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Subcutaneous Adipose Tissue from Obese and Lean Adults Does Not Release Hepcidin *In Vivo*

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Hepcidin is the main regulator of systemic iron homeostasis and is primarily produced by the liver but is also expressed, at the mRNA-level, in periphery tissues including the subcutaneous and visceral adipose tissue. Obesity is associated with elevated hepcidin concentrations and iron depletion suggesting that the exaggerated fat mass in obesity could contribute significantly to circulating hepcidin levels consequently altering iron homeostasis. The objective of this study was to determine if abdominal subcutaneous adipose tissue (AbScAT) releases hepcidin *in vivo* and if release is modified by obesity. Arterio-venous differences in concentrations of hepcidin were measured across AbScAT in 9 obese and 9 lean adults. Overall ($n = 18$), mean plasma hepcidin concentrations were significantly higher in arterialized compared to AbScAT venous samples [mean difference (arterialized-AbScAT venous plasma hepcidin) $= 4.9 \pm 9.6$ ng/mL, $P = 0.04$]. Net regional release was not calculated because mean venous plasma hepcidin concentrations were lower than mean arterialized concentrations indicating no net release. Significant correlations between AbScAT venous and arterialized plasma hepcidin concentrations with anthropometric variables were not observed. Findings from this vein drainage study suggest there is no net release of hepcidin from the AbScAT depot and thereby no ability to signal systemically, even in obesity.

KEYWORDS: hepcidin, obesity, adipose tissue, *in vivo* secretion, iron homeostasis

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1. INTRODUCTION

Hepcidin is a small peptide hormone that functions as the homeostatic regulator of systemic iron regulation and as the link between host defense and iron metabolism [1]. Hepcidin directs the flux of iron into plasma by regulating the ferroportin-1 (Fpn) exporter. Ferroportin-1 facilitates the export of iron from intestinal enterocytes, hepatocytes, reticuloendothelial macrophages, and the placenta [2]. When hepcidin binds to Fpn, the two proteins are internalized and degraded within lysosomes [3]. Sensing of circulating iron and iron stores is thought to occur in the liver which is the primary site of hepcidin production [2, 3]. Hepcidin produced by the liver is simultaneously regulated by iron stores, anemia, hypoxia, and inflammation. When body iron levels are elevated or inflammation or infection is present, liver hepcidin production is increased, resulting in diminished Fpn expression and decreased iron bioavailability (i.e., cellular iron sequestration and decreased dietary iron absorption) [2–4]. Conversely, when body iron levels are depleted or anemia or hypoxia exists, liver hepcidin expression is minimal, allowing for increased dietary iron absorption and mobilization from body stores via active Fpn transporters. However, at any given time, hepcidin expression can be determined by the interplay of these pathways and the relative strength of each of the individual signals [2–7]. The bioactive form of hepcidin, hepcidin-25, is measurable in human plasma, serum, and urine [8, 9].

Hepcidin is also expressed in other tissues including the heart, placenta, kidneys, and adipose tissue [4]. Expression within these tissues is believed to be regulated by hypoxia and inflammation and not body iron stores [4]. The impact of acute inflammation on hepcidin mRNA expression in these tissues has been studied in both surgical and injury models. In a recent study, subcutaneous adipose hepcidin mRNA expression and cytokines were significantly increased immediately following cardiac surgery compared to subcutaneous adipose explants assessed before surgery [10]. Also, in an animal model, acute myocardial infarction was associated with increased hepcidin mRNA expression in the ischemic myocardium [11]. However, it is not known if hepcidin expressed in these periphery tissues is actively secreted or has the potential to pose systemic impact.

