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The Murphy Roths Large (MRL) mouse strain is naturally resistant to high fat diet-induced hyperglycemia

Aaron J. Mull^{#a}, Tirsit K. Berhanu^{#a}, Nathan W. Roberts^a, and Ahlke Heydemann^{a,b}

^aDepartment of Physiology and Biophysics, The University of Illinois at Chicago, Chicago, IL 60612, USA

^bCenter for Cardiovascular Research, The University of Illinois at Chicago, Chicago, IL 60612, USA

[#] These authors contributed equally to this work.

Abstract

Objective—Due to their previously identified naturally and chronically increased levels of skeletal muscle pAMPK we hypothesized and now investigated whether the MRL/MpJ (MRL) mice would be resistant to high fat diet (HFD)-induced metabolic changes.

Materials/Methods—Three-week old male MRL and control C57Bl/6 (B6) mice were randomly assigned to 12 weeks of high fat diets (HFD) or control diets (CD). Weekly animal masses and fasting blood glucose measurements were acquired. During the last week of diet intervention, fasted animals were subjected to glucose and insulin tolerance tests. At harvest, tissues were dissected for immunoblots and serum was collected for elisa assays.

Results—The MRL mouse strain is known for its ability to regenerate ear punch wounds, cardiac cryoinjury, and skeletal muscle disease. Despite gaining weight and increasing their fat deposits the MRL mice were resistant to all other indicators of HFD-induced metabolic alterations assayed. Only the HFD-B6 mice displayed fasting hyperglycemia, hyperinsulinemia and hypersensitivity to glucose challenge. HFD-MRL mice were indistinguishable from their CDMRL counterparts in these metrics. Skeletal muscles from the HFD-MRL contained heightened levels of pAMPK, even above their CD counterparts.

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To whom correspondence should be addressed: Ahlke Heydemann, Ph.D. University of Illinois at Chicago COMRB 2035, MC 901 835 South Wolcott Ave. Chicago, IL 60612-7352 Phone: 312-355-0259 Fax: 312-996-1414 ahlkeh@uic.edu RO1: RHL102322A.

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9. Author Contributions AM wrote the manuscript, prepared the figures, conducted the statistics, performed the weekly fasting glucose, glucose and insulin tolerance tests. TB wrote the manuscript, performed the weekly fasting glucose, glucose and insulin tolerance tests, performed the western blots, and maintained the mouse colony.

NR harvested mice and isolated serum. AH wrote the manuscript and provided experimental design and guidance and is guarantor of all data included herein.

Conclusions—The MRL mouse strain is the first naturally occurring mouse strain that we are aware of that is resistant to HFD-induced metabolic changes. Furthermore, the increased pAMPK suggests a proximal mechanism for these beneficial metabolic differences. We further hypothesize that these metabolic differences and plasticity provide the basis for the MRL mouse strain's super healing characteristics. This project's ultimate aim is to identify novel therapeutic targets, which specifically increase pAMPK.

Keywords

AMPK; Animal model; Genetic resistance; Skeletal muscle metabolism; High fat diet

1. Introduction

The MRL/MpJ (MRL) mouse strain was originally bred for autoimmunity research and was subsequently found to have robust regenerative potential. The mouse strain heals many types of wounds faster and more completely than commonly used laboratory mouse strains; the MRL mice completely heal ear hole punches without fibrosis or scarring [1], have reduced scarring in cryoinjured cardiac ventricles [2], and have reduced fibrosis in a genetic model of muscular dystrophy [3]. These beneficial alterations are all present in wild type MRL mice without genetic manipulations. The molecular mechanisms behind the MRL super healing are not fully understood. Unlike the C57Bl/6 (B6) control mice, MRL mice maintain some characteristics of embryonic metabolism, such as increased glycolysis rates [4]. We hypothesized that the embryonic-like metabolism in the MRL mice is likely responsible for their super healing characteristics and have therefore begun investigating various critical aspects of MRL metabolism. In this pursuit we have identified that MRL mice have increased pAMPK in their skeletal muscles and that this feature has consequences which would alter the mouse's metabolism, HFD susceptibility, and healing abilities [5].

