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ARTICLE

Increased insulin secretory capacity but decreased insulin sensitivity after correction of iron overload by phlebotomy in hereditary haemochromatosis

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Abstract
Aims/hypothesis We recently demonstrated that humans with hereditary haemochromatosis have decreased insulin secretory capacity with a compensatory increase in insulin sensitivity. We therefore determined how these measures change after correction of tissue iron overload.
Subjects and methods Five non-diabetic subjects who had been studied previously at the time of initial diagnosis by means of the OGTT and frequently sampled intravenous glucose tolerance tests (FSIVGTT) underwent phlebotomy to normalise their serum ferritin. After normalisation of ferritin they were studied again (33±4 months after the initial studies) by OGTT and FSIVGTT.
Results Normalisation of tissue iron stores resulted in an average 1.8-fold increase in the integrated area under the insulin curve during OGTT ($p<0.0001$), but no significant change in the area under the glucose curve (10% decrease, $p=0.32$). After phlebotomy, there was a 2.2-fold increase in insulin secretory capacity as determined by FSIVGTT (acute insulin response to glucose [AIRg], $p<0.02$) but a concomitant 70% fall in insulin sensitivity (Si, $p<0.05$). The disposition index (AIRg×Si) was unchanged (5% increase, $p=0.90$). BMI and fasting glucose were unchanged. At the time of diagnosis of haemochromatosis, four of the subjects had IGT. After normalisation of ferritin, two achieved NGT and two remained with IGT, despite 2.5- and 3.7-fold increases in insulin secretory capacity.
Conclusions/interpretation Insulin secretory capacity improves after normalisation of iron stores in subjects with hereditary haemochromatosis. Glucose tolerance status improves incompletely because of decreased insulin sensitivity after phlebotomy. We conclude that tissue iron levels are an important determinant of insulin secretion and insulin action.

Keywords Diabetes · Haemochromatosis · Impaired glucose tolerance · Insulin resistance · Insulin secretion

Abbreviations

AIRg	acute insulin response to glucose
FSIVGTT	frequently sampled intravenous glucose tolerance test
Si	insulin sensitivity

Introduction

Hereditary haemochromatosis is transmitted as an autosomal recessive trait and occurs in approximately 0.5% of Europids of Northern European descent [1, 2]. Most patients with haemochromatosis are homozygous for a single nucleotide substitution (C282Y) in the haemochromatosis gene (*HFE*) [3]. Normal HFE expression is required for the regulation of hepcidin, a small hepatic peptide whose expression is induced by increased

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hepatic iron and inflammation [4]. Failure to induce hepcidin in haemochromatosis results in unregulated entry of iron into the circulation from the gastrointestinal tract and macrophages [5].

There is controversy about the frequency of morbidity that accompanies haemochromatosis. In relatively small clinical studies, the prevalence of diabetes in haemochromatosis has been found to be in the range of 7–40% [6, 7]. We recently reported a significantly increased prevalence of diabetes (23%) and IGT (30%) in adult subjects with haemochromatosis compared with matched controls (0% diabetes and 14% IGT) [8]. These rates of diabetes agreed well with the prevalence of diabetes (26%) assessed by chart review of more than 200 subjects with haemochromatosis who had previously been studied by our group. Subjects with haemochromatosis and controls had similar insulin secretory capacity (acute insulin response to glucose [AIRg]) and insulin sensitivity (Si), as assessed by frequently sampled intravenous glucose tolerance testing (FSIVGTT). Haemochromatosis subjects with IGT exhibited a 68% decrease in AIRg compared with those with NGT. They were not insulin-resistant, instead exhibiting a 62% increase in Si. The subjects with diabetes were overweight (14%) or obese (86%), suggesting the possibility that secondary insulin resistance could not be compensated because of decreased insulin secretory capacity. The phenotype of these subjects was mirrored in a mouse model of hereditary haemochromatosis with targeted deletion of *Hfe* or replacement of deleted wild-type *Hfe* with the cognate human mutation C282Y [9]. These mice exhibited decreased insulin secretory capacity secondary to oxidative stress, decreased glucose-stimulated insulin secretion and beta cell apoptosis. Like the humans with haemochromatosis, the mice also had increased Si and they did not develop diabetes on a normal chow diet.

