INCREASED GLUCOSE DISPOSAL AND AMP-DEPENDENT KINASE SIGNALING IN A MOUSE MODEL OF HEMOCHROMATOSIS*

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Running head: Increased glucose disposal in hemochromatosis.

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Hereditary hemochromatosis is an inherited disorder of increased iron absorption that can result in cirrhosis, diabetes and other morbidities. We have investigated the mechanisms underlying supranormal glucose tolerance despite decreased insulin secretion in a mouse model of hemochromatosis with deletion of the hemochromatosis gene (Hfe−/−). Hfe−/− mice on 129Sv or C57BL/6J backgrounds have decreased glucose excursions after challenge compared to controls. In the C57BL/6J/Hfe−/− mice, for example, incremental area under the glucose curve is reduced 52% (p<0.001) despite decreased serum insulin, and HOMA insulin resistance is decreased 50% (p<0.05). When studied by the euglycemic clamp technique 129Sv/Hfe−/− mice exhibit a 20% increase in glucose disposal (p<0.05) at submaximal insulin but no increase at maximal insulin compared to wild types. [1,2-13C]-D-glucose clearance from plasma is significantly increased in Hfe−/− mice (19%, p<0.05), and lactate derived from glycolysis is elevated 5.1-fold in Hfe−/− mice (p<0.0001). Basal but not insulin-stimulated glucose uptake is elevated in isolated soleus muscle from Hfe−/− mice (p<0.03). Compared to controls Hfe−/− mice exhibit no differences in serum lipid, insulin, glucagon, or thyroid hormone levels; adiponectin levels are elevated 41% (p<0.05) and adiponectin message in adipocytes is increased 83% (p=0.04). Insulin action measured by phosphorylation of Akt is not enhanced in muscle, but phosphorylation of AMP-dependent kinase (AMPK) is increased. We conclude that supranormal glucose tolerance in iron overload is characterized by increased glucose disposal that does not result from increased insulin action. Instead, the Hfe−/− mice demonstrate increased adiponectin levels and activation of AMPK.

Hereditary hemochromatosis Type 1, an autosomal recessive disorder, occurs at a frequency of approximately 0.5% in populations of Northern European extraction (1,2). Most patients with hemochromatosis are homozygous for a missense mutation (C282Y) in the Hfe protein (3). This results in an inability of serum iron to normally regulate secretion of the peptide hepcidin, allowing unlimited egress of dietary iron from duodenal cells and macrophages into the circulation (4,5). The fully penetrant clinical syndrome is characterized by skin pigmentation, cirrhosis, arthritis, cardiomyopathy, diabetes and other endocrinopathies, although phenotypic expression varies greatly (1). Iron excess is also associated with non-hemochromatotic diabetes and other aspects of the metabolic syndrome, although causality and pathogenic mechanisms for that association are not established (6-9).

Diabetes is part of the classic presentation of hemochromatosis, and we have recently reported that the prevalence of diabetes in persons with hemochromatosis over the age of 45 exceeds 20% (10). In a mouse model with targeted deletion of the Hfe gene (Hfe−/−), insulin secretory capacity is diminished secondary to loss of beta cell mass and desensitization of glucose-induced insulin secretion (11). Mitochondrial function is also impaired in the Hfe−/− mice (H. Jouihan and D.A. McClain, submitted). These defects, however, are well compensated and affected mice have supranormal glucose tolerance. Humans with
hemochromatosis have a similar phenotype, namely decreased insulin secretion that tends to be compensated by increased insulin sensitivity (10). Most individuals with hemochromatosis and diabetes are overweight, suggesting that when obesity-related insulin resistance intervenes, these individuals are not able to compensate with increased insulin secretion due to the loss of beta cell mass (10). Normalization of iron stores results in increased insulin secretion but decreased insulin sensitivity (12). These results suggest that iron plays a role in regulating metabolism and glucose disposal. We therefore examined glucose homeostasis in the Hfe<sup>−/−</sup> mouse in order to determine the mechanism for the enhanced glucose tolerance. We report that the increased glucose disposal is associated with increased adiponectin production and activation of AMP-dependent kinase in skeletal muscle.

**EXPERIMENTAL PROCEDURES**

*Experimental animals.* Targeted mutagenesis produced a knockout of the Hfe gene (Hfe<sup>−/−</sup>) (13). The mutation was bred onto the 129/SvEvTac and C57BL/6J genetic backgrounds for over ten generations. Normal chow (Harlan Teklad TD-8640) contained 4.5% of calories as fat and 0.33 g/kg of iron. Age- and sex-matched wild type 129SvEvTac/Hfe<sup>+/−</sup> or C57BL/6J/Hfe<sup>+/−</sup> littermates were used as controls. Procedures were approved by the Institutional Animal Care and Use Committee of the University of Utah.