As increasing pAMPK is central to many type 2 diabetes treatments, including metformin, [6] we decided to challenge the MRL mice with a HFD, hypothesizing that they would be resistant to many of the HFD-induced metabolic consequences due to their naturally elevated pAMPK. Chronic HFD in humans and mice leads to insulin resistance, hyperinsulinemia, impaired glucose tolerance, and impaired carbohydrate and lipid metabolism making mice a useful model of T2DM [7-9]. As resting skeletal muscle metabolizes 10-20% of hepatic glucose output [10], our current investigations have focused upon HFD-mediated metabolic alterations in skeletal muscle.

AMPK is a master regulator of skeletal muscle metabolism [11]. Low ATP levels induced by exercise, calorie restriction, or pathology activate AMPK by phosphorylation; pAMPK then activates pathways that replenish ATP levels, and deactivates central pathways involved in ATP consumption. In addition, through Glut4 translocation, pAMPK triggers uptake of glucose into skeletal muscles [12]. pAMPK also increases β -oxidation of free fatty acids by enhancing fatty acid uptake by mitochondria [13]. Therefore, clinicians are using AMPK activators, such as metformin and resveratrol, in patients with T2DM [6, 14]. These compounds also have numerous AMPK independent functions, indicating the need for a new

animal model to investigate naturally upregulated pAMPK. The MRL mice now provide such a model.

In the current manuscript we report that the MRL mouse strain is resistant to HFD-associated pathology. We also suggest a causative mechanism for the MRL strain's HFD resistance, and identified that the MRL mice on HFD had much higher levels of active, phosphorylated AMPK in their skeletal muscles than the HFD-B6 mice and the CD-MRL mice. We have therefore identified another unique aspect of the MRL mouse strain and provide the research community with another, very unique model to investigate T2DM.

2. Methods

2.1 Ethics

All protocols adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and by the University of Illinois at Chicago IACUC, (10-203).

2.2 Mice

Wild type MRL/MpJ (000486) and C57BL/6J (000664) male mice were purchased from Jackson Laboratories (Bar Harbor, ME). Beginning at 3 weeks of age mice were fed regular chow diet (CD, Harlan 7912, 58% kcal carbohydrate, 17% kcal fat) or high fat diet (HFD, Research Diets D12492, 21% kcal carbohydrate, 60% kcal fat (9% soybean oil and 91% lard)) ad libitum for 12 weeks. Food intake per cage was weighed twice a week for 12 weeks and is reported as absolute grams consumed and as grams consumed/final grams of total animal mass in the cage. Animals were weighed each week, and weekly fasting blood glucose levels were taken 6 hours after food withdrawal with a glucometer (One Touch Ultra; Lifescan, Milpitas, CA). In the final four days of the 12-week diet regiment glucose tolerance tests were performed on 6-hour fasted mice by taking a basal blood glucose test followed by intraperitoneal injection of 2mg/g D-glucose. After two additional days insulin tolerance tests on 6-hour fasted mice by taking a basal blood glucose test followed by intraperitoneal injection of 1mU/gr of humalog (E.L.Lilly, Indianapolis, Indiana). Blood glucose was measured at 30, 60, 90, 120, 150 and 180 minutes after injections. At 15 weeks old, after a six-hour fast, serum was collected and tissues were harvested for immunoblotting or immunofluorescence.

2.3 Dual-energy X-ray absorptiometry (DXA)

DXA was employed to determine the amount of fat gained in the body, excluding head and tail, as percent of body mass [15].

2.4 Immunoblotting

Immediately following excision, mouse triceps brachii and quadriceps were frozen in liquid nitrogen until use. Muscles were prepared and analyzed as previously reported (5). Primary antibodies are against pAMPK and AMPK (2535 & 2793, Cell Signaling, Beverly, MA, 1:1000). Grey values of bands from multiple exposures were quantified with ImageJ software and normalized to the coomassie stained sister gel.