Obesity is associated with a multitude of metabolic abnormalities, including altered iron homeostasis. In both adults and children, obesity is linked to diminished iron stores and impaired iron absorption despite adequate dietary iron intake [12–22]. Several researchers have demonstrated that serum levels of hepcidin are significantly elevated in obese compared to lean women and children [18, 19, 23], suggesting that hepcidin may play a significant role in iron dysregulation observed in obesity. As individuals become obese, their adipose tissue enlarges and undergoes a series of alterations, including increased production of several proinflammatory factors such as TNF-α and IL-6 [24]. Previous research has demonstrated that several of these proteins, referred to as adipokines, including IL-6 and leptin, are actively secreted by the adipose tissue and can act as endocrine signals bringing about systemic metabolic impact [25, 26]. Recently, Bekri et al. [27] reported that subcutaneous and visceral adipose hepcidin mRNA expression was significantly higher in obese compared to lean women, while liver mRNA expression was similar. This discovery led to the hypothesis that the altered systemic iron homeostasis and increased serum levels of hepcidin observed in obese individuals are, in part, a result of inflamed, exaggerated fat mass secreting bioactive hepcidin into circulation. However, to date, there are no data demonstrating *in vivo* release of hepcidin from human adipose tissue. Therefore, the objective of this study was to determine if abdominal subcutaneous adipose tissue (AbScAT) releases hepcidin *in vivo,* and if this release is modified by obesity.

2. METHODS

2.1. Subjects

Nine obese (body mass index (BMI) $\geq 30.0\,\text{kg/m}^2$) and nine lean (BMI: 18.5–$24.9\,\text{kg/m}^2$) adult volunteers were recruited from the greater Oxford community by advertisement or from the Oxford BioBank [28]. Participants attended the Oxford Center for Diabetes, Endocrinology and Metabolism Clinical Research

Unit at the National Institute for Health Research, Oxford Biomedical Research Center. The participants were fasted from 10 PM the night prior and asked to refrain from strenuous exercise and alcohol consumption for 24 hours before the study day. None of the subjects took medication known to affect iron or inflammatory status, and all were normoglycemic based on fasting glucose levels. The protocol was approved by the National Health Service/National Research Ethics Service, and all participants gave written, informed consent.

2.2. Anthropometric Measures

Subjects were asked to remove shoes, outdoor clothing, and excess items from their pockets before anthropometric measures were obtained. Weight was measured to the nearest 0.01 kg using an electronic scale. Height was measured to the nearest 0.5 cm using a wall-mounted stadiometer. Waist circumference was measured at the midpoint between the bottom of the rib cage and the iliac crest using a flexible tape measure.

2.3. Cannulas and Blood Sampling

Arterio-venous differences were measured across AbScAT. Under local anesthesia, a superficial epigastric vein draining AbScAT and an arterialized dorsal hand vein (with the hand kept in a warming box at 60°C) were cannulated [29]. The cannulae were kept patent with an intravenous infusion of 0.9% saline. After a resting period of ~45 min, blood samples were taken simultaneously from the two sites. Blood was drawn into lithium heparin syringes, and plasma was prepared rapidly at 4°C and stored at −80°C until analysis. Previous studies have shown that venous blood from epigastric veins represents the efflux from the adipose tissue bed, and arterio-venous differences across AbScAT yield results that are similar to microdialysis studies [25, 30].

2.4. Plasma Hepcidin Assay

Plasma hepcidin was determined using a competitive enzyme-linked immunosorbent assay at Intrinsic LifeSciences in La Jolla, Calif, USA. Detailed methods and performance of this assay have been published elsewhere [31]. The reference ranges for plasma hepcidin using this assay are 29–254 ng/mL in healthy men and 17–286 ng/mL in healthy women.

2.5. Blood Flow Measurements and Calculations

Abdominal subcutaneous adipose tissue blood flow was calculated from the washout of ^{133}Xe assuming a partition coefficient of 10 mL/g. This method is based on the principle that disappearance of ^{133}Xe is proportional to adipose tissue blood flow [32]. Abdominal subcutaneous adipose tissue plasma flow was calculated as the AbScAT blood flow * (1-hematocrit) [33]. Hematocrit was measured by microcapillary method.