*Glucose tolerance testing.* Following a 6 h fast mice were injected intraperitoneally with 1 mg/g glucose in 0.9% saline. Glucose levels were measured from tail vein blood (3 µl, Glucometer Elite, Bayer Corp., Tarrytown, New York) at 0, 5, 15, 30, 60 and 120 min. Blood (30 µl) was collected at 0 and 30 min for insulin determinations. Values for the homeostasis model assessment of insulin resistance (HOMA-IR) were calculated from the product of fasting serum glucose (mmol/l) and insulin (µU/ml) divided by 22.5 (14).

*Euglycemic clamp procedure.* The jugular vein was catheterized under avertin anesthesia, using Micro-Renathane tubing (Braintree Scientific, Inc., MRE 025). After a 48 h recovery, mice were fasted overnight and infused through the catheter with [3H]glucose (20µCi bolus, 0.1 µCi/min). A dual infusion pump (Harvard Apparatus, Pump 33) was used to infuse insulin at a constant flow rate and 50% dextrose at a variable rate to maintain glucose at 100-150 mg/dl. Glucose was measured at 10 minute intervals as described above for glucose tolerance testing. After steady state was reached for 3 successive glucometer readings five tail vein blood samples were collected every 20 minutes to measure specific activity. These samples were air dried to remove tritiated water and counted in scintillation counter. The glucose infusion rate was calculated as: flow rate(ml/hr)/60/kg/2. Glucose turnover rate (mg/kg/min) was calculated as the tracer infusion rate (dpm/min) divided by the plasma glucose-specific activity (dpm/min/mg) corrected for body weight. Hepatic glucose output was calculated as the difference between the tracer-derived rate of glucose appearance and the infusion rate of glucose.

*Ex vivo glucose uptake into isolated soleus muscle.* 2-deoxy-D-glucose (2-DG) uptake was measured as previously described (15). Both soleus muscles were excised from Hfe<sup>−/−</sup> and wild type mice and preincubated for one hour in 2 ml of Krebs Ringer buffer (KRB) containing BSA (1.25%), mannitol (32 mM) and glucose (8 mM). The muscles were then placed in a wash solution for 10 minutes containing KRB with BSA (1.25%), mannitol (40 mM) and pyruvate (2 mM). Muscles were then transferred to transport media for 20 minutes containing KRB, mannitol (39 mM), 2-deoxy-D-glucose (1 mM), <sup>3</sup>H-2-DG (1.5 µCi/ml) and <sup>14</sup>C-mannitol (0.256 µCi/ml). One soleus muscle from each mouse was placed in transport media for basal measurement and the second muscle in transport media plus insulin (13.3 nM). All incubations were performed in a shaking water bath at 29°C. After the final incubation muscles were flash frozen, weighed and digested for 10 min at 70°C with KOH (1.0 M, 0.5 ml) and then neutralized with HCl (1.0 M, 0.5 ml). Aliquots were collected for scintillation counting. Presence of <sup>14</sup>C-mannitol was used to correct for the amount of extracellular 2-DG that was nonspecifically taken up into the tissue but not transported into muscle cells. Counts were normalized to muscle weight.
Stable isotope tracer studies. The [1,2-$^{13}$C$_2$]-D-glucose tracer (Cambridge Isotope Laboratories Inc., Andover, MA, >98% isotopic purity and positional accuracy) was injected intraperitoneally (1 mg/g) 3 h prior to harvest of tissues. One and 2 h following tracer injection 50 µl of serum were collected from the tail vein. At 3 h animals were anesthetized and blood collected by cardiac puncture. Tissues were collected and frozen in liquid nitrogen after sacrifice. Serum was deproteinized and the derived glucose treated with hydroxylamine hydrochloride and acetic anhydride to create the aldonitrile pentaacetate derivative for GC/MS analysis. Glucose molecular ion and its positional isotopomers were monitored at the m/z328 ion cluster. Lactate was extracted by ethyl acetate after acidification with HCl and derivatized to its propylamine-hepafluoro-buty! ester form and the m/z328 (carbons 1-3 of lactate, chemical ionization) was monitored for the detection of m1 (recycled lactate through the pentose cycle) and m2 (lactate produced by glycolysis) for the estimation of glucose-triose cycling (16,17). Mass spectral data were obtained on the HP5973 mass selective detector connected to an HP6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA). The settings were as follows: GC inlet 230°C, transfer line 280°C, MS source 230°C, MS Quad 150°C. An HP-5 capillary column was used for glucose and lactate analysis. Mass spectral analyses were carried out by three independent automatic injections of 1 ml samples and accepted only if the standard sample deviation was less than 1% of the normalized peak intensity.