We therefore sought to determine the response of these subjects to phlebotomy and normalisation of tissue iron stores. We report that insulin secretory capacity increased significantly, but a parallel decrease in Si resulted in incomplete resolution of abnormalities in glucose tolerance.

Subjects and methods

Subjects

The study participants were recruited from all consecutive referrals to the Hemochromatosis Research Clinic at the University of Utah School of Medicine from 2000 to the present. All of these referred patients agreed to serve in the study and informed consent was obtained from those who agreed. *HFE* genotyping was performed using allele-specific PCR primers as previously described [8]. Five non-diabetic subjects who underwent study at the time of diagnosis agreed to repeat studies after normalisation of iron stores, as determined by serum ferritin, although one later declined the intravenous glucose tolerance test. The characteristics of this study population are shown in Table 1. All subjects' ferritin values were above normal before phlebotomy and were normalised by phlebotomy, although the change was not statistically significant because of the wide range of initial ferritin values (1230–14300 pmol/l). The average interval between the pre- and post-phlebotomy studies was 33±4 (range 18–41) months. The average interval from normalisation of serum ferritin to the post-phlebotomy study was 20±5 (range 7–36) months. The study was approved by the University of Utah Institutional Review Board and the Advisory Committee to the General Clinical Research Center.

Clinical studies

After a 12-h overnight fast, an intravenous catheter was placed and subjects received an OGTT using a 75-g oral glucose load. Glucose and insulin values were determined at 0, 30, 60, 90 and 120 min. AUCs were determined assuming linear changes between time points. Glucose tolerance status was defined according to WHO criteria [10]. Insulin was assayed by radioimmunoassay using a kit that has 20% cross-reactivity with proinsulin (Diagnostic Products, Los Angeles, CA, USA). The next morning, also after a 12-h fast, the subjects underwent an intravenous glucose tolerance test [11]. The subjects were given a 300 mg/kg glucose load over 1 min; blood samples for insulin and glucose determination were collected at intervals of 1 min for 6 min, then at 2-min intervals until 18 min. At 20 min, 0.03 U/kg of regular insulin was infused over a 1-min interval. Blood was then drawn at 1-min intervals for 4 min, then every 10 min until a total of 180 min had elapsed. Results were analysed using Minmod software [12] to determine AIRg, a measure of insulin secretory capacity, and Si.

Table 1 Characteristics of the study population (n=5)

	Before phlebotomy	After phlebotomy
Age (years)	49.0±4.6	50.8±4.9
Genotype	100% C282Y/C282Y	–
Sex	Two female, three male	–
BMI (kg/m^2)	24.7±2.1	24.3±1.5
Ferritin (pmol/l)	4955±2386	162±11
Glucose tolerance status	NGT n=1, IGT n=4	NGT n=3, IGT n=2
Fasting glucose (mmol/l)	4.87±0.12	4.98±0.08

Statistics

Statistical analyses were performed using SPSS software (SPSS, Chicago, IL, USA). Pre- and post-phlebotomy variables were analysed by paired *t* test.

Results

Oral glucose tolerance testing

Fasting glucose values were unchanged after phlebotomy (Table 1). The results of OGTT are shown in Fig. 1. Although two of the subjects who were originally glucose-intolerant exhibited normal glucose tolerance after phlebotomy, two others remained glucose-intolerant and there was no significant change in the average AUC for serum glucose. The insulin AUC, however, uniformly and significantly increased by an average of 1.8-fold ($p<0.0001$).

Changes in insulin secretion and insulin sensitivity assessed FSIVGTT

The OGTT data, namely an increase in insulin levels without a comparable decrease in glucose excursions, suggested increased insulin secretory capacity but decreased insulin sensitivity as a result of phlebotomy. We therefore performed FSIVGTT to better quantify these variables. Testing confirmed a significant, 2.2-fold increase in insulin secretion (Fig. 2a; AIRg, $p<0.02$) and a 70% decrease in insulin sensitivity (Fig. 2b; Si, $p<0.05$). The disposition index, the product of AIRg and Si, showed a variable response to phlebotomy, with no average change (Fig. 2c) but large variance among the subjects. The two subjects who remained glucose-intolerant after phlebotomy did so despite 2.5- and 3.7-fold increases in AIRg; they exhibited 75 and 76% decreases in Si respectively, despite no change in BMI.