2.5 ELISA

Serum was kept on ice for 30 minutes after terminal retro-orbital bleeds. Serum was spun in serum separation tubes (Becton Dickinson, Franklin Lakes, NJ) at 11.5×RPM for 1.5 minutes and frozen at −80°C until use. Serum insulin and adiponectin were quantified with a sandwich ELISA kit as instructed by the manufacturers (Alpco, Salem, NH; and R&D Systems, Minneapolis, MN, respectively). Serum high density lipoproteins (HDL), low/very low density lipoproteins (LDL), triglycerides (TG) and free fatty acids were also determined according to the manufacturer's instructions (Sigma, St. Louis, MO).

2.6 Immunofluorescence

Quadriceps tissues were frozen in liquid nitrogen cooled isopentane and sectioned at 10µm; sections from all four treatment groups were affixed to the same slide to ensure similar staining conditions. Staining was performed as previously described [16], with the following primary antibodies Glut4 (Millipore, Billerica, MA, 1:100) and dystrophin (Novocastra labs, Buffalo Grove, IL, 1:200). Pictures were acquired on an Observer.Z1 microscope (Zeiss, Jena, Germany) at 400 × initial magnification with identical exposure times for each stain.

2.7 Electron Microscopy

Freshly isolated quadriceps muscles from one animal of each group were delivered to the Electron Microscopy Facility of the UIC RRC. Micrographs were captured in a blinded manner at 10,000 and 30,000 magnifications.

2.8 Statistics

Glucose tolerance tests and weekly blood glucose assays were analyzed as areas under the curves (AUC) calculated with Prism software (Graphpad Software, LaJolla, CA) for each animal. The results were analyzed by one-way ANOVA with Bonferroni posttest, unless otherwise indicated in the results section. Averages are reported with standard errors and the numbers of animals used for each assay are indicated in the figure legends.

3. Results

The MRL mouse strain has been reported to exhibit unusual metabolic characteristics [4, 5]. In previous experiments we identified that the MRL mice have increased mitochondrial area, mitochondrial genomes, and pAMPK in their skeletal muscles compared to B6 control mouse strain [5]. As it is well established that metformin and other prescriptions to combat type-2 diabetes provide therapeutic benefits by increasing pAMPK levels; we have now evaluated the hypothesis that wild type MRL mice would be naturally resistant to HFD-induced metabolic changes. Despite a robust increase in percent body fat, the HFD-MRL mice were resistant to the metabolic perturbations caused by chronic HFD.

3.1 Mouse morphometry

As expected, both mouse strains regardless of diet composition, demonstrated increased mass throughout the 12 week diet intervention. Despite eating less, the two HFD mouse groups gained slightly more weight, than their corresponding control groups (Figure 1A,

averages). At fifteen weeks of age the HFD-MRL mice were 7.5 grams heavier than the CD-MRL mice and the HFDB6 mice weighed 10.6 grams more than the CD-B6 mice. The differences in mass never achieved significance between the HFD and CD groups in cumulative area under the curve (AUC) comparisons (Figure 1B).

As the CD-MRL mice are significantly larger than the B6 mice, we also analyzed the relative weight gain in grams over the initial body mass. The MRL mice gained proportionately less mass on the HFD than the B6 mice did (Figure 1C), which is clearly demonstrated in the cumulative AUC bar graph (Figure 1D). The B6 difference is statistically significant $p=0.0005$, while the MRL difference is not, $p=0.089$. Proportionately the HFD-B6 mice gained significantly more weight compared to their CD counterparts than the HFD-MRL mice did.

Because of the surprising difference in proportionate mass gain between the HFD groups, we also analyzed the amount of food consumed by each of the four groups in absolute grams (Figure 1E) and as a ratio to final body mass (Figure 1F). The HFD-B6 mice consumed less food than their CD counterparts over the 12 week study period (B6 difference 10.83 g, $p<0.05$). There was no difference in food consumption between the MRL mouse groups (MRL difference 3.369g, $p>0.9999$). We were surprised to see that both MRL diet groups consumed proportionately less food and therefore fewer calories than their B6 counterparts, the CD difference was significant (CD difference 13.25g, $p=0.0271$) while the HFD difference was not significant (HFD difference 5.787g, $p=0.3024$).

