Vaspin promotes insulin sensitivity in elderly muscle and is upregulated in obesity

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Abstract

Adipokines have emerged as central mediators of insulin sensitivity and metabolism, in part due to the known association of obesity with metabolic syndrome disorders such as type 2 diabetes. Recent studies in rodents have identified the novel adipokine vaspin, as playing a protective role in inflammatory metabolic diseases by functioning to promote insulin sensitivity during metabolic stress. However, at present the skeletal muscle and adipose tissue expression of vaspin in humans is poorly characterised. Furthermore, the functional role of vaspin in skeletal muscle insulin sensitivity has not been studied. Since skeletal muscle is the major tissue for insulin-stimulated glucose uptake understanding the functional role of vaspin in human muscle insulin signalling is critical in determining its role in glucose homeostasis. The objective of this study was to profile the skeletal muscle and subcutaneous adipose tissue expression of vaspin in humans of varying adiposity and to determine the functional role of vaspin in mediating insulin signalling and glucose uptake in human skeletal muscle. Our data shows that vaspin is secreted from both human subcutaneous adipose tissue and skeletal muscle, and is more highly expressed in obese older individuals compared to lean older individuals. Furthermore, we demonstrate that vaspin induces activation of the PI3K/AKT axis, independent of insulin receptor activation, promotes GLUT4 expression and translocation and sensitises older obese human skeletal muscle to insulin-mediated glucose uptake.

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Introduction

The recognition that adipose tissue is an endocrine organ, capable of secreting a plethora of adipose-secreted cytokines (adipokines) that mediate tissue cross-talk, has transformed our understanding of metabolism, with implications for the treatment of metabolic diseases such as type 2 diabetes (Bluher 2014; Bluher and Mantzoros 2015). It has long been established that the accumulation of abdominal fat (central adiposity) is associated with insulin resistance (Carey, et al. 1996; Kahn and Flier 2000; Pratley, et al. 1995) and it is now known that in obese individuals the adipose tissue becomes inflamed, with infiltrated T-lymphocytes (Zeyda, et al. 2011) and macrophages (Weisberg, et al. 2003; Wentworth, et al. 2010; Zeyda, et al. 2007; Zeyda and Stulnig 2007) promoting the release of pro-inflammatory and anti-inflammatory adipokines from adipocytes (Tilg and Moschen 2006; Zeyda et al. 2007). In this regard, several studies have profiled adipokine expression and secretion in visceral fat tissue (VAT) and recent proteomic analysis of adipose tissue secretome has identified over 600 adipokines (Lehr, et al. 2012). In addition, adipokines are also secreted from subcutaneous adipose tissue (SAT) (Blaber, et al. 2012; Pellegrinelli, et al. 2015; Skurk, et al. 2007). Considering that SAT represents a large proportion of total adipose tissue (Rosqvist, et al. 2017; Rossi, et al. 2011) SAT-derived adipokines are likely important contributors to tissue cross talk and systemic inflammatory burden.

The functional role of the adipokines leptin and adiponectin as mediators of metabolic health and insulin sensitivity are well described. However, at present the functional role of many novel adipokines in regulating human insulin sensitivity and glucose handling is poorly understood (Nicholson, et al. 2018). Partly this is because despite skeletal muscle being the major organ for insulin-stimulated glucose
uptake (Thiebaud, et al. 1982), the expression and functional role of these adipokines in human skeletal muscle has not been studied (Nicholson et al. 2018). Given that age is a risk factor for the onset of type II diabetes (Kalyani, et al. 2017), understanding the effect of these different adipokines on skeletal muscle insulin sensitivity, particularly in older individuals, is important in determining the potential to target them therapeutically. Of the recently identified novel adipokines, one of the most interesting is vaspin (SERPINA12), a 47 kDa protein, which was first reported in the VAT of the genetically obese OLETF rat (Hida, et al. 2000). Importantly, several different animal models have recently implicated vaspin as an adipokine that functions to promote insulin sensitivity during metabolic stress. In a diabetic mouse model, administration of recombinant vaspin increased insulin sensitivity and glucose tolerance, whilst reducing the expression of pro-inflammatory adipokines including TNF-α and resistin and increasing the expression of adiponectin (Hida et al. 2000). Similar increases in insulin sensitivity have been reported in both db/db and C57BL6 mice following administration with recombinant vaspin (Heiker, et al. 2013), whilst transgenic mice overexpressing vaspin display improved glucose tolerance and are protected from obesity when challenged with a high fat diet (Nakatsuka, et al. 2012). Currently, very little is known with regards to the expression and secretory profile of vaspin in humans, or its functional role in human skeletal muscle tissue. However, serum concentrations of vaspin have been reported to be associated with insulin resistance in both non-diabetic and type 2 diabetic patient cohorts (Aust, et al. 2009; Jian, et al. 2014; Teshigawara, et al. 2012; Youn, et al. 2008), and its expression has been detected in the SAT of obese but not lean individuals (Kloting, et al. 2006), supporting the notion that vaspin is a key adipokine in mediating metabolic health and insulin sensitivity (Jian et al. 2014). Therefore, the aim of this study was to
104 examine the expression of vaspin in human skeletal muscle and subcutaneous adipose tissue in elderly individuals of varying adiposity, and to determine the functional effect of recombinant human vaspin on insulin signalling and glucose uptake in human skeletal muscle tissue.
Materials and methods