Acute insulin treatment and Western blotting for activation of Akt and AMPK. Age-matched (6-8 month) male mice were injected with insulin (0.75unit/kg) or saline. After 30 min, hindlimb muscle was collected and tissue homogenates prepared for Western blot analysis. Phosphorylation of Akt (Ser473), glycogen synthase kinase 3β (Ser21), acetyl-CoA carboxylase (Ser79) and AMPK (Thr172), and total Akt and AMPK were detected by immunoblotting (Cell Signaling Technology, Danvers, MA).

Quantitation of non-heme iron in isolated muscle tissue. Non-heme iron in muscle tissue was quantified as described (18). Briefly, muscle (10 mg) tissue was lysed in hydrochloric acid and trichloroacetic acid. After heating for one hour at 95°C, non-heme iron was released into the supernatant, where it was reacted with ferrozine in the presence of thioglycolic acid quantified by spectrophotometry.

Adipocyte preparation, sizing, and quantitation of muscle and adipocyte transcript levels by RT-PCR. After a 24 h fast, mice were sacrificed and hindlimb muscle and epididymal fat pads were dissected. Muscle was submerged in approximately 8-10-fold volume of RNA-Later (Ambion, Austin, Texas) and stored at −20°C. After homogenization, RNA extraction was performed according to the TRI Reagent manufacturer's protocol (MRC). Epididymal fat pads were digested with 4 mg/ml Collagenase type VIII (Sigma) in HBSS for 30 minutes in a rotating water bath (180 rpm) at 37°C. The adipocytes were strained through a 100 µm nylon mesh, washed with 10 ml HBSS and centrifuged at 800 rpm for 5 minutes. Adipocyte size was determined in aliquots of the isolated adipocytes fixed in osmium tetraoxide as described (19). For RNA preparation, the fat cake was transferred to a clean tube and the infranatant removed. RNA was extracted using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen) according to the manufacturer's protocol. First-strand cDNA synthesis was carried out using Superscript III reverse transcriptase (Invitrogen, Carlsbad, California) according to the manufacture's protocol, with 3.0 µg of RNA and 240 ng of oligo-dT (Invitrogen) in 20 µl. Real-time PCR was performed with a thermal cycler (LightCycler, Roche Diagnostics) as described (20,21). Primers were designed using Primer 3 software (http://primer3.sourceforge.net). Reactions (10 µl) were performed using 8 ng cDNA. For each transcript, analyses of the melting curves and visualization after agarose gel electrophoresis confirmed the absence of non-specific products. Quantitation of cDNA products was accomplished by the LightCycler software. Standard curves of log cDNA amount vs. crossing point cycle number were constructed for each run. The following primers were used (designed using Primer3 Software): adiponectin (AdipoQ) 5'-GGAACTTTGCAAGTTGGAT-3' and 5'-GCTTCTCCAGGCTCTCTT-3', cyclophilin A (Ppia) 5'-AGCAGTGAGAAAGATTGG and 5'-TCTTCTTGGTGCTCGCCATT, and
ribosomal protein L13a (Rpl13a) 5'-GGAGAAACGGAAGGAAAAGG and 5'-ACAGGAGCAGTGCCCTAAGGA. Sample concentrations were calculated automatically by the LightCycler software using the second derivative maximum method. AdipoQ levels were normalized to the geometric mean of the Ppia and Rpl13a levels for the same sample. All samples were repeated in triplicate for each primer set.

Statistical procedures. Descriptive statistics are represented as average ± standard error of the mean. The student t-test (two tail) was used to compare differences between groups.

RESULTS

Improved glucose tolerance in Hfe<sup>−/−</sup> mice. Glucose tolerance was assessed in male mice aged 6-8 months, when significant iron has accumulated in tissues (11). The phenotypes of the 129SvEvTac strain of Hfe<sup>−/−</sup> mice have been described and include decreased serum glucose excursions after challenge despite decreased insulin levels (11). We first replicated these findings in a separate cohort (Fig. 1A) to allow comparison to the C57BL/6J strain as described below. Fasting glucose values were 17% lower (p<0.01, see also Table 3 below) and total and incremental areas under the glucose curve (AUC<sub>G</sub>) were respectively 27% and 56% lower in the 129SvEvTac Hfe<sup>−/−</sup> mice compared to wild types (not shown, p<0.0001). The differences in glucose tolerance were not accounted for by differences in body weight (normal chow: wild type 28.7 ± 0.4g, Hfe<sup>−/−</sup> 28.5 ± 0.4g). No differences in body composition were detected by dual photon absorptiometry; specifically, the fat mass was 15 ± 4% in the wild types and 15 ± 2% in the Hfe<sup>−/−</sup> mice (not shown).