Discussion

Our previously published results demonstrate that hereditary haemochromatosis secondary to mutations in the *HFE* gene is accompanied by a significant prevalence of abnormalities in glucose homeostasis [8]. A substantial fraction of subjects, however, also had NGT, suggesting that there exist other genetic or environmental modifiers of the phenotype. At the stage of IGT, the primary abnormality appeared to be a decrease in insulin secretory capacity, which in a mouse model is associated with decreased beta cell mass and increased oxidative stress and apoptosis in the beta cells [9]. In both humans and in the mouse model, the loss of insulin secretory capacity was accompanied by increased insulin sensitivity. Thus, the mice with haemochromatosis did not develop diabetes on a normal chow diet, and in humans diabetes only occurred in the subpopulation that was overweight or obese [8, 9]. This suggests that secondary insulin resistance, resulting not

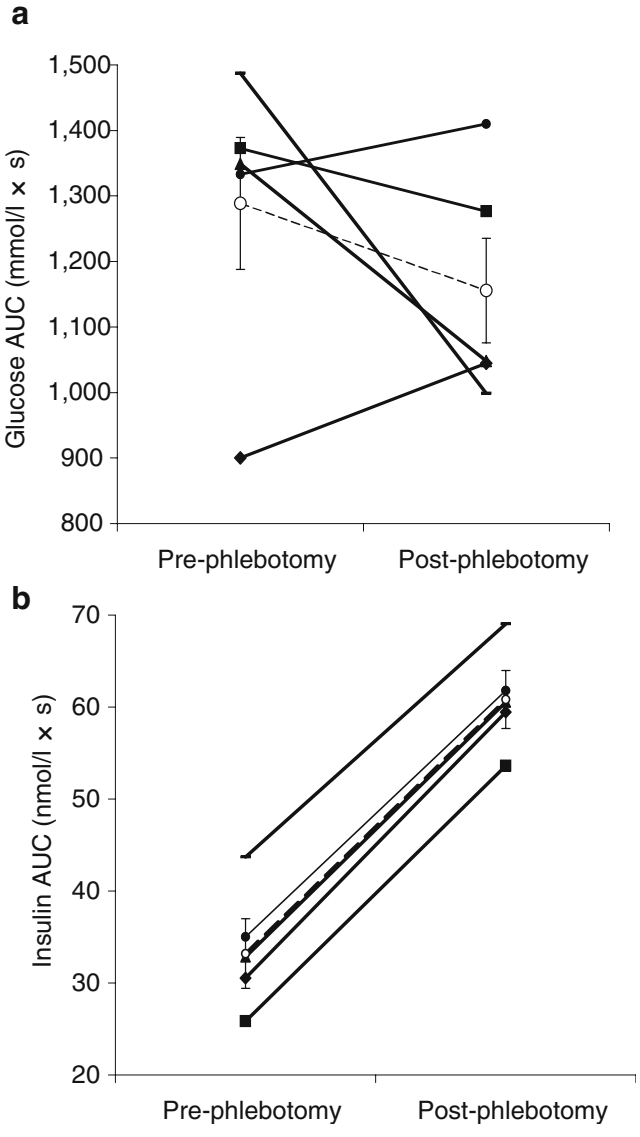
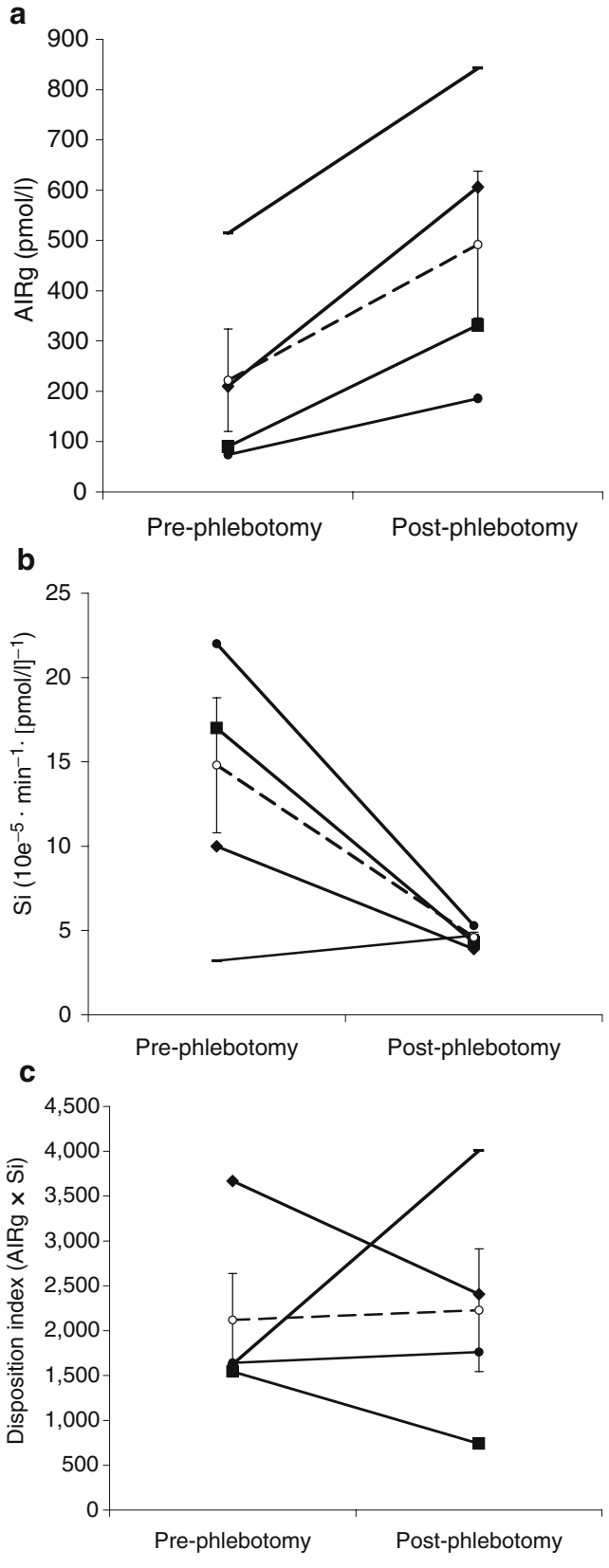