1. Human samples

Gluteus maximus skeletal muscle, subcutaneous adipose tissue (SAT) and blood samples were obtained from 21 lean (BMI 22.8 ± 0.3, age 71 ± 1.6 yr), and 17 obese (BMI 34.5 ± 0.9, age 66 ± 2 yr) elderly age-matched individuals undergoing elective hip-replacement surgery at the Royal Orthopaedic Hospital, Birmingham, UK, and Russell’s Hall Hospital, Dudley, UK. Diabetic patients and those taking anti-inflammatory medication within two weeks prior to surgery were excluded from the study. Skeletal muscle biopsies were also collected from n=7 young lean individuals. Informed consent was obtained from all patients prior to sample collection and full ethical approval for the study was obtained from the Research Ethics Committee (NRES 13/NE/0222) in line with the Declaration of Helsinki.

Sample collection, processing, storage and subsequent experimental procedures were carried out in compliance with Human Tissue Authority guidelines under the Human Tissue Act (2004). Serum was obtained from blood samples (5 ml) taken at the time of surgery by allowing them to clot at room temperature for 20-30 min, followed by centrifugation at 1620g for 10min. Patient characteristics are detailed in Table 1.

2. Isolation of primary human myoblasts and their differentiation into myotubes

Skeletal muscle samples (~200 mg) were sliced with a scalpel, placed in 5 ml of 0.05% trypsin-EDTA solution and rotated in an incubator (37°C, 5% CO₂) for 15 min. Trypsin was inactivated by the addition 5 ml of myoblast growth media (Ham’s F10 nutrient mix, supplemented with 20% HyClone™ research grade foetal bovine serum
and 1% penicillin and streptomycin). The solution was centrifuged for 5 min at 2000g and the resulting pellet re-suspended in 12 ml of myoblast growth medium before transfer to an uncoated T75 cell culture flask for 20 min. Finally, the solution was transferred to a T75 cell culture flask pre-coated with 0.2% gelatine. Myoblast growth medium was replaced every 48h.

For experimentation, myoblasts were grown to confluence (5-7 days) and differentiation into myotubes was induced by replacing myoblast growth media with differentiation media [Ham’s F10 nutrient mix (Sigma-Aldrich, Dorset, UK), supplemented with 6% horse serum (Sigma-Aldrich, Dorset, UK) and 1% penicillin and streptomycin]. Differentiation media was replaced every second day. All experiments were conducted on the 8th day of differentiation. Myotubes were immunostained (Figure 3A) for desmin as previously described (O'Leary, et al. 2017).

3. Generation of Adipose and Muscle Conditioned Media

SAT or skeletal muscle derived from lean and obese subjects was incubated at 37°C, 5% CO₂ in serum free culture media (Ham’s F10 nutrient mix, Sigma-Aldrich, Dorset, UK) at a ratio of 1g:10ml for up to 24 h. In the case of adipose tissue, larger samples were divided into segments of ~ 1 g to ensure that the surface area of adipose tissue exposed to medium remained approximately constant. For timecourse analysis of secreted vaspin, 50 µl aliquots of conditioned media were taken hourly for 6 h and then again at 24 h. Supernatants were removed and stored at -80°C for western blot analysis.
4. RNA extraction and gene expression analysis

Snap-frozen skeletal muscle and subcutaneous adipose tissue samples (~100mg) were first ground into a fine powder using a pestle and mortar under liquid nitrogen. Powdered tissue was immediately transferred to 1ml of Trizol® reagent (Invitrogen) and homogenised using a Qiagen Tissue Ruptor (Qiagen, Manchester, UK). Primary human myotubes were lysed in 1ml of TRlzol® reagent (Life technologies, Paisley, UK).

RNA was extracted as described in the manufacturer’s protocol. RNA was re-suspended in 30 µl of RNase free water (Life technologies, Paisley, UK) and heated at 55°C for 10 min. RNA was quantified using a Nanodrop 2000 (Life technologies, Paisley, UK). Relative mRNA expression was determined by Quantitative Real-Time Polymerase Chain (qRT-PCR), performed using PrecisionTM OneStepPLUS qRT-PCR (with SYBR-green) mastermix (Primerdesign, UK), 5 ng total RNA and primers as described in supplementary Table 1. All reactions were performed using a Biorad sfx cycler (BioRad).

5. Immunoblotting analysis

Aliquots of powdered skeletal muscle tissue, subcutaneous adipose tissue and primary human myotubes were lysed in RIPA buffer (Life technologies, Paisley, UK) containing Phosphatase Inhibitor Cocktail 3 (1:100, Sigma-Aldrich, Dorset, UK) and Protease Inhibitor Cocktail (1:100, Sigma-Aldrich, Dorset, UK). After addition of RIPA buffer, samples were placed on ice for 20 min. Total protein in cell and tissue lysates was quantified by performing a BCA assay (Life technologies, Paisley, UK) and protein samples prepared for gel electrophoresis by diluting in loading buffer consisting of, 2-mercaptoethanol (Sigma-Aldrich, Dorset, UK), 4x Laemmli Sample
Buffer and double distilled H$_2$O, followed by boiling for 10 min in a heat block. For adipose and muscle-conditioned media samples, 10 µl of conditioned media diluted in loading buffer was loaded per sample. For cell lysates, 10-15µg of protein was loaded per sample depending on the particular western blot.