3.2 Both HFD groups gain equivalent fat mass

Dual-energy X-ray absorptiometry (DXA) was utilized to quantify the amount of fat gained as percent of body mass. In figure 2A, the light blue regions represent fat deposits imaged from animals in the prone position between the right fore and hind limbs. Both of the HFD mouse groups gained statistically more fat mass as a percentage than their CD counterparts (Figure 2B). From the DXA images and comparisons made during harvest time, both of the HFD mouse groups markedly increased their visceral fat. This indicates that both of these mouse groups are consuming enough to elicit fat weight gain; however the MRL mice do not succumb to the pathogenic metabolic phenotypes of diseased mice.

3.3 Glucose regulation

Evidence that the MRL mice are resistant to HFD-induced metabolic change is illustrated in the weekly fasting blood glucose data (Figure 3A). As expected, the HFD-B6 mice became hyperglycemic. The hyperglycemia in the B6 strain was evident after 6 weeks of HFD feeding (CD B6 versus HFD-B6 $p=0.0064$, two-way ANOVA). Furthermore, the HFD-B6 mice had significantly higher fasting blood glucose measurements compared to the CD-B6 mice in cumulative AUC measurements (Figure 3B, CD-B6 versus HFD-B6 $p=0.0024$). The HFD-MRL mice never displayed hyperglycemia when compared to the CD MRL in weekly comparisons or in cumulative AUC measurements (CD-MRL versus HFD-MRL $p>0.999$). In addition, the HFD-MRL mice had significantly lower blood glucose measurements compared to the HFD-B6 mice, beginning at 3 weeks of diet (HFD-B6 versus HFD-MRL $p=0.0009$) and continuing through the rest of the diet intervention as demonstrated by AUC

measurements (HFD B6 versus HFD-MRL $p<0.0001$). The CD-MRL mice also had significantly lower fasting blood glucose levels than the CD-B6 mice ($p<0.0001$).

To further support the data indicating hyperglycemia resistance in the HFD-MRL mice, glucose tolerance tests were performed during the final week of diet intervention. These tests demonstrated that the HFD-B6 mice were unable to clear the injected glucose (Figure 3C, averages). The HFD-B6 mice were hyperglycemic immediately following the injections and did not return to normoglycemia until 4 hours post-injection. The CD-B6, CD-MRL, and HFD-MRL mice were hyperglycemic for approximately the first hour post-injection and by 2 hours normoglycemia was reestablished (normoglycemia defined as 238mg/dL, for B6 males, age 8 weeks, after 4 hours fasting, Jax mouse phenome database, reference; Lake1). The HFD-MRL blood glucose values following the glucose bolus were significantly lower than their HFD-B6 counterparts, (Figure 3D, HFD differences $p<0.0001$). The blood glucose values of the two MRL mouse groups were not statistically different from each other after the twelve week diet regimens (CD-MRL versus HFD-MRL $p=0.9999$). The blood glucose values between the two CD groups were also not significantly different, (CD differences $p>0.9999$). These data confirm that the MRL mice metabolically adapt to the HFD to resist hyperglycemia unlike the B6 mouse strain. Similarly, the HFD-MRL mice respond to insulin challenge in an unusual, adaptive manner. The HFD-MRL mice appear hyper-responsive to insulin challenge, their blood glucose values significantly decline more than the three other mouse groups, and remain depressed longer (Figure 3E). The significant drop is illustrated in the cumulative AUC calculations (Figure 3F, HFD differences $p<0.005$).

3.4 Hormone levels

Serum insulin levels were measured by ELISA to further characterize the HFD-elicited changes in the two mouse strains (Figure 4A). Serum (taken from six-hour fasted mice) insulin measurements after 12 weeks of HFD trended to the expected increase of insulin levels in HFDB6 mice (CD-B6 versus HFD-B6 $p=0.126$). No difference in serum insulin was detected between the MRL mice (CD-MRL versus HFD-MRL $p=0.332$). The CD-MRL mice had significantly more serum insulin per mL than the CD-B6 mice ($p=0.001$).