Fig. 1 Integrated areas under the serum glucose (**a**) and insulin (**b**) curves (AUC) after oral glucose tolerance testing. Values for each subject before and after phlebotomy are shown as *solid lines* with *closed symbols*, and the group average as the *dashed line* and *open circles* (±SE). Individual subjects are identified in this and subsequent figures with the different closed symbols: Subject 1, *closed squares*, IGT before and after phlebotomy; Subject 2, *closed circles*, IGT before and after phlebotomy; Subject 3, *closed triangles*, IGT before and NGT after phlebotomy; Subject 4, *closed diamonds*, NGT before and after phlebotomy; Subject 5, *horizontal bar*, IGT before and NGT after phlebotomy. $p=0.32$ for the 10% decrease in area under the glucose curve after phlebotomy; $p<0.0001$ for the increase in insulin values



◀ **Fig. 2** Insulin secretion, Si and glucose disposition indices in subjects with haemochromatosis before and after phlebotomy determined by the FSIVGTT. Values for each subject before and after phlebotomy are shown as *solid lines* and *closed circles* and the group average as the *dashed line* and *open circles* (±SE). Data were analysed by the Bergman minimal model using Minmod software [12]. Individual subjects are identified with the following symbols: Subject 1, *closed squares*, IGT before and after phlebotomy; Subject 2, *closed circles*, IGT before and after phlebotomy; Subject 3 depicted in Fig. 1 declined the FSIVGTT; Subject 4, *closed diamonds*, NGT before and after phlebotomy; Subject 5, *horizontal bar*, IGT before and NGT after phlebotomy. **a** Acute insulin response to glucose (AIRg, $p<0.02$ for the difference after phlebotomy). **b** Si, $p<0.05$ for the difference after phlebotomy. **c** Disposition index (DI, $p=0.9$)

from the haemochromatosis itself but from obesity and/or other factors, such as cirrhosis [8], is also a factor in the development of diabetes with haemochromatosis.