Samples were loaded into a 5% stacking gel and separated by performing 12% SDS-page. Protein was transferred to a methanol-activated PVDF membrane (0.2 µm) using a Trans-Blot Turbo™ system (Bio-Rad, Hertfordshire, UK). Membranes containing protein samples from tissue and primary human myotubes were blocked with TBS-T containing 5% BSA. Membranes containing protein samples from serum and adipose conditioned media were blocked with 5% milk, 2% BSA, 0.5% H$_2$O$_2$.

Membranes were immuno-probed overnight at 4°C, with primary antibodies as described in supplementary Table 2, Followed by incubation for 2h at RT with the appropriate secondary antibody [HRP-linked anti-rabbit/ anti-mouse IgG secondary antibody (1:10,000; GE Healthcare, Buckinghamshire, UK)]. Blots were developed using Amersham ECL Prime (GE Healthcare, Buckinghamshire, UK), and imaged using a ChemiDoc™ MP System (Bio-Rad, Hertfordshire, UK) (Supplementary Figures 1-4). Densitometry analysis of bands was performed using an open-source, public domain software package (Image J v1.47).

6. Vaspin ELISA

Serum vaspin was measured using a commercially available human vaspin ELISA kit (AdipoGen Life Sciences), following the manufactures instructions. Serum samples were diluted 1:4 in ELISA buffer and the plate was read on a BioTek EL808 microtiter plate reader (BioTek, Swindon, UK).
7. MesoScale immunoassays

Myotubes were cultured in 96-well plates and stimulated with 10 nM human insulin (I0908, Sigma-Aldrich, Dorset, UK), 100 ng/ml of recombinant human vaspin (R&D systems, Minneapolis, USA) or 100 ng/ml recombinant human vaspin immediately followed by 10 nM insulin. Cells were lysed in 90µl MSD lysis buffer (MesoScale Discovery, Gaithersburg, Massachusetts) containing Phosphatase Inhibitor Cocktail 3 (1:100 dilution, Sigma-Aldrich, Dorset, UK) and a Protease Inhibitor Cocktail (1:100 dilution, Sigma-Aldrich, Dorset, UK). Insulin receptor, IGF1 receptor and AKT (thr308) phosphorylation, as a percentage of total protein, was determined using Mesoscale Discovery phospho and total protein assays, performed according to the manufacturer’s instructions and detected on the SECTOR Imager 6000 (MesoScale Discovery, Gaithersburg, Massachusetts).

8. Glucose uptake assays

Glucose uptake in primary human myotubes was quantified using either a radiometric or a fluorometric assay. For the radiometric assay, obese myotubes were cultured in 6-well plates and either left unstimulated or were pre-incubated for 24 h with vaspin (100 ng/ml). The next day myotubes were washed twice in warm PBS and incubated in serum-free media (Ham’s F10) for 2 h. Myotubes were washed a further 2 times before incubation with 2 ml reaction buffer (138 mM NaCl, 1.85 mM CaCl2, 1.3 mM MgSO4, 4.8 mM KCl, 50 mM HEPES pH 7.4, 0.2% (W/V) BSA) for 45 min at 37°C. Myotubes were then incubated with or without insulin (100 nM) for 30 min on a plate warmer at 37°C before the addition of 250 µL of 27.8 kBq [3H]-2-DG and 10 µM 2-DG at timed intervals to each well for 15 min. The buffer was aspirated with a vacuum pump and myotubes were washed 3 times with ice cold...
PBS containing 10 mM glucose. Myotubes were then lysed in 500 μL 0.5 M NaOH and 0.1% SDS then transferred to scintillation vials and 5 ml liquid scintillation fluid was added. Samples were vortexed and assayed for [3H]-2-DOG uptake expressed as disintegrations/min/well using a β-counter.

For the fluorometric assay, lean and obese myotubes cultured in 96 well plates were washed 3 times in PBS before incubation with glucose free DMEM media (Gibco, Loughborough, UK) for 3 h. Myotubes were washed a further 3 times before incubation with 100 μM 2-NBDG glucose (Sigma), diluted in glucose free media with or without 100nM insulin, for 30 min. Myotubes were subject to a further 3 washes with PBS before final addition of 100μl PBS/well. Fluorescence was measured by excitation at 485 nm and emission at 528 nm using a Synergy™ 2 Multi-Detection Microplate Reader (BioTek, Swindon, UK). Untreated myotubes were included to correct for myotube auto-fluorescence. 5 biological replicates were performed per condition for each cell line.