The serum was further analyzed for adiponectin levels; increased levels of adiponectin usually indicate metabolic homeostasis (Figure 4B). There was a significant decrease in serum adiponectin between the B6 mice (CD-B6 versus HFD-B6 $p=0.0157$). The difference between the two MRL groups was not significant. Furthermore, both of the MRL cohorts had significantly lower adiponectin levels than the B6 mice (CD-B6 versus CD-MRL $p=0.0001$; HFD-B6 versus HFD-MRL $p=0.0003$). This data provide further evidence that the MRL mice achieve metabolic homeostasis and do not decrease their adiponectin levels below basal, whereas the HFD causes detrimental adiponectin decreases in the B6 mouse strain.

3.5 Serum lipid analysis

Serum analysis of triglyceride levels revealed no differences between any of the animal groups (Figure 4C). There was a statistically significant difference between the MRL animals for free fatty acids (Figure 4D). Cholesterol levels were separately assessed for high

density lipoproteins and low density/very low density lipoproteins. There were no significant differences in high density lipoproteins levels in any of the relevant mouse group comparisons (Figure 4E). There were also no significant comparisons for low density and very low density lipoproteins (data not shown). However, both of the HFD groups trended to an increase in low density and very low density lipoproteins over the strain-specific CD mice (data not shown). The ratio of HDL/LDL was not significantly higher for the CD-MRL compared to the CD-B6 animals (Figure 4F).

3.6 AMP-activated protein kinase levels

MRL and B6 mice differentially regulate pAMPK and AMPK in response to HFD. Immunoblotting was performed to assess the levels of pAMPK and AMPK in the triceps brachii tissues to initiate the investigations into the molecular mechanisms of altered MRL response to HFD (Figure 5A). The bands were quantified and normalized to a coomassie stained sister gel (Figure 5B and C). Because of the large differences in expression, multiple exposures were utilized to quantify all of the bands. As previously reported [5], the CD-MRL mice had a trend to increased pAMPK levels compared to the CD-B6 mice. In addition, the levels of pAMPK increased even more in the HFD-MRL mice (CD-MRL compared to HFD-MRL $p=0.0007$). In the control B6 mouse strain, the HFD did not alter the pAMPK levels, indicating the metabolic inflexibility of the B6 mice (CD-B6 compared to HFD-B6 $p=0.999$). The elevated levels of pAMPK in HFD-MRL skeletal muscle were confirmed in quadriceps tissues from the same cohort of animals, (Figure 5D). As increasing pAMPK is critically important in T2DM treatment, this natural increase in pAMPK is potentially the mechanism behind the MRL mouse strain's HFD resistance.

We also detected an increase in AMPK in the MRL triceps brachii compared to the B6 tissues, (Figure 6A and B). AMPK levels were not significantly altered by HFD in either of the mouse strains. Quadriceps tissues were also analyzed for AMPK levels (Figure 6C). Again a significant increase of AMPK in the MRL tissues was evident. The ratios of pAMPK/AMPK for the 4 mouse groups are shown and further indicate increased adaptability of the MRL tissues (Triceps, Figure 6D and Quadriceps Figure 6E).

3.7 Increased membrane localized Glut4 in MRL muscles

Further investigations of increased pAMPK were demonstrated by utilizing a well-known pAMPK target; increased Glut4 membrane localization. Immunofluorescence clearly shows Glut4 membrane localization in the two MRL groups while the two B6 cohorts demonstrate more cytoplasmic staining (green stain, Figure 7). Glut4 differences were undetectable with the HFD feeding protocol. Dystrophin staining was used to indicate the sarcolemmal membranes (red stain, Figure 7).

3.8 Muscle ultrastructure

Electron microscopy was utilized to analyze subcellular morphology. HFD-B6 mice demonstrated lipid accumulations between myofibrils in the quadriceps muscles (Figure 8). The HFD-B6 quadriceps muscles contained many of these spaces, while no lipid-like accumulations were present in the quadriceps muscles from the three other mouse groups. This confirms MRL have effectively adapted to HFD feeding.