To examine whether the enhanced glucose tolerance is strain-specific, we crossed the Hfe<sup>−/−</sup> knockout onto the diabetes- and obesity-prone C57BL/6J strain. The glucose tolerance of the wild type C57BL/6J mice is worse than that of the wild type 129SvEvTac strain, but the Hfe<sup>−/−</sup> mutation also resulted in improved glucose tolerance in the C57BL/6J strain (Fig. 1B). There was a 34% decrease in the total AUC<sub>G</sub> and a 52% decrease in the incremental AUC<sub>G</sub> in the C57BL/6J/Hfe<sup>−/−</sup> mice (not shown, p=0.001). Fasting glucose was 18% lower in the C57BL/6J/Hfe<sup>−/−</sup> compared to wild types (p=NS). Body weights did not differ between the C57BL/6J Hfe<sup>−/−</sup> and wild types (wild type 22.0 ± 0.6g, Hfe<sup>−/−</sup> 21.0 ± 0.4g).

Increased glucose disposal rates and insulin sensitivity in Hfe<sup>−/−</sup> mice. Our previously published data demonstrate that improved glucose tolerance is maintained in the face of lower insulin levels, suggesting that the Hfe<sup>−/−</sup> mice are more insulin sensitive (11). We therefore performed euglycemic clamp studies in the 129SvEvTac strain. The glucose disposal rate (GDR) of Hfe<sup>−/−</sup> mice at submaximal (5 mU/kg/min) insulin was 36% higher than in wild types (p<0.05). In the Hfe<sup>−/−</sup> mice the GDR did not increase further at maximal (10 mU/kg/min) insulin, whereas wild type mice showed a 20% increase in glucose disposal rate (GDR) at 10 mU/kg/min insulin compared to 5 mU/kg/min insulin (Fig. 2, p=0.05). Wild type and Hfe<sup>−/−</sup> mice had comparable GDRs at maximal insulin.

The increased insulin sensitivity seen in the 129SvEvTac strain was also present in the C57BL/6J/Hfe<sup>−/−</sup> mice. The insulin resistance indices determined by HOMA-IR were significantly lower in both strains of Hfe<sup>−/−</sup> mice compared to wild types of that same strain (Table 1). The magnitude of the effect of Hfe deletion was greater in the C57BL/6J than the 129 strain: 129SvEvTac/Hfe<sup>−/−</sup> showed a 25% reduction in HOMA-IR (p<0.05) and a 14% reduction (p=NS) in fasting insulin values compared to wild type controls, whereas the C57BL/6J/Hfe<sup>−/−</sup> showed a 50% reduction in HOMA-IR (p<0.05) and a 40% reduction in fasting insulin (p<0.05) compared to controls. We previously reported that post-challenge insulin levels are decreased in 129SvEvTac/Hfe<sup>−/−</sup> mice, and the same trend is seen in this smaller cohort for both 129SvEvTac and C57BL/6J strains (Table 1).

Increased glucose disappearance and glycolysis revealed by stable isotope tracer studies. We next determined the metabolic fate of absorbed [1,2-<sup>13</sup>C<sub>2</sub>]-D-glucose administered (as for glucose tolerance testing) intraperitoneally at 1 mg/g. Consistent with the glucose clamp data, Hfe<sup>−/−</sup> mice exhibited a 19% increase in [1,2-<sup>13</sup>C<sub>2</sub>]-D-glucose clearance compared to controls (Table 2, p<0.05). The metabolism of the [1,2-<sup>13</sup>C<sub>2</sub>]-D-glucose was further tracked and demonstrates increased glycolysis. In vivo metabolism of [1,2-<sup>13</sup>C<sub>2</sub>]-D-glucose to lactate produces two mass
We next investigated the insulin stimulation produced to a labeled, derived from the pentose cycle (one $^{13}$C substitution, [3-$^{13}$C$_1$]-lactate) produced by glycolysis (17). Absolute serum lactate levels were 2.6-fold higher in $Hfe^{-/-}$ mice (Table 2, $p=0.0001$), consistent with increased glycolysis. Levels of the $m1$ isotopomer (singly labeled, derived from the pentose cycle) were 2.7-fold higher in the $Hfe^{-/-}$ mice ($p<0.0001$) but were present in similar proportion to total lactate in wild type (13.2%) compared to $Hfe^{-/-}$ mice (13.7%). The $m2$ isotopomer product of glycolysis, however, was produced to a significantly greater degree in the $Hfe^{-/-}$ mice (5.1-fold higher in $Hfe^{-/-}$ compared to wild type mice, $p<0.001$) such that the proportion of total lactate derived from glycolysis was approximately doubled in the $Hfe^{-/-}$ mice (wild type: 4.4%; $Hfe^{-/-}$: 8.4%; $p=0.05$).