9. Quantification of GLUT4 translocation by flow cytometry

Quantification of GLUT4 translocation to the cell membrane was determined by flow cytometry in myoblasts as previously described (Koshy, et al. 2010). In brief, primary human myoblasts (10⁵ cells/condition) from obese subjects (n=5) were treated with either vaspin (100ng/ml, 15min) or insulin (100nM, 15 min). Post-treatment, cells were washed with PBS and stained for GLUT4 (Anti-GLUT4 antibody ab65267, Abcam. 0.05µg/10⁵ cells, diluted in 2% BSA PBS) on ice for 20min. A second wash with PBS was then preformed, before addition of the secondary antibody (Alexa Flour 488 goat anti-mouse IgG, Invitrogen, Thermofisher Scientific. 5 μg/ml diluted in 2% BSA PBS) for 20 min on ice. Cells were then washed
in PBS and fixed with paraformaldehyde (Medium A, Life Technologies) for 20 min at RT. Following a single wash in PBS cells were re-suspended in PBS and the relative surface expression of GLUT4 quantified by measuring the mean fluorescence intensity (MFI) of a minimum 1500 cells/condition using an AccuriC6TM bench top flow cytometer (BD Biosciences).

**Statistical analysis**

Data analysis was carried out using Graphpad Prism v5 statistical package. The normality of data was established by a Shapiro-Wilk test. Significance was determined by parametric t-tests and by ANOVA with Dunnett’s post-hoc tests where appropriate, and are detailed in the figure legends. The correlation of vaspin and GRP78 expression with BMI was assessed by determining the Pearson correlation coefficient. Data is presented as mean ± SEM, with a p value of < 0.05 considered statistically significant.
Results

1. The expression of vaspin and its putative receptor GRP78 are increased in the skeletal muscle and subcutaneous adipose tissue of obese individuals.

We first examined the mRNA expression of vaspin and its putative plasma membrane receptor GRP78 (HSPA5) in both skeletal muscle and SAT in humans of varying BMI. Vaspin mRNA expression was significantly greater in both SAT (2.5 fold, p<0.01) and skeletal muscle (1.5 fold, p<0.05) of obese subjects in comparison to lean subjects (Figure 1A, 1B) and demonstrated a significant positive correlation with BMI (Figure 1E) and skeletal muscle (Figure 1F). Similarly, expression of GRP78 was significantly greater in both SAT (1.5 fold, p<0.05) and skeletal muscle (1.8 fold, p<0.05) of obese subjects in comparison to lean subjects (Figure 1C, 1D) and was positively correlated with BMI in both tissue types (Figure 1G, 1H).

2. Vaspin is rapidly secreted from human adipose tissue and muscle tissue and is detectable systemically in both lean and obese individuals.

To investigate the potential for vaspin to signal between human SAT and skeletal muscle we first examined by immunoblotting the ex-vivo secretion of vaspin from SAT and skeletal muscle into culture media over a 24 h time course. Vaspin was detected in serum free culture media that had been conditioned with either SAT or skeletal muscle for 1 h, indicating that vaspin is rapidly secreted from both tissue types. The amount of vaspin detected in both SAT and skeletal muscle conditioned media increased up to 6 h and was maintained for the duration of 24 h (Figure 2A, 2B). Further, a significantly greater level of vaspin was detected in SAT conditioned media of obese (n=16) in comparison to lean (n=17) at 24 h (Figure 2C). We then examined by ELISA whether vaspin could be detected systemically in human sera,
and whether there was a difference in the systemic concentration of vaspin between lean (n=16) and obese (n=15) individuals. Vaspin was detected in the serum of both lean and obese individuals but there was no significant difference between levels found in obese individuals in comparison to lean individuals in either males or females (Figure 2D). However, serum vaspin was on average higher in obese females compared to lean females. Furthermore, although there was no significant correlation between serum vaspin and BMI in males (Figure 2E), in females we found a significant positive correlation ($R^2 = 0.267$, $P<0.05$) with BMI (Figure 2F).

3. The effect of vaspin on insulin signalling pathways in primary human myotubes.

We next examined the effect of stimulating primary human myotubes (Figure 3A) with recombinant human vaspin on insulin signalling pathways. Firstly, lean primary myotubes were stimulated with recombinant vaspin (100 ng/ml) acutely for either 5, 10 or 15 min and AKT activity (phosphorylation) quantified using MesoScale immunoassays (Mesoscale Discovery). Myotubes stimulated with vaspin for either 10 or 15 min showed a significant 3-fold increase in phosphoAKT (Threonine 308)/total AKT expression (Figure 3B), which along with activation of the other AKT phosphorylation site (serine 473) was validated by immunoblotting (n=4 patient replicates) (Figure 3C-E). To investigate the mechanism of the vaspin-mediated induction in basal AKT activity we examined whether acute or chronic stimulation of human myotubes with vaspin led to activation of either the insulin receptor or the IGF receptor. As expected, 15 min of insulin stimulation alone induced a significant increase in the phosphorylation of both the insulin receptor and the IGF receptor (Figure 3F, 3G). However, stimulation of myotubes with vaspin for either 15 min or...
chronic stimulation for 24 h induced no phosphorylation of either the insulin receptor
or the IGF receptor (Figure 3F, 3G), indicating that vaspin did not directly affect
activation of upstream insulin signalling. Given these findings, we then examined
whether vaspin affected the activity of downstream insulin signalling by determining
the activity status PI3K by immunoblotting. Acute stimulation of primary human
myotubes (n=4 patients) with vaspin (100 ng/ml) induced a transient increase in
phosphorylation of PI3K between 10-15 min as determined by immunoblotting
(Figure 3H, 3I).