4. Discussion

This research clearly demonstrates that the super healing MRL mouse strain is resistant to high fat diet (HFD)-mediated metabolic pathologies. This resistance is characterized by the absence of hyperglycemia, hyperinsulemia, rapid achievement of normoglycemia after glucose challenge, hypersensitivity to insulin challenge, and lack of lipid droplets within skeletal muscle cells. Further data prove that the HFD-MRL mice are receiving excessive calories, which elevate their percent body fat; therefore intestinal absorption is unimpaired. Data are presented which indicate that the beneficial MRL adaptations are within their cellular metabolism. Further, we provide data that is consistent with the absolute increases in pAMPK causing the HFD resistance in the MRL mouse strain.

The MRL mouse demonstrates increases in pAMPK, this increase is central not only to metabolic adaptations but also to the extraordinary healing demonstrated by this mouse strain. There are a number of studies indicating that increasing pAMPK levels is a therapeutic target and is arguably necessary for T2DM treatments (reviewed in [17]). Metformin is the most widely prescribed medicine for type 2 diabetes patients [18]. It has been demonstrated that Metformin treatment increases pAMPK levels [19]. Similarly, animal models indicate that an increase in pAMPK either genetically or through pharmaceuticals alleviate T2DM symptoms [20]. Alternatively, mouse models of AMPK deficiency are more susceptible to HFD-induced metabolic pathologies [21-23]. Our novel findings with the current non-genetically manipulated mouse model significantly support the data that increasing pAMPK causes resistance to HFD-induced metabolic pathologies.

HFD induces systemic pathologies by multiple molecular mechanisms. Of the possibilities four dominant, and not exclusive, hypothesis are 1) lipotoxicity, 2) toxic effects of hyperglycemia due to insulin resistance, 3) pathologically excessive immune response, and 4) increased reactive oxygen species. Multiple investigations have demonstrated that increased pAMPK reduces all of these pathogenic scenarios, although pAMPK's exact role in these reductions are still under dispute. pAMPK has been demonstrated to phosphorylate acetyl-CoA carboxylase, which inhibits malonyl-CoA and results in increased β -oxidation; pAMPK also decreases triglyceride synthesis [13]. pAMPK also increases the mitochondrial content of cells, which further increases β -oxidation [24]. There were no significant differences in serum free fatty acid levels, although both HFD groups were trending to decreased levels. This unusual result [25] may arise because the mice in this study were fasted for 6 hours before serum was collected and the CD mice are likely conducting lipolysis for fuel at this point, while the cells of the HFD mice contain sufficient energy. Increased pAMPK also decreases the effects of hyperglycemia as it is known to increase glycolysis through Glut4 membrane translocation and increased hexokinase 2 activity [26]. A pathogenic inflammatory response from HFD feeding is well accepted. Increased pAMPK is known to decrease this inflammatory immune response [27]. Importantly pAMPK has been demonstrated to shift macrophages to the alternatively activated, type 2, anti-inflammatory phenotype [28]. pAMPK is also known to inhibit TGF β 1 signaling, which would further reduce pathologic inflammation and fibrosis [29]. Lastly, pAMPK also inhibits reactive oxygen species [30], reducing the pathologic consequences of an over active immune response even further. Therefore, the naturally increased pAMPK found in

the MRL skeletal muscle tissue can inhibit HFD pathologies at multiple points. Future experiments are being designed to identify which of these pathways are being inhibited in the MRL mouse strain.

4.1 HFD-MRL mice have normal glycemic control

The HFD-MRL mice are resistant to basal hyperglycemia and have a higher glucose infusion tolerance than HFD-B6 mice and even CD-B6 mice. One possible explanation, consistent with previous reports [31], is that the increased pAMPK causes the demonstrated Glut4 membrane translocation, which increases glucose influx. Figure 5 indicates increased pAMPK, is demonstrably more important than an increased pAMPK/AMPK ratio in delivering Glut4 to the membrane. An alternative mechanism is the increased serum insulin identified in the MRL mice. Glucose influx is the first rate limiting step of glucose metabolism and therefore blood clearance. In addition, the increased glycolysis seen in MRL mice [4, 5] contributes to their maintained glucose tolerance. As anaerobic glycolysis is less efficient than aerobic oxidative phosphorylation, animals that are relatively more glycolytic would eliminate glucose faster. Increased glycolysis has been demonstrated in the CD-MRL [4, 5].