If AbScAT venous plasma hepcidin concentrations are found to be higher than levels in arterialized samples (arterio-venous difference), the net regional release of hepcidin from the AbScAT (ng·100 g adipose tissue^{-1}· min^{-1}) will be calculated using the Fick principle, which is the product of the arterio-venous plasma hepcidin difference and AbScAT plasma flow (net regional hepcidin release = ([AbScAT venous plasma hepcidin] − [arterialized plasma hepcidin] * AbScAT plasma flow)) [25]. This value will be calculated for all subjects combined (overall), for the obese and lean groups separately, and on an individual basis.

2.6. Statistical Analysis

Demographic data are presented as means (with standard deviations) or frequencies. Anthropometric and biochemical data are presented as means (with standard deviations). Comparisons between the

TABLE 1: Demographic and clinical characteristics of Caucasian obese and lean adults ($n = 18$).

	Obese ($n = 9$)	Lean ($n = 9$)
Age (years)	45.8 ± 5.3	44.2 ± 7.9
Gender (male/female) [% (n)]	55.6 ($n = 5$)/44.4 ($n = 4$)	22.2 ($n = 2$)/77.78 ($n = 7$)
Body mass index (kg/m^2)†	36.8 ± 3.7	22.6 ± 1.4
Waist circumference (cm)†	115.1 ± 10.9	77.7 ± 5.9
Arterialized plasma hepcidin (ng/mL)	68.7 ± 48.7	62.0 ± 67.1
AbScAT venous plasma hepcidin (ng/mL)	64.7 ± 47.4	56.1 ± 57.3
Hematocrit (%)	41.4 ± 0.03	39.3 ± 0.05

All biochemical analysis was performed on fasted samples.
Data are presented as means $\pm$ standard deviations or as percentages.
Differences between groups were assessed using Student's t-test for continuous variables and Fisher's exact test for categorical variables.
†Differed significantly between obese and lean participants ($P < 0.0001$).
AbScAT: Abdominal subcutaneous adipose tissue.

arterialized and venous sites were assessed using paired t-tests, and differences between groups were assessed using Student's t-tests for continuous variables and Fisher's exact test for categorical variables. To determine if linear relationships existed between arterialized and venous plasma hepcidin concentrations and anthropometric variables, Spearman's correlation coefficients were calculated. All P values were two sided, and the type I error rate was set at 0.05. All analyses were performed using SAS software (version 9.2, 2008, SAS Institute Inc., Cary, NC, USA).

3. RESULTS

The demographic and clinical characteristics are presented in Table 1. All participants were identified as Caucasian. Mean age did not differ significantly between obese and lean participants (45.8 versus 44.2 years, resp.). Although there were more males in the obese compared to the lean group (obese: 55.6% ($n = 5$) versus lean: 22.2% ($n = 2$), resp.), the difference was not statistically significant. As expected, mean BMI and waist circumference were significantly greater (36.8 kg/m^2 versus 22.6 kg/m^2 and 115.1 cm versus 77.7 cm, resp.) in the obese group. Hematocrit was not statistically different between the obese compared to the lean group; however, when hematocrit was assessed by gender (data not shown), obese men had lower hematocrit compared to lean men (obese: 41.2 versus lean: 46.5%; $P = 0.48$) and obese women had higher hematocrit compared to lean women (obese: 41.8 versus lean: 37.3%; $P = 0.01$). Significant differences between obese and lean groups for AbScAT venous or arterialized plasma hepcidin concentrations were not observed. Also, no differences were observed for AbScAT venous or arterialized plasma hepcidin concentrations when assessed by obese and lean gender groups (data not shown).