4. Vaspin induces the expression and translocation of GLUT4 protein and
promotes glucose uptake in the presence of insulin in human myotubes.

Following these data, and with the observation that vaspin induced activation of
downstream insulin signalling components, we then investigated the functional
metabolic role of vaspin by examining whether vaspin affected GLUT4 expression
and glucose uptake in primary human myoblasts and myotubes from obese subjects.
Firstly, we examined whether there was a difference in skeletal muscle GLUT4
mRNA expression between old obese (n=8), old lean (n=8) and young (n=7)
subjects. Compared to young subjects, GLUT4 expression was 69% lower in the
skeletal muscles from old obese subjects (p<0.05; Figure 4A). In line with the
relatively low expression of GLUT4 in old obese muscle, myotubes derived from old
obese subjects also displayed a blunted glucose uptake response to insulin (100 nM,
30 min), in comparison to myotubes cultured from old lean subjects (Figure 4B).
Stimulation of obese myotubes with vaspin (100ng/ml) for 24 h induced a significant
(p<0.05) ~5-fold increase in the expression of GLUT4 mRNA (Figure 4C), which
translated into a significant (p<0.05) increase in GLUT4 protein (Figure 4D).
Furthermore, stimulation of primary human myoblasts with either insulin (100nM) or vaspin (100 ng/ml) for 15 min caused a significant (p<0.05) increase in GLUT4 protein surface expression, compared to non-stimulated myoblasts, indicative of an increase in GLUT4 protein translocation (Figure 4E).

We then investigated whether these vaspin-mediated effects on both the expression and translocation of GLUT4 were sufficient to impact on glucose uptake in previously insulin-insensitive obese myotubes. Primary human myotubes from old obese subjects that had not been pre-treated with vaspin showed no increase in glucose uptake in response to 30 min of insulin stimulation (100nM), compared to unstimulated control (Figure 4F). However, 30 min insulin stimulation significantly increased glucose uptake in vaspin pre-treated myotubes, compared to control myotubes (Figure 4F). Finally, since p38 and AMPK activation have been implicated in promoting GLUT4 expression we examined the effect of vaspin stimulation on their activation status. Acute stimulation of primary human myotubes (0-15 min) with 100ng/ml vaspin induced a significant activation of both p38 (n=4 biological replicates) and AMPK (n=4 biological replicates), as measured by immunoblotting (Figure 4G-I).
Discussion

With the current dearth of human data on the adipokine vaspin, this study is the first to characterise the expression of vaspin in both skeletal muscle and adipose tissue in elderly individuals of varying BMI and to report the functional effect of vaspin on human skeletal muscle insulin signalling pathways and glucose uptake. Previously, vaspin mRNA has been reported to be detectable in only around 15% of human SAT samples, with no expression reported in SAT of lean subjects (Kloting et al. 2006). In the present study, we detected vaspin mRNA in both SAT and skeletal muscle from both lean and obese individuals. However, vaspin was more highly expressed in the SAT and skeletal muscles from obese individuals and in both tissue types its expression positively correlated with BMI. We also found that vaspin protein was rapidly secreted from both human SAT and skeletal muscle tissue, with the quantity secreted from obese tissues being greater than from lean tissues. Together these data support the findings from rodent studies that describe an increase in vaspin tissue expression with obesity (Heiker et al. 2013; Hida et al. 2000). It is important to note that our finding that vaspin expression in adipose and muscle tissue was increased with obesity was in patients without type II diabetes. Gene array data in the GEO database does not reveal any significant difference in vaspin adipose tissue expression between patients with or without insulin resistance or in those with type II diabetes compared to individuals with normal glucose tolerance (Edgar, et al. 2002). Furthermore, a recent report found that circulatory levels of vaspin in morbidly obese individuals declined following weight loss induced by laparoscopic sleeve gastrectomy (Ibrahim, et al. 2018). These findings, together with our data here, support the notion that vaspin expression in humans is dependent on BMI irrespective of diabetic status.
Contrary to previous reports (Youn et al. 2008), we found no significant difference in serum levels of vaspin in obese individuals compared to lean. This was despite the greater expression and secretion of vaspin from obese SAT of these individuals. This might suggest that elevated secretion of vaspin from SAT in obese individuals is more likely to have localised effects rather than systemic effects on distal tissues. However, it should be noted that serum concentrations of vaspin were variable and therefore with a larger cohort the higher on average concentration of vaspin in obese individuals may have reached statistical significance.