The $m1$ glucose isotopomer, in which $^{13}$C glucose occupies the carbon-1 position, is produced via metabolism of $m2$ glucose through the pentose cycle followed by gluconeogenesis using the derived $m1$ lactate as substrate. Therefore, hepatic glucose recycling can be monitored by the reappearance of $m1$ glucose. Consistent with the increased lactate production, the $Hfe^{-/-}$ mice exhibited a 36% increase in hepatic glucose recycling ($p<0.05$) as revealed by the appearance in serum of the $m1$ glucose isotopomer.

Increased basal but not insulin-stimulated glucose uptake into isolated soleus muscle from $Hfe^{-/-}$ mice. Because skeletal muscle is a major site for glucose disposal, accounting for approximately 75% of total glucose disappearance during a hyperinsulinemic clamp study (22), we investigated glucose uptake and hormone signaling in isolated soleus muscles from the wild type 129SvEvTac and 129SvEvTac $Hfe^{-/-}$ mice. We first verified that muscle from the $Hfe^{+/+}$ mice was iron-overloaded, with a 2.1-fold increase in non-heme iron (not shown, $p=0.02$). Isolated soleus muscles from $Hfe^{+/+}$ mice exhibited an 18% increase in 2-deoxyglucose uptake compared to controls in the absence of insulin ($p<0.03$). Controls did not differ from $Hfe^{-/-}$ mice in maximally insulin-stimulated uptake (Fig. 3).

Expression of mRNAs for the glucose transporters GLUT1 and GLUT4 were not altered in the $Hfe^{-/-}$ muscle and Western blotting revealed no change in GLUT1 or GLUT4 protein (not shown).

Increased adiponectin and AMPK signaling in $Hfe^{-/-}$ mice. We next investigated the basis for the observed increased glucose tolerance, glucose disposal, and insulin sensitivity of the $Hfe^{-/-}$ mice by measuring a series of fasting serum hormone and metabolite levels in males of the 129SvEvTac strain (Table 3). The only statistically significant differences noted were in fasting glucose and serum adiponectin, with adiponectin levels being 41% higher in the $Hfe^{-/-}$ mice ($p<0.01$). Triglycerides and free fatty acids trended lower in the $Hfe^{-/-}$ mice, but not significantly. Intramyocellular lipid levels were also unchanged in the $Hfe^{-/-}$ mice (not shown).

The increased adiponectin was attributable to an approximately 2-fold up regulation of adiponectin mRNA levels in isolated primary adipocytes from epididymal fat pads of male 129SvEvTac mice (Fig. 4A). Because of the inverse association of adiponectin levels with obesity (23) and adipocyte size (24), we also measured adipocyte size in cells isolated from epididymal fat pads. Adipocytes from $Hfe^{-/-}$ mice were 27% smaller in diameter (Fig. 4B, $p<0.005$), despite the absence of a change in total fat mass (see above).

Because of the decreased HOMA-IR in the $Hfe^{-/-}$ mice, we examined insulin signaling. Phosphorylation of Akt, however, did not differ between wild type and $Hfe^{-/-}$ mice, neither in the basal state nor after in vivo insulin stimulation (Fig. 5A). Likewise, no changes were observed in the basal and insulin-stimulated phosphorylation of the Akt target glycogen synthase kinsase 3β ($\beta$-GSK3 $\beta$). Because of the known effect of adiponectin to activate AMPK, we also examined phosphorylation of AMP-dependent kinase. Phosphorylation of AMPK was increased 81% in muscle from $Hfe^{-/-}$ mice (Fig. 5B and C, $p=0.01$) without a change in total AMPK protein. We verified activation of AMPK was by examining phosphorylation of a downstream target of AMPK, acetyl-CoA carboxylase (ACC). It too showed increased phosphorylation in muscle from $Hfe^{-/-}$ mice (Fig. 5B and C, $p<0.05$).

**DISCUSSION**
We previously reported that prediabetic humans with hereditary hemochromatosis exhibit decreased insulin secretory capacity that tends to be compensated by increased insulin sensitivity (10,12). Diabetes develops in 23% of humans with hemochromatosis, but usually only in the setting of obesity, suggesting that the increased insulin demands of obesity cannot be met because of relative insulin deficiency (10). Our studies of a mouse model of hereditary hemochromatosis with inactivation of the \( Hfe \) gene have revealed a similar phenotype: Decreased insulin secretion due to apoptotic loss of beta cell mass, decreased glucose-responsiveness, and increased oxidative stress in the affected islets (11) and mitochondrial dysfunction (H. Jouihan and D.A. McClain, unpublished data). Similar to most normal-weight humans with hemochromatosis, the mice did not develop diabetes and instead exhibited supranormal glucose tolerance. We report herein that the enhanced glucose tolerance is observed in 129SvEvTac and in C57BL/6J mice, a strain prone to diabetes and obesity. The \( Hfe^{-/-} \) mice and their isolated muscles exhibit increased glucose disposal rates at submaximal but not maximal insulin and increased rates of glycolysis. Increased muscle glucose uptake cannot be accounted for by increased insulin signaling but is instead accompanied by increased activation of AMP-dependent kinase (AMPK). In addition, adiponectin production by adipocytes is increased, consistent with the increased AMPK activation.