Overall (all participants), postabsorptive concentrations of plasma hepcidin were significantly higher in the arterialized compared to the AbScAT venous samples (mean difference (arterialized−venous plasma hepcidin) = 4.9 ± 9.6 ng/mL, $P = 0.04$). When differences in arterialized and venous plasma hepcidin concentrations were assessed by group (obese and lean separately), venous concentrations were lower than arterialized (Figure 1), but these differences failed to reach statistical significance. This may be due to the small sample size within each group. Net regional hepcidin release was not calculated overall or by group because mean venous plasma hepcidin concentrations were lower than mean arterialized plasma concentrations indicating no net hepcidin release. However, in five subjects (Table 2), AbScAT venous concentrations of plasma hepcidin were higher than in the arterialized samples, although the reverse was true for a greater number of participants ($n = 12$, adipose tissue blood flow could not be determined for one lean female). Notably, there was no consistent pattern to the hepcidin arterio-venous difference with the obese individuals ($n = 3$) no more likely to show net release than lean participants ($n = 2$) (Fisher's exact test, $P = 0.99$); although women ($n = 4$) were more likely to show net release compared to men ($n = 1$), this
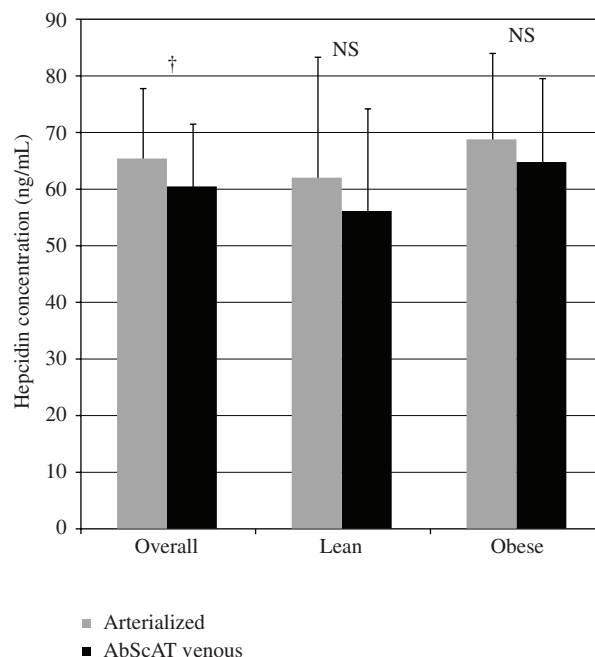
FIGURE 1: Postabsorptive arterialized dorsal hand vein and abdominal subcutaneous adipose tissue (AbScAT) venous plasma concentrations of hepcidin in obese ($n = 9$) and lean ($n = 9$) adults. Comparison between arterialized and venous sites was assessed using paired *t*-tests. †Significantly different at 0.05 level. NS: not significantly different at 0.05 level.

was not statistically significant (Fisher's exact test, $P = 0.60$). For those demonstrating net hepcdin release from AbScAT, release did not differ between the obese and lean individuals (obese: 9.8 ± 5.6 ng $\cdot$ 100 g adipose tissue$^{-1} \cdot$ min^{-1} versus lean: 9.9 ± 7.0 ng $\cdot$ 100 g adipose tissue$^{-1} \cdot$ min^{-1}; Student's *t*-test, $P = 0.98$). Significant correlations between AbScAT venous and arterialized plasma hepcidin concentrations with anthropometric variables were not observed (data not shown).

4. DISCUSSION

These data demonstrate, for the first time, that there is no overall net secretion of hepcidin, *in vivo*, by AbScAT, from fasted obese or lean individuals. Also, no significant correlations were observed between anthropometric measures and arterialized or venous plasma hepcidin concentrations. Collectively, these findings are somewhat surprising as subcutaneous and visceral adipose hepcidin mRNA expression has been reported to be significantly elevated in obese compared to lean women [27], and adipose-derived hepcidin was hypothesized to be associated, in part, with the increased hepcidin concentrations and the iron depletion observed in obesity [27]. Nonetheless, like IL-6 [34], hepcidin has a high turnover rate in plasma [35], and one would anticipate an arterio-venous difference across a tissue if the tissue was consistently and actively releasing significant amounts of the protein [25]. Further, in this study, when assessed overall, venous hepcidin concentrations were significantly lower than arterialized concentrations suggesting that net removal or degradation of hepcidin by AbScAT may be possible. Yet, arterio-venous studies cannot rule out the possibility that subcutaneous adipose-derived hepcidin could still have an autocrine or paracrine effect on tissue-level iron regulation, such as promotion of iron sequestration within the adipose tissue. This effect, however, may be minor considering that a recent study reported that in morbidly obese women, tissue iron content, assessed by Perl's Prussian blue stain, a semiquantitative technique, was minimal in AbScAT [18]. Additional studies including a quantitative approach to assessing adipose tissue iron content would