A number of previous publications have examined systemic levels of vaspin in males and females of varying BMI, with contrasting results. In individuals aged 17-79 it was found that serum vaspin was significantly higher in obese male subjects compared to lean male subjects, but was not significantly different between obese and lean females (Youn et al. 2008). However, the study by Esteghamati et al. found that serum vaspin levels positively correlated with BMI in both males and females (Esteghamati, et al. 2014). In addition, higher levels of serum vaspin were reported in females compared to males in individuals with normal glucose tolerance, but no gender difference was seen in individuals with T2D (Youn et al. 2008). However, Seeger and colleagues examined serum vaspin levels in diabetic and non-diabetic patients in both controls and in those patients on chronic hemodialysis (CD) with BMI between 26-30 and found that vaspin was higher in females and furthermore that gender was an independent predictor of circulating vaspin concentrations (Seeger, et al. 2008). Together these observations could reflect sex dimorphism in SAT distribution and quantity (Fuente-Martin, et al. 2013; White and Tchoukalova 2014). It is known for example that females predominantly accumulate more SAT than males (Kotani, et al. 1994), and thus vaspin may play a greater role.
in mediating glucose homeostasis in females than in males. Indeed, some studies have previously reported that the prevalence of metabolic syndrome is higher in females than in males (Beigh and Jain 2012), with central adiposity being a greater risk factor for metabolic diseases in females (Holliday, et al. 2011; Li, et al. 2006).

Contrary to the above studies, we found no significant difference between serum vaspin concentration between males and females in either normal-weight or obese individuals. Furthermore, and contrary to the findings of Esteghamati et al. 2014 we did not find a significant correlation between BMI and vaspin levels in males. However, we did observe that serum vaspin in females significantly correlated with BMI. These disparities may be a reflection of the relatively smaller sample size used in our study but also likely reflects differences between subject cohorts. For example, the male subjects studied by Youn et al. 2008 had a mean age of 39 years, whilst both the male and female subjects studied by Esteghamati et al. 2014 had a mean age of 49 years, considerably lower than the mean age of subjects in this study.

Vaspin-mediated activation of PI3K/AKT has been reported previously in several other cell types, including pancreatic cells (Liu, et al. 2017), 3T3-L1 preadipocytes (Liu, et al. 2015) and endothelial progenitor cells (Sun, et al. 2015). Furthermore, it was recently reported that vaspin promotes the PI3K/AKT signalling pathway leading to increased GLUT4 protein expression in rats fed a high fat diet (Liu, et al. 2018) and can improve glucose tolerance in mice (Nakatsuka et al. 2012). Therefore, our demonstration that vaspin induces activation of PI3K-AKT signalling and increases insulin sensitivity in obese human myotubes is significant. These vaspin-induced effects appear to occur independently of upstream insulin signalling, since vaspin had no direct effect on either the insulin receptor or IGF-1 receptor phosphorylation.
status. Activation of PI3K/AKT signalling is known to promote GLUT4 translocation and glucose uptake in skeletal muscle cells (Dugani and Klip 2005), and here we report that vaspin promotes GLUT4 expression and translocation and facilitates glucose uptake in human obese myotubes. Notably, 15 min stimulation of primary human myoblasts derived from obese subjects with vaspin was able to significantly increase GLUT4 surface translocation to a similar magnitude to that observed with insulin stimulation. This finding may be of clinical significance given that GLUT4 translocation is impaired in T2D patients with insulin insensitivity (Maria, et al. 2015). Indeed, since our data shows that vaspin induces insulin signalling independent of activating the insulin receptor this could provide a novel route to therapeutically improve glucose maintenance in insulin resistant individuals.

Despite being able to promote GLUT4 translocation we found that insulin stimulation of obese myotubes did not result in glucose uptake. Previous studies in rats have demonstrated that GLUT4 expression declines with age (dos Santos, et al. 2012) and obesity (Kahn and Pedersen 1993). Here we report for the first time in humans that muscle tissue derived from elderly obese individuals displayed a significantly lower expression of GLUT4 mRNA in comparison to young lean individuals. The retention of this phenotype in primary human myotubes from elderly obese subjects may therefore explain the blunted insulin response observed at baseline.

Importantly, prolonged 24 h pre-treatment of obese myotubes with vaspin sensitised them to insulin-mediated glucose uptake. This sensitisation is likely due to vaspin increasing the bioavailable intracellular pool of GLUT4. Previous studies in 3-T3-L1 adipocytes (Liu et al. 2015) and in mice (Hida, et al. 2005) have reported that vaspin induces GLUT4 expression. Here, we report for the first time that vaspin significantly induces GLUT4 mRNA and GLUT4 protein expression in human skeletal muscle.
Importantly, we also demonstrate that vaspin is able to activate both AMPK and p38 signalling in primary human myotubes. AMPK activation has previously been reported to upregulate GLUT4 expression in primary human myotubes (McGee, et al. 2008), while p38 is known to regulate transcription factors that control the expression of GLUT4 (Montessuit, et al. 2004; Niu, et al. 2003). Activation of these signalling pathways may therefore be a potential mechanism to explain the vaspin induced upregulation in GLUT4 mRNA and protein expression observed.

However, it should be noted that primary human myotubes in culture elicit relatively low insulin stimulated glucose uptake (Aas, et al. 2004; Al-Khalili, et al. 2003; Montell, et al. 2001), compared to skeletal muscle tissue. This could be due to low basal expression of GLUT4, which has been attributed to denervation (Chowdhury, et al. 2005; Jensen, et al. 2009) and the subsequent absence of contraction. Therefore, in our model system, treatment of myotubes with vaspin may only be increasing the pool of bioavailable GLUT4 towards physiological levels. Whether vaspin administration in humans in vivo would have the same effect remains to be seen.