We therefore sought in the present study to determine whether this phenotype of decreased insulin secretory capacity with increased insulin sensitivity would be reversed by phlebotomy and normalisation of tissue iron levels. Five subjects, four of whom had IGT, were therefore restudied by both oral and intravenous glucose tolerance testing an average of 20±5 months after normalisation of serum ferritin. Both the area under the insulin curve during OGTT and the AIRg during FSIVGTT increased significantly after phlebotomy. However, the area under the glucose curve did not change significantly, and there was a decrease in Si as determined by FSIVGTT, such that the glucose tolerance status of two of the subjects with IGT did not improve despite 2.5- and 3.7-fold increases in AIRg.

The results suggest that there are complex inter-relationships among iron status, insulin secretion and insulin sensitivity. It might have been predicted, for example, that individuals with IGT based solely on an insufficient insulin secretory response would regain normal glucose tolerance with a 2- to 4-fold increase in insulin secretory capacity, but this did not occur. The results are more consistent with one or both of two possibilities: that insulin secretion rates control insulin sensitivity, and/or that iron is an independent factor in regulating both insulin secretion and insulin sensitivity. Regarding the former hypothesis, it is most often thought that insulin secretion responds to insulin sensitivity rather than vice versa. For example, the hyperinsulinaemia of obesity and early type 2 diabetes is thought to be a compensatory mechanism for insulin resistance [13]. However, there are also examples in human pathology and a wide variety of experimental models in which insulin secretion determines insulin sensitivity. That is, low levels of insulin secretion can be compensated to maintain NGT, and conversely primary hyperinsulinaemia can induce insulin resistance [14–18]. However, such a relationship between insulin secretion and insulin sensitivity does not explain the lack of improvement of IGT despite increased

insulin secretion. Analysis of mice with iron overload suggests instead that iron itself plays a central role in the regulation of metabolism and insulin sensitivity. We have noted, for example, increased glucose disposal rates, activation of AMP-dependent kinase (AMPK) and resistance to diet-induced obesity with increased thermogenesis in iron-overloaded mice (DA McClain and RC Cooksey, unpublished results). In lower organisms, especially in the yeast *Saccharomyces cerevisiae*, the connections between iron and metabolism are well established. For example, Snf1 (AMP-dependent kinase) is involved in signalling the shift from oxidative to fermentative metabolism and also controls the induction of iron transport genes [19]. In humans, the mechanisms for the regulation of insulin action and glucose homeostasis by iron are unknown, however.

The present data do not support insulin resistance as being a primary consequence of iron overload. Previous studies have suggested that both insulin deficiency and insulin resistance are contributing factors in the diabetes of haemochromatosis [20–22]. Some of this work is difficult to interpret because subjects with established diabetes were studied, in which case the attendant hyperglycaemia may itself have resulted in insulin resistance and insulin secretory abnormalities [23]. Furthermore, the studies most clearly demonstrating iron effects on insulin sensitivity in patients without diabetes are those of subjects with transfusional or idiopathic iron overload, and not of subjects with haemochromatosis [21, 24]. The tissue distribution of iron may differ significantly between dietary iron overload and haemochromatosis, and lead to different phenotypes of fuel homeostasis. For example, in dietary iron overload there is iron accumulation in macrophages, which is not the case in haemochromatosis because macrophages express ferroportin, and the lack of downregulation of ferroportin due to the deficiency of hepcidin allows free egress of iron from these cells [25]. This may explain why increased iron stores in the absence of haemochromatosis are associated epidemiologically with the development of typical type 2 diabetes rather than the insulin-sensitive phenotype reported here [26–29].

In summary, we report that after phlebotomy therapy to reduce iron overload in hereditary haemochromatosis, insulin secretory capacity improves significantly but insulin sensitivity decreases. The net result of these changes is that glucose intolerance may either resolve or remain in individual subjects. The results suggest that iron, more specifically the iron status of specific tissues, may play a key role in determining insulin secretion, insulin action and metabolic regulation.

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