TABLE 2: Demographic, body mass index, arterialized plasma and abdominal subcutaneous adipose tissue (AbScAT) venous plasma hepcidin concentrations, and *in vivo* net regional hepcidin release from AbScAT in obese ($n = 9$) and lean ($n = 9$) subjects.

Gender	Age (years)	BMI (kg/m^2)	Arterialized plasma hepcidin (ng/mL)	AbScAT venous plasma hepcidin (ng/mL)	Net regional AbScAT hepcidin release (ng·100 g adipose $tissue^{-1} \cdot min^{-1}$)*
Male	51	36.4	9.9	7.5	−9.219912
Male	46	41.3	59.8	67.9	16.0832952
Male	48	41.5	140	133.6	−19.305216
Male	44	35.9	148.7	144.4	−17.088888
Male	40	30.2	76.2	59.1	−65.28951
Male	44	22.3	9	7.4	−5.53728
Male	32	24.3	119	99.3	−18.024318
Female	56	33.8	74.1	61.6	−9.828
Female	39	36.1	59.1	61.4	5.231787
Female	43	35.4	30.4	31.9	8.0784
Female	45	40.4	20.4	15.3	−17.136
Female	45	21.7	21	19.3	−1.978596
Female	57	21.3	93.9	84.9	NA
Female	41	24.7	90.6	93.5	4.871826
Female	56	23.9	6.4	6.2	−0.450996
Female	43	22.8	8.5	8	−1.96371
Female	40	21	12.9	19.2	14.829696
Female	40	21.6	196.3	167	−144.338832

All biochemical analysis was performed on morning fasting samples.
*Net regional hepcidin release = [(AbScAT venous plasma hepcidin] − (Arterialized plasma hepcidin)] ∗ AbScAT plasma flow.
BMI: body mass index; NA: not available due to unreliable adipose tissue blood flow calculation.

be needed to examine the autocrine and paracrine effect of adipose-derived hepcidin in obesity. Also, it is important to note that net secretion of hepcidin was evident in five individuals, but obese were no more likely to show net release compared to lean subjects. Also, no clinical features could clearly distinguish these individuals from those displaying no net secretion although four of the individuals were younger women. Additional larger scale studies are required to better understand this disparity. However, similar results have been reported for tumor necrosis factor-α (TNF-α), in which the researchers concluded that there was no overall net secretion of TNF-α from AbScAT in a group of obese and lean individuals. Although, like with hepcidin, a few subjects within the group did display net secretion, the authors reported that no clinical or demographic characteristics could explain why TNF-α net secretion was observed in these individuals [25].

Two previous studies [18, 27] have demonstrated that hepcidin mRNA expression in AbScAT and visceral adipose tissue from obese women did not differ significantly. However, it is well understood that adipose tissue is not homogenous [36]. Therefore, despite reporting that the AbScAT does not release hepcidin, we cannot exclude the possibility that the visceral adipose depot secretes hepcidin and has an influential role in systemic or localized iron regulation.

Collectively, previous research and findings from this study suggest that liver- and not adipose-derived hepcidin may be associated with the increased systemic hepcidin concentrations and the iron dysregulation observed in obesity. First, hepatic hepcidin mRNA expression was reported to be significantly