A cell surface receptor for vaspin has yet to be fully elucidated. One possible mechanism is that vaspin signals through GRP78, a protein that has an important intracellular regulatory role during cellular ER stress (Lee 2005; Matsuo, et al. 2013). However, recent evidence shows GRP78 also functions as a cell surface receptor, with membrane localised GRP78 binding vaspin and in turn promoting AKT and AMPK signalling in H-4-II-E-C3 cells (Nakatsuka et al. 2012). Here, we show GRP78 expression is present in both SAT and skeletal muscle in older individuals. Expression of GRP78 was found to be significantly greater in both SAT and skeletal muscle of obese subjects and displayed a positive correlation with BMI in each case.
This is concurrent with the results of Khadir et al, who also found increased GRP78 expression in SAT of obese humans (Khadir, et al. 2016). Chronic ER stress can be induced by obesity (Yilmaz 2017) and is associated with inflammatory adipose tissue (Kawasaki, et al. 2012), and this may explain the increased GRP78 expression in muscle and adipose tissue of obese individuals in our present study. Additionally, ER stress has been shown to promote re-localisation of GRP78 to the cell membrane, thus increasing ligand binding capacity (Zhang, et al. 2010). Whether the functional effects of vaspin we report here in human myotubes are dependent on GRP78 signalling requires further study.

In summary, this study shows that vaspin is directly secreted from both human SAT and skeletal muscle and its expression is increased with increasing adiposity in older people. Functionally, vaspin induces PI3K/AKT activation, increases both GLUT4 expression and translocation and promotes insulin-stimulated glucose uptake in primary obese older human myotubes. This data support a role for vaspin as a protective adipokine in the development of insulin resistance in elderly obese individuals. Further studies are needed to identify the mechanism of action of vaspin as this may provide a novel therapeutic target to improve insulin sensitivity independent of the insulin receptor.

Declaration of interest

The authors declare that they have no conflicts of interest with the contents of this article.
Funding

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Acknowledgments

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Carey DG, Jenkins AB, Campbell LV, Freund J & Chisholm DJ 1996 Abdominal fat and insulin resistance in normal and overweight women: Direct measurements reveal a strong relationship in subjects at both low and high risk of NIDDM. *Diabetes* **45** 633-638.


Zeyda M, Huber J, Prager G & Stulnig TM 2011 Inflammation correlates with markers of T-cell subsets including regulatory T cells in adipose tissue from obese patients. *Obesity (Silver Spring)* **19** 743-748.


Figure legends

Figure 1. Vaspin and GRP78 mRNA expression are upregulated in subcutaneous adipose tissue and skeletal muscle in obese elderly individuals. (A) The mRNA expression of vaspin in SAT of lean (n=21) and obese (n=17) elderly individuals. (B) The mRNA expression of vaspin in skeletal muscle of lean (n=4) and obese (n=4) elderly. (C) The mRNA expression of GRP78 in SAT of lean (n=8) and obese (n=9) elderly. (D) The mRNA expression of GRP78 in skeletal muscle of lean (n=7) and obese (n=9) elderly. (E) Correlation of vaspin mRNA expression in SAT with BMI. (F) Correlation of vaspin mRNA expression in skeletal muscle with BMI. (G) Correlation of GRP78 mRNA expression in SAT with BMI. (H) Correlation of GRP78 mRNA expression in skeletal muscle with BMI. Expression of mRNA was quantified by qRT-PCR, and normalised using GAPDH. Data are represented as mean ± SEM. * signifies p<0.05, *** signifies p=<0.001, as determined by unpaired t-tests.

Figure 2. Vaspin is rapidly secreted from human subcutaneous adipose tissue and systemically is elevated in obese individuals.

Detection of vaspin protein in (A) SAT conditioned media and (B) skeletal muscle conditioned media in lean and obese subjects over a 24 h time course, as measured by immunoblotting. 10 µl adipose or skeletal muscle conditioned media was loaded per sample lane. Bars represent mean densitometric units ± SEM of immunoblots from n=3 subjects analysis. *=p<0.05, **=p<0.01, ***=p<0.001, significantly different from time=0 media control sample as determined by one-way ANOVA with Dunnett’s post-hoc tests. (C) Densitometric analysis of vaspin protein expression in adipose conditioned media at 24h from lean (n=17) and obese (n=16) subjects as measured.

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by immunoblotting. 10 µl of adipose conditioned media was loaded per sample lane. *p<0.05, as determined by unpaired t-test. (D) Detection of vaspin protein in the serum of male (n=8 lean; n=8 obese) and female (n=8 lean; n=7 obese) subjects by ELISA. (E) Pearson correlation of serum vaspin with BMI in males. (F) Pearson correlation of serum vaspin with BMI in females.