The glucose tolerance tests demonstrated additional beneficial MRL adaptations. The HFD-MRL mice responded identically to the glucose bolus as their strain specific CD counterparts, while the HFD-B6 mice responded as expected and were metabolically unable to clear the glucose efficiently. The MRL-HFD mice also responded normally to the insulin challenge and their blood glucose values dropped significantly compared to the B6-HFD mice.

4.2 Unique molecular signaling adaptations in the MRL mice

Upon HFD feeding, serum insulin levels are expected to increase to compensate for the subsequent hyperglycemia [32]. The serum insulin levels trend to an increase in the HFD-B6 animals compared to their CD controls. The HFD-MRL mice had a trend to lower insulin levels than the CD-MRL mice. This difference further illustrates increased metabolic adaptations in the HFD-MRL mice, which maintain homeostasis without increasing insulin levels. Equally intriguing is the higher serum insulin levels observed in the CD-MRL mice compared to their B6 counterparts. This may allow the MRL mice to process more glucose and thus resist hyperglycemia under normal CD conditions. The insulin may also be important in regulating many other aspects of metabolism. Insulin has been shown to be instrumental in carbohydrate and lipid metabolism. Insulin also has functions as a growth factor [33] and may be central to the enhanced regeneration and size displayed by the MRL mice.

Consistent with published results [34], the HFD-B6 mice have less serum adiponectin than their CD B6 controls. Decreased adiponectin occurs with decreased insulin sensitivity and impaired glucose tolerance [35]. It is therefore unexpected that adiponectin levels are lower in both MRL groups, which display increased insulin sensitivity and better glucose tolerance. The serum adiponectin levels do not change with HFD in MRL mice. This further indicates that the MRL mice are resistant to HFD-induced metabolic changes. In addition, it

may be that the MRL mice do not require the protective effects of adiponectin and therefore produce less.

In most HFD protocols increased insulin and decreased adiponectin levels are consistent with a state of insulin resistance. It is therefore surprising that the HFD MRL animals in this protocol demonstrate increased insulin and decreased adiponectin levels but appear HFD resistant by all other characteristics. In addition, the CD MRL animals also have increased insulin and decreased adiponectin. As we are just beginning to unravel the metabolic differences between these animal strains a complete picture is difficult to assemble. However, the multiple functions of insulin and adiponectin and the overall improved health of the HFD-MRL animals are at least two confounding factors.

CD-MRL mice have higher levels of pAMPK than CD-B6 mice; the MRL mice have even higher levels of pAMPK while on HFD. Consistent with previous reports, we hypothesized this increased pAMPK is the causative factor in the MRL mouse strain's HFD resistance. It is rather unusual that the MRL mice also contain increased AMPK. In acute situations to maintain energy homeostasis, AMPK levels do not change and only pAMPK levels change. The important difference is that the MRL mice have chronically elevated AMPK and pAMPK. Therefore, in this situation the important metric is the absolute levels of active pAMPK. Even the CD comparisons reveal the same inverse correlation; increased pAMPK and reduced basal fasting glucose in the CD-MRL mice compared to the CD-B6 mice.

5. Conclusion

The ultimate goal of this study was the characterization of the MRL mouse strain's metabolic adaptation to HFD. We have now demonstrated that a non-genetically manipulated mouse strain is resistant to HFD-induced metabolic changes. The finding that MRL mice are resistant to hyperglycemia on a HFD has important clinical applications. A future goal is to identify why pAMPK is naturally increased in the MRL muscle tissues. This knowledge will generate novel T2DM therapeutic targets. Future studies will also examine how increased pAMPK imparts resistance to HFD, resistance to muscular dystrophy related fibrosis, and increased wound healing. Additional investigations examining HFD-mediated pathologic differences in the liver and adipose tissues, and immune systems will further elucidate resistance to HFD.

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Abbreviations

AMPK	AMP-activated protein kinase
AUC	area under the curve
B6	C57BL/6J mouse strain
CD	chow diet
DXA	dual-energy X-ray absorptiometry
GTT	glucose tolerance test
HFD	high fat diet
ITT	insulin tolerance test
MRL	MRL/MpJ mouse strain
T2DM	Type 2 Diabetes Mellitus

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Figure 1.