greater than that observed in AbScAT and visceral adipose tissue from obese women [18, 27]; in one study, it was reported to be more than 700 times greater [18]. Also, Tussing-Humphreys and colleagues [18] reported that liver hepcidin mRNA expression was positively correlated with serum hepcidin concentrations, whereas adipose mRNA expression was not. However, Bekri and colleagues [27] reported that liver hepcidin mRNA expression was similar in hepatic biopsies from obese and lean premenopausal women suggesting that the liver is not the source of increased hepcidin in obesity. But this observation is somewhat flawed as the iron status of the lean women was not described [27] and could have differed considerably from the obese women. As reported by Bekri et al. [27], iron status was depleted, based on transferrin saturation ($<20\%$), in 60% ($n = 15$) of the obese women. Matching the iron status of obese and lean subjects is critical as hepatic hepcidin expression is simultaneously regulated by body iron stores, inflammation, hypoxia, and anemia [2–6]. It is also critical that when matching on iron status, gender and age are also considered, as iron status differs between men and women and also with menopause [37]. Several studies have investigated the regulation of hepatic hepcidin by opposing stimuli and suggest that the strength of the individual stimuli, rather than the hierarchy of signaling pathways, determines hepatic hepcidin production [6, 7]. Thus, restricting a comparison group to healthy, noninflamed, lean men or women (limit to either premenopausal or menopausal) with similar iron status can allow one to evaluate the impact of the inflammatory signal on hepcidin expression in obesity. For example, in a previous study, in which obese and lean premenopausal women were matched on iron status (hemoglobin, transferrin saturation, and ferritin concentrations), median serum hepcidin concentrations (obese: 103.6 ng/mL versus lean: 16.3 ng/mL; $P < 0.0001$) and hepcidin regulation appeared to differ significantly between the two groups [18]. In the obese women, serum hepcidin was positively correlated with central adiposity and inflammation and weakly correlated with markers of iron status including transferrin saturation, serum transferrin receptor, and serum iron. Conversely, in the lean women, serum hepcidin was strongly correlated with the markers of iron status assessed. This suggests that in obesity, the inflammatory signal counterregulates the signal related to iron depletion which could lead to a modest increase in liver hepcidin production. Interestingly, serum hepcidin concentrations in obese individuals are similar to those reported in healthy, iron-replete individuals [18, 31]. Therefore, if the lean women assessed in the Bekri et al. [27] study were iron replete, it would not be surprising that their liver hepcidin mRNA expression was similar to the obese women and suggests that hepcidin expression in obesity is upregulated, despite underlying iron depletion. Hence, to confirm that liver-derived hepcidin is the source of increased systemic hepcidin concentrations in obesity, obese and lean individuals with similar iron status would need to participate in a hepatic vein catheterization study to assess if hepatic hepcidin release is significantly greater in obesity despite similar iron levels.

This study is not without limitation. First, although the sample size was relatively small and heterogeneous for age and gender, it was not unlike samples reported in other arterio-venous drainage studies investigating adipose tissue physiology [25, 38–40]. Also, the small sample size did not impact our ability to demonstrate that collectively there was no net secretion of hepcidin by AbScAT *in vivo* from obese and lean individuals as the mean AbScAT venous plasma hepcidin concentrations were significantly lower than arterialized samples, and secretion was not modified by obesity. However, the small sample size likely impeded our ability to observe a similar association when assessed separately within the obese and lean (Figure 1) as the sample size was reduced to only nine individuals per group. Second, this study does not rule out the possibility that AbScAT releases hepcidin at different times of the day or in response to food consumption, exercise, or starvation warranting further investigation. Finally, a comprehensive assessment of iron status and data pertaining to menopause and menstruation for females may have allowed for a more thorough characterization of the findings.

5. CONCLUSION

In summary, there was no overall net secretion of hepcidin by AbScAT from obese or lean individuals. The mechanism linking obesity to elevated hepcidin concentrations and iron depletion remains unclear

although it is attractive to speculate that liver-derived hepcidin stimulated by inflammation and not adipose-derived hepcidin plays an important role in this phenomenon. Additional research is required to confirm this hypothesis. Gaining an understanding of the relationship between obesity and diminished iron status, in light of the burgeoning obesity epidemic, is critical since the clinical significance of suffering simultaneously from both morbidities remains unknown [41].

ACKNOWLEDGMENTS

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