Figure 3. Vaspin activates downstream insulin signalling pathways in primary human myotubes. (A) Representative isolated human myoblasts and differentiated myotubes immunofluorescently stained with desmin and viewed using a 20X objective. (B) Effect of acute stimulation (0, 5, 10, 15 min) of primary human myotubes with recombinant human vaspin (100ng/ml) on AKT\textsuperscript{thr308} phosphorylation measured by mesoscale analysis (n=4 biological replicates, 2 from male and 2 from female). (C) Representative immunoblots of AKT\textsuperscript{ser473} and AKT\textsuperscript{thr308} phosphorylation in untreated and vaspin (100 ng/ml) stimulated myotubes after 0, 5, 10 and 15 min stimulation (n=4 primary donors 2 male, 2 female, 15 ug of total protein was loaded per sample). (D) Densitometric analysis of AKT\textsuperscript{Thr308} western blots normalised to Total AKT protein (n=4). (E) Densitometric analysis of AKT\textsuperscript{ser473} western blots relative to Total AKT protein (n=4). (F) Effect of insulin (0-30 nM, 30min) and acute (15 min) or chronic (24 h) vaspin stimulation (100 ng/ml) on the phosphorylation of insulin receptor and (G) IGF receptor as measured by mesoscale analysis (n=16 biological replicates from 8 primary donors, 4 male, 4 female). (H) Timecourse of acute vaspin stimulation (100 ng/ml) on the activation of PI3K assessed by immunoblotting (n=4 primary donors1 male, 3 female). 15 ug total protein was loaded per sample) (I). Densitometric analysis of Phospho-PI3K western blots normalised to total PI3K,
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**Figure 4. Vaspin induces GLUT4 expression and sensitises insulin-mediated glucose uptake in primary human myotubes**

(A) GLUT4 mRNA expression relative to GAPDH in skeletal muscle tissue derived from lean young (n=7) lean old (n=8) and obese old (n=8) subjects. *=p<0.05, significantly different between young and old obese as determined by one way ANOVA with Dunnett’s post-hoc tests. (B) Effect of insulin (100 nM, 30 min) on 2-NBDG glucose uptake in primary human myotubes derived from lean (n=4 females) and obese (n=3 females) subjects. *=p<0.05, significantly different from unstimulated control as determined by 2-way ANOVA. (C) GLUT4 mRNA expression in primary human myotubes from obese subjects stimulated for 24 h with recombinant vaspin (100 ng/ml) or left untreated (n= 5 primary donors, 2 male, 3 female). Expression was determined by qRT-PCR and normalised to GAPDH. *=p<0.05, significantly different from unstimulated control, as determined by t-test. (D) GLUT4 protein expression as measured by immunoblotting in primary human myotubes from obese subjects stimulated for 24 h with vaspin (100 ng/ml) or left untreated (n=6 primary donors, 2 male, 4 female). *=p<0.05, significantly different from unstimulated control, as determined by t-test. (E) Primary human myoblast membrane localised GLUT4 expression in response to insulin (100 nM 15 min), or Vaspin (100 ng/ml 15 min), (n=5 obese primary donors, 2 male, 3 female). Relative surface expression of GLUT4 was quantified by measuring the mean fluorescence intensity (MFI) in a minimum of 1500 cells/ condition by flow cytometry. *=p<0.05, significantly different
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Densitometric analysis of vaspin protein expression in adipose conditioned media at 24h from lean (n=17) and obese (n=16) subjects as measured by immunoblotting. 10 µl of adipose conditioned media was loaded per sample lane. *=p<0.05, as determined by unpaired t-test. (D) Detection of vaspin protein in the serum of male (n=8 lean; n=8 obese) and female (n=8 lean; n=7 obese) subjects by ELISA. (E) Pearson correlation of serum vaspin with BMI in males. (F) Pearson correlation of serum vaspin with BMI in females.
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Table 1. Patient characteristics

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### Supplementary Table 1. PCR Primer Sequences

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| Vaspin| Applied Biosystems | Forward Primer: 5’-GGG AGC CTT GGC ATG ATG-3’  
                      | Reverse Primer: 5’-AGC AAA GGC AAG GGC AGA T-3’ |
| GRP78 | Applied Biosystems | Forward Primer: 5’-GGCCGCACGTGGAATG-3’  
                      | Reverse Primer: 5’- TTGAACGGCAAGAACTTGATGT-3’ |
| GAPDH | Primer Design      | Proprietary               |
| GLUT4 | Primer Design      | Proprietary               |
## Supplementary Table 2: Antibodies

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<td>Total PI3K</td>
<td>CST 4257</td>
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Supplementary Figure 1

Full size of immunoblots of Vaspin. Predicted MW - 47 kDa
Supplementary Figure 2

Full size of immunoblot of pAKT\textsuperscript{thr308}. Predicted MW - 60 kDa

Full size of immunoblot of pAKT\textsuperscript{ser473}. Predicted MW - 60 kDa

Full size of immunoblot of Total AKT. Predicted MW - 60 kDa

Full size of immunoblot of beta actin. Predicted MW - 42 kDa
Supplementary Figure 3

Full size of immunoblot of pPI3K. Predicted MW - 60 kDa

Full size of immunoblot of Total PI3K Predicted MW - 60 kDa

Full size of immunoblot of beta actin. Predicted MW - 42 kDa
Supplementary Figure 4

Full size of immunoblot of pAMPK.
Predicted MW - 62 kDa

Full size of immunoblot of total AMPK.
Predicted MW - 62 kDa

Full size of immunoblot of phospho P38.
Predicted MW - 38 kDa

Full size of immunoblot of Total P38.
Predicted MW - 38 kDa