Although we have not demonstrated directly that the activation of AMPK is mediated by adiponectin, the increased glucose uptake and glycolysis in the \( Hfe^{-/-} \) mice are consistent with the reported actions of adiponectin and activation of AMPK which result in increased translocation of the glucose transporter GLUT4 (25-30). The mechanism by which adiponectin activates AMPK is not yet completely understood, nor do the current data rule out other mechanisms by which AMPK might be activated. For example, the beta cell phenotype in the \( Hfe^{-/-} \) mice—decreased glucose stimulated insulin secretion and apoptosis—is consistent with mitochondrial dysfunction (11), and we have observed increased oxidative stress and decreased substrate oxidation in isolated mitochondria from livers of \( Hfe^{-/-} \) mice (H. Jouihan and D.A. McClain, unpublished data). Thus, mitochondrial dysfunction might also contribute to activation of AMPK by causing increased AMP/ATP ratios. The association of mitochondrial defects caused by iron with increased insulin sensitivity would be somewhat surprising in that mitochondrial dysfunction has been hypothesized to be responsible for aspects of the metabolic syndrome including insulin resistance and diabetes (31). Our findings therefore suggest either: (a) Modest mitochondrial dysfunction may contribute to mechanisms such as the activation of AMPK that could, at least under circumstances of normal fuel intake, protect from diabetes as has been suggested by population studies of mitochondrial gene mutations (32), (b) the effects of iron in impairing mitochondrial function may only be seen in selected tissues with high levels of iron uptake or with high degrees of dependence on mitochondrial metabolism (such as islets), or, (c) the effects of iron on insulin sensitivity and metabolism represent regulatory responses to iron—for example the upregulation of adiponectin synthesis—that are separate from iron’s “toxic” effect on mitochondrial function. The multiple effects of iron on production of insulin and other hormones and on fuel handling in different tissues are complex and the observed phenotype of iron-overloaded mice is not likely to be explained on the basis of a single event. Further studies are underway to characterize mitochondrial function under situations of iron overload in different tissues and its contribution to the metabolic changes seen in the \( Hfe^{-/-} \) mice.

The mechanism for the up regulation of adiponectin in the \( Hfe^{-/-} \) mice is not clear. Adiponectin secretion is inversely correlated with adiposity (23) and adipocyte size (24) although the mechanisms underlying that association are also not known. The \( Hfe^{-/-} \) mice do have smaller adipocytes, however overall fat mass as measured by dual photon absorptiometry is unchanged. Why their adipocytes are smaller is not clear and testing mRNA levels of an extensive array of nuclear proteins known to be involved in adipocyte differentiation and function has not suggested potential mechanisms (not shown). It is known that iron stimulates lipolysis in isolated adipocytes, and the inhibition of this effect by acetyl-L-cysteine suggests that it is signaled by oxidant stress rather than directly by iron (33). Adiponectin secretion itself, however, is negatively regulated by oxidant stress (34,35), so
there may exist competing signals that regulate adiponectin, perhaps as a function of the degree of iron overload. The details of these signaling pathways linking iron, adipocyte size, and adiponectin secretion remain to be elucidated.

The observed changes in metabolism in the Hfe<sup>−/−</sup> mice are consistent with the close coupling of metabolism with iron availability in lower organisms. In yeast, for example, stimulation of AMPK regulates both glucose oxidation and iron uptake (36). Less is known about iron regulation of metabolism in higher organisms, although the data that demonstrate regulation of mammalian adipocyte lipolysis by iron suggest that at least some of the regulation by iron observed in yeast may be conserved (33). A significant role for iron in the metabolic regulation of higher organisms would therefore not be surprising and warrants further study.

In summary, we have demonstrated here and in previous work that iron has a multitude of effects on metabolism that both predispose and protect from diabetes (10,11). Excess iron results in decreased insulin secretion, but also results in increased glucose uptake into skeletal muscle and increased glycolysis mediated at least in part by activation of AMPK. These effects are important in considering the phenotype of individuals with hereditary hemochromatosis, and may also be relevant to the reported relationships between iron and the risk of diabetes and the metabolic syndrome in the non-hemochromatotic population (6-9).

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FOOTNOTES

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†Both authors contributed equally to this manuscript.
The abbreviations used are: AMPK, AMP-dependent kinase; HOMA-IR, homeostasis model assessment of insulin resistance; 2-DG, 2-deoxy-D-glucose.

