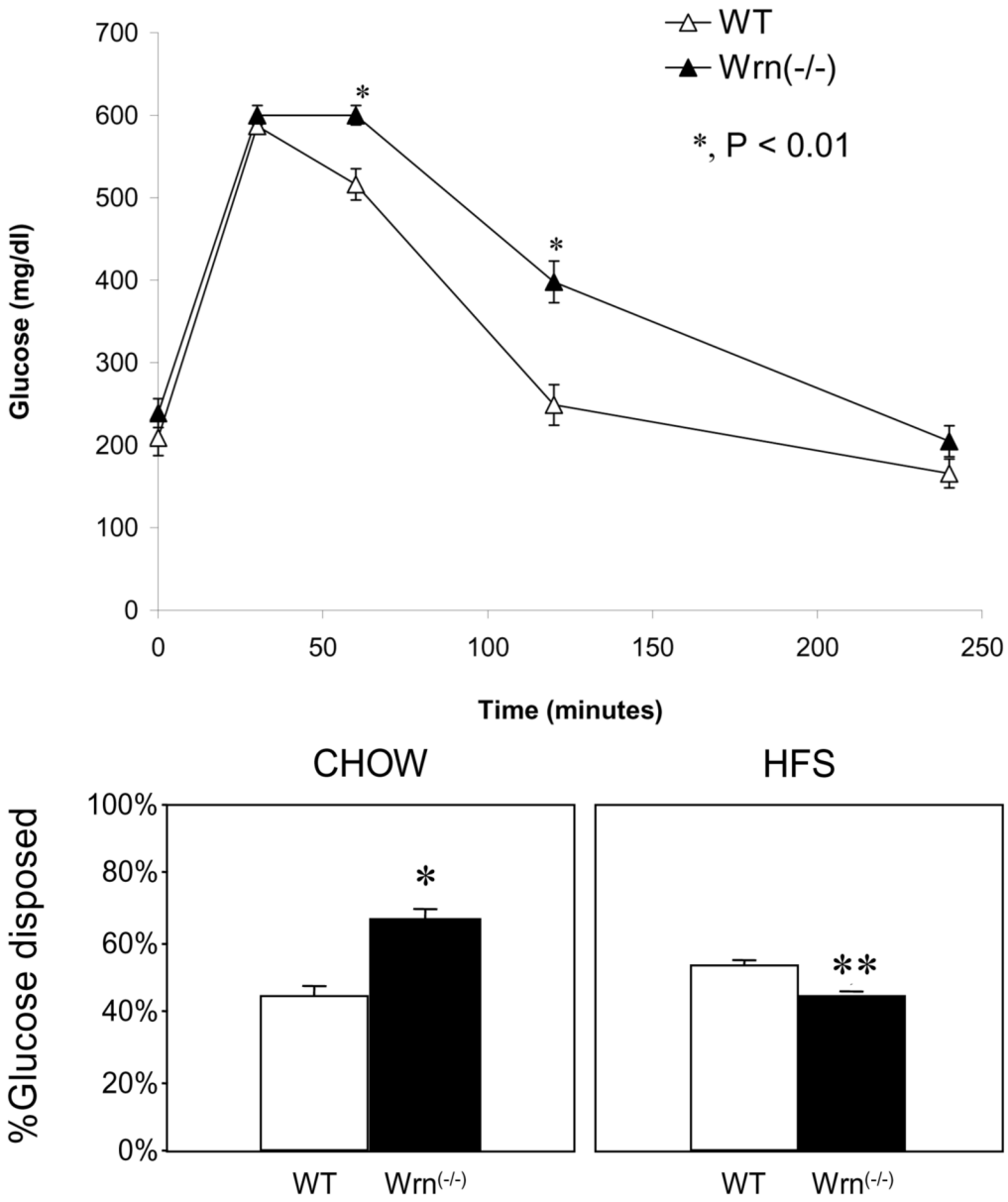

Fig. 5.

(A) Intraperitoneal glucose tolerance test (IPGTT) for WT (open triangles) and $Wrn^{(-/-)}$ (closed triangles) mice fed HFS. Values are presented as means ± SEM for 5 to 8 mice per cohort. At 5 months of age and after one month on the diet, mice were fasted overnight before the IPGTT was performed. P value (< 0.01*) denotes significant difference between WT and $Wrn^{(-/-)}$ mice fed HFS 60 minute after injection of glucose. (B) Insulin-mediated glucose disposal for $Wrn^{(-/-)}$ mice fed HFS diet. Values are presented as means ± SEM for 5 to 8 mice per cohort. At 10 months of age and after 6 months on the diet, mice fed HFS or CHOW were subjected to the assay. P value denotes significant difference between WT and $Wrn^{(-/-)}$ mice fed HFS (**< 0.004).