MRL mice gained less mass than B6 controls after 12 weeks of high fat diet (HFD) and consumed absolutely and proportionately less food. **A.** Weekly body mass of MRL and B6 mice on normal chow diet (CD) and HFD. **B.** Absolute body masses from cumulative area under the curve (AUC) calculations of each group, sum from 3 weeks to 15 weeks old. **C.** Weekly body mass as ratio to starting mass. **D.** Cumulative AUC of each group normalized for initial mass. Standard errors and significant differences are indicated. (n=10, 14, 15, 18). **E.** Food consumption of cages of MRL or B6 mice on CD or HFD. (cages; n=2, 2, 2, 3) **F.** Food consumed in each cage over 12 weeks normalized to final body mass. (cages; n=2, 6, 3, 4).

Figure 2.

HFD elicits increases of fat mass in both animal strains. Top panel; the sedated animals are lying prone, anterior on the right. The light blue region indicates visceral adipose deposits. Bottom panel is quantitation of the percent fat mass between head and tail. (n=4, 12, 7, 4)

Figure 3.

MRL mice are resistant to HFD-induced increases in blood glucose, have enhanced glucose tolerance and are hyperresponsive to insulin. **A.** Fasting (6 hours) blood glucose was measured weekly for the 12 weeks of the diet protocols. **B.** Cumulative (for the full 12 weeks) area under the curve calculations illustrate the blood glucose increase in the HFD B6 mice. (n=10, 11, 10, 15). **C.** Glucose tolerance test (IP injection of 2mg/g D-glucose) was performed after 12 weeks of diet and after a 6 hour fast. Blood glucose was measured every 30 minutes for up to 6.5 hours. **D.** Cumulative area under the curve calculations for 90 minutes illustrates the blood glucose increase in the HFD-B6 mice. (n=10, 22, 14, 15). **E.** Insulin tolerance test (IP injection of 1mU/gr) was performed after 12 weeks of diet and after a 6 hour fast. Blood glucose was measured every 30 minutes for up to 6.5 hours. **F.** Cumulative area under the curve calculations for 90 minutes illustrates the hyperresponsiveness of the HFD-MRL mice to insulin. (n=10, 22, 14, 15).

Figure 4.

Serum cytokine and lipid levels of 6-hour fasted mice after 12 weeks of diet were measured by ELISA or colorimetric assays. **A.** Serum insulin levels were increased in the MRL CD mice (n=6). **B.** MRL mice had lower levels of serum adiponectin compared to the B6 mouse strain (n=6). **C.** No significant changes were detected in serum triglycerides (n=6). **D.** Reduced serum free fatty acid levels in the HFD animals. (n=11). **E.** No significant changes were detected in serum HDL (n=6). **F.** or HDL/LDL ratios (n=6).

Figure 5. Significantly increased pAMPK protein levels in MRL triceps brachii and quadriceps tissues. **A.** Immunoblot with pAMPK specific antibody on triceps brachii tissues. **B.** Coomassie stain of a sister gel. **C.** Quantification of triceps brachii pAMPK band intensities (n=3). **D.** Quantification of quadriceps pAMPK band intensities (n=3).

Figure 6.

AMPK is also increased in MRL skeletal muscle tissue. **A.** Immunoblot with AMPK specific antibody on triceps brachii tissues. **B.** Quantification of triceps brachii AMPK band intensities (n=3). **C.** Quantification of quadriceps AMPK band intensities (n=3). **D.** Triceps pAMPK/AMPK ratios. **E.** Quadriceps pAMPK/AMPK ratios.

Figure 7.

Glut4 is increased at the quadriceps sarcolemma of MRL mice. Immunofluorescence demonstrates increased membrane localization of Glut4 (in green). Membrane is identified by dystrophin staining (red). Original magnification was 400x, scale bar is 50 μ M

Figure 8.
Lipid-like droplets are found only in the B6 HFD mice quadriceps by electron micrograph.
Original magnification is 11,600x, scale bar is 1 μ M