FIGURE LEGENDS

Fig. 1. Glucose tolerance testing of 129SvEvTac and C57BL/6J strains of Hfe<sup>−/−</sup> mice. Male mice aged 6-8 months were studied after 8 weeks of either a normal chow or high iron diet. A. Glucose excursions after intraperitoneal glucose tolerance testing in the 129SvEvTac strain, Hfe<sup>−/−</sup> and wild type (N=9 or 10 per group). All glucose values at each time point differ significantly (p<0.05) between wild type and Hfe<sup>−/−</sup> mice as do areas under the glucose curve (see text). B. Glucose tolerance testing of the C57BL/6J strain of Hfe<sup>−/−</sup> and wild type mice (5-8 per group). Glucose values at 5, 30, 60, and 120 min differ between wild type and Hfe<sup>−/−</sup> mice (p<0.05) and area under the glucose curve differs significantly between the Hfe<sup>−/−</sup> mice and wild types (see text).

Fig. 2. Euglycemic clamp studies of 129SvEvTac/Hfe<sup>−/−</sup> mice and wild type mice. Male mice aged 6-8 months (N=6/group) were studied on normal chow diets. Euglycemic glucose clamps were performed at insulin infusion rates of 5 and 10 mU/kg/min. Total glucose disposal rates (glucose infusion plus endogenous glucose production) are shown, with hepatic glucose production having been determined by isotope dilution. * p<0.05

Fig. 3. Increased basal but not insulin-stimulated glucose uptake into isolated soleus muscle of Hfe<sup>−/−</sup> mice. Soleus muscle from 6-8 month-old male 129SvEvTac wild type and Hfe<sup>−/−</sup> mice (5/group) was exposed to [3H] 2-deoxy-D-glucose in the presence or absence of 13 nM insulin. Results for glucose uptake are corrected for extracellular tissue absorption using mannitol and normalized to muscle weight. *p<0.05

Fig. 4. Adiponectin mRNA and adipocyte size in isolated adipocytes from 129SvEvTac wild type and Hfe<sup>−/−</sup> mice. A. Adiponectin mRNA levels were quantified by RT-PCR and normalized to Ppia and Rpl13a levels (N=4, p=0.04). B. Adipocyte size was determined in isolated adipocytes fixed in osmium tetroxide (N=5 collagenase digestions of each mouse line, with 20-40 cells counted for each digestion, p<0.01).

Fig. 5. Activation of Akt and AMPK in hindlimb muscle tissue of 129SvEvTac wild type and Hfe<sup>−/−</sup> mice. A. Insulin stimulation of Akt phosphorylation. Age-matched (6–8 months old) male 129SvEvTac wild type and Hfe<sup>−/−</sup> mice were injected with insulin (0.75unit/kg) or saline. After 30 min, muscle tissues were collected and tissue homogenates were prepared for Western blot analysis. Phosphorylation of Akt (Ser473), phosphorylation of the Akt substrate glycogen synthase kinase 3β (pGSK3β), and total Akt in a typical experiment is shown. B. AMPK phosphorylation and activation in 129SvEvTac wild type and Hfe<sup>−/−</sup> mice. Hindlimb muscle was collected from age-matched (6–8 months old) male 129SvEvTac wild type and Hfe<sup>−/−</sup> mice. Phosphorylated AMPK (Thr172), total AMPK, and phosphorylation of the AMPK substrate acetyl-CoA carboxylase (pACC) were detected by Western blotting. C. Quantification of AMPK phosphorylation by densitometry. Levels of phosphorylated AMPK were normalized to total AMPK within each of three independent experiments. Total AMPK did not differ between wild type and mutant mice. Data are expressed as the mean ± S.E. * p<0.01, † p<0.05 for Hfe<sup>−/−</sup> compared to wild type.
Table 1: Serum insulin levels and HOMA-IR values in wild type and Hfe<sup>−/−</sup> mice.

| Strain/treatment     | Fasting serum insulin (ng/ml) | Serum insulin, 30 min post GTT (ng/ml) | HOMA-IR  |
|----------------------|-------------------------------|-----------------------------------------|----------|
| 129SvEvTac           | 0.29 ± 0.03                   | 0.37 ± 0.03                             | 1.60 ± 0.14 |
| 129SvEvTac/Hfe<sup>−/−</sup> | 0.25 ± 0.03                   | 0.30 ± 0.03                             | 1.35 ± 0.14* |
| C57BL/6J             | 0.40 ± 0.06                   | 0.42 ± 0.09                             | 2.31 ± 0.42 |
| C57BL/6J/Hfe<sup>−/−</sup> | 0.24 ± 0.02 *                 | 0.32 ± 0.02                             | 1.15 ± 0.09† |

*p<0.05 or †p<0.01 for Hfe<sup>−/−</sup> compared to wild type control of the same strain. All mice were males aged 5-7 months. HOMA-IR was calculated as (fasting glucose [mmol/l] x fasting insulin [microU/ml])/22.5.
Table 2: Metabolic fate of glucose after intraperitoneal challenge with [1,2\(^{13}\)C\(_2\)]D-glucose in 129SvEvTac wild type and Hfe\(^{-/-}\) mice.

| Serum analyte                                      | Wild type       | Hfe\(^{-/-}\)  |
|----------------------------------------------------|----------------|---------------|
| Plasma glucose clearance (loss of double-labeled glucose, %) | 22.1 ± 0.9      | 26.3 ± 1.7*   |
| Total lactate (total lactate peak heights, 120 min)  | 282 ± 14        | 743 ± 15 ‡    |
| Lactate from pentose cycling (m1 isotopomer, peak height, 120 min) | 37.3 ± 2.0      | 102.3 ± 2.0   |
| Percent of lactate from pentose cycling             | 13.2%           | 13.7%         |
| Lactate from glycolysis (m2 isotopomer, peak height, 120 min) | 12.3 ± 0.8      | 62.8 ± 1.2 ‡  |
| Percent of total lactate from glycolysis            | 4.4%            | 8.4% *        |
| Glucose recycling (singly/double labeled glucose, %/hr) | 11.8 ± 0.5      | 16.0 ± 0.8*   |

N=3 per group, each measured in triplicate. * p<0.05 ‡ p<0.0001 for Hfe\(^{-/-}\) vs. wild type.
Table 3: Fasting serum chemistries in *129SvEvTac* wild type and *Hfe<sup>—/—</sup>* mice.

|                          | Wild type     | *Hfe<sup>—/—</sup>* |
|--------------------------|---------------|---------------------|
| Glucose (mg/dl)          | 103.7 ± 2.1   | 85.8 ± 1.1 *        |
| Insulin (ng/ml)          | 0.29 ± 0.03   | 0.26 ± 0.03         |
| Adiponectin (µg/ml)      | 6.8 ± 0.4     | 9.6 ± 0.5 *         |
| Glucagon (pg/ml)         | 77.4 ± 5.1    | 86.1 ± 9.7          |
| Leptin (ng/ml)           | 7.1 ± 1.1     | 5.5 ± 1.5           |
| Resistin (ng/ml)         | 3.1 ± 0.4     | 3.6 ± 0.3           |
| Free T4 (µg/ml)          | 0.26 ± 0.02   | 0.32 ± 0.06         |
| Triglycerides (mg/dl)    | 52.3 ± 5.3    | 31.5 ± 8.6          |
| Free fatty acids (mM)    | 0.7 ± 0.1     | 0.5 ± 0.2           |

Fasting sera from 6-12 mice per group, all males aged 5-7 months, were analyzed. * p<0.01 for *Hfe<sup>—/—</sup>* vs. wild type.
Fig. 1

A

B

Blood Glucose (mg/dl)

Time (min)

Blood Glucose (mg/dl)

Time (min)
Fig. 2

![Graph showing GDR (mg/kg/min) for 129 Wild Type and 129 Hfe -/- mice with 5 and 10 mU doses.](image-url)

- * indicates a significant difference.

GDR (mg/kg/min)

129 Wild Type | 129 Hfe -/-
Fig. 3

![Bar chart showing 2-deoxyglucose uptake (pmol/mg/min) under Basal and Insulin conditions for 129 wild type and 129 hfe -/- genotypes. The chart indicates a significant difference (*) in uptake between the two genotypes under Insulin conditions.](http://www.jbc.org/)

2-Deoxyglucose uptake (pmol/mg/min)

- **Basal**
  - 129 wild type: 50 pmol/mg/min
  - 129 hfe -/-: 70 pmol/mg/min

- **Insulin**
  - 129 wild type: 90 pmol/mg/min
  - 129 hfe -/-: 110 pmol/mg/min

*Significantly different (p < 0.05).
Fig. 4

A

Relative Adiponection mRNA

129 Wild Type  129 Hfe-/-

B

Adipocyte Diameter (microns)

129 Wild Type  129 Hfe-/-
Fig. 5

A

|          | basal | + insulin |
|----------|-------|-----------|
| 129 WT   |       |           |
| 129 Hfe<sup>−/−</sup> |       |           |
| 129 WT   |       |           |

- pAkt (S473)
- pGSK3β
- Total Akt

B

- pAMPK
- Total AMPK
- pACC

C

|                | pAMPK | pACC |
|----------------|-------|------|
| 129 Wild Type  |       |      |
| 129 Hfe<sup>−/−</sup> | *    | †    |

* Significant difference
† Significant difference by comparison

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Increased glucose disposal and AMP-dependent kinase signaling in a mouse model of hemochromatosis

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