Serum Levels of Retinol Binding Protein 4 (RBP4) and Adipose Tissue Expression Levels of Protein Tyrosine Phosphatase 1B (PTP1B) are Increased in Obese Men Resident in North East of Scotland Without Any Changes in Endoplasmic Reticulum (ER) Stress Response Marker Genes

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Abstract

Background: Retinol-binding protein 4 (RBP4) is an adipokine identified as a marker of insulin resistance in mice and humans. Protein tyrosine phosphatase 1B (PTP1B) expression levels, as well as other genes involved in the endoplasmic-reticulum (ER) stress response, are increased in adipose tissue of obese, high-fat diet fed mice. In this study, we investigated if serum- and/or adipose-tissue RBP4 protein levels, and expression levels of PTP1B and other ER stress-response genes, are altered in obese and obese/diabetic men resident in North East of Scotland.

Methods: We studied three groups of male volunteers: 1) normal/overweight (body mass index (BMI) <30), 2) obese (BMI>30) and 3) obese/diabetic group (BMI>30) controlling their diabetes either by diet or anti-diabetic drug, metformin. We analysed their serum- and adipose-tissue RBP4 protein levels, as well as adipose tissue mRNA expression of PTP1B, BIP (binding immunoglobulinprotein), ATF4 (activated transcription factor 4) and GRP94 (glucose-regulated protein 94), alongside other markers of adiposity (% body fat, leptin, cholesterol, triglycerides) and insulin resistance (oral glucose tolerance tests (OGTT), insulin, HOMA-IR, C-reactive protein (CRP), adiponectin).
Results: We found that obese Scottish subjects had significantly higher serum-RBP4 protein levels in comparison to the normal/overweight subjects ($p<0.01$). Serum-RBP4 levels were normalized in obese/diabetic subjects treated with diet or metformin ($p<0.05$). Adipose-tissue RBP4 protein levels were comparable between all three groups of subjects and so were serum- and adipose-transthyretin (TTR) levels. Adipose-tissue PTP1B mRNA levels were increased in obese subjects in comparison to normal/overweight subjects ($p<0.05$), however diet and/or metformin treatment did not reverse this effect. Adipose-tissue BIP, ATF4 and GRP94 expression levels were unchanged in obese and obese/diabetic subjects.

Conclusions: Human obesity results in an increase in serum, but not adipose-tissue, RBP4 protein levels and these are normalized in obese/diabetic subjects, which exhibit improvements in insulin sensitivity through diet or metformin treatment. However, whilst adipose-tissue PTP1B mRNA levels do increase in obese Scottish subjects, these remain high in obese/diabetics on diet or metformin treatment.

Abbreviations: retinol-binding protein 4 (RBP4), transthyretin (TTR), oral glucose tolerance test (OGTT), c-reactive protein (CRP), homeostasis-model-assessment of insulin resistance (HOMA-IR), body mass index (BMI), protein tyrosine phosphatase 1B (PTP1B), BIP (binding immunoglobulinprotein), ATF4 (activated transcription factor 4) and GRP94 (glucose-regulated protein 94)

Introduction

Obesity is a major risk factor for a number of metabolic diseases such as type 2 diabetes, cardiovascular disease, cancer, Alzheimer's disease and others. Recent studies clearly demonstrate that adipose tissue acts as an endocrine and paracrine organ, by secreting various proteins (adipokines) [1]. These bioactive molecules, including leptin, adiponectin, visfatin, omentin, tumor necrosis factor-α (TNF-α), resistin, retinol-binding protein 4 (RBP4) and many others influence metabolic processes such as food intake, glucose and lipid metabolism, inflammation and insulin resistance (reviewed in [2]). Adipose tissue is an important inflammatory source in obesity and type 2 diabetes, due to the cytokines produced by the adipose tissue itself as well as macrophages that have infiltrated the adipose tissue during the progression of obesity [3].

RBP4 is an adipokine recently discovered to be elevated in insulin-resistant states and obesity in mice [4] and humans [3,5]. RBP4 is a transport protein for retinol (vitamin A) and is normally bound to transthyretin (TTR) in circulation. In many studies, the level of serum RBP4 elevation correlates highly with the degree of insulin resistance in both mice and humans, and serum RBP4 levels are suggested to be highly predictive of metabolic syndrome risk in a large population-based study [6]. However, not all studies agree with these findings and find that either serum RBP4 levels are unaltered in insulin resistance/diabetes, or that RBP4 mRNA levels in human abdominal subcutaneous tissue levels are even down-regulated in obesity (for commentary see [7]).

Protein-tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed non-receptor tyrosine phosphatase and a key negative regulator of leptin and insulin signalling [1]. High-fat diet feeding in mice has been shown to increase PTP1B levels in a number of tissues such as the brain, muscle, liver and adipose tissue [1,8-10], and global PTP1B$^{-/-}$ mice exhibit reduced adiposity and improvements in insulin sensitivity [11,12]. However, this improvement in insulin sensitivity is tissue-specific as adipose-specific PTP1B$^{-/-}$ mice exhibit an increase in leptin production and mild glucose intolerance [1]. Low-grade inflammation, such as that caused by obesity and prolonged high-fat diet feeding, has been shown to lead an induction of the endoplasmic-reticulum stress [13-15] and result in an increase in PTP1B protein and mRNA expression levels [10,16]. Increase in ER stress response genes, such as BIP (binding immunoglobulinprotein), ATF4 (activated transcription factor 4) and GRP94 (glucose-regulated protein 94) in mice is associated with obesity and insulin resistance.

In this study we aimed to investigate if serum and adipose tissue RBP4 protein levels are altered in human obesity and type 2 diabetes in a cohort of men resident in North East of Scotland and if this can be manipulated by either diet or metformin treatment in obese/diabetic subjects. As the method of measuring RBP4 levels has been suggested to account for some of the discrepancies in findings between different studies and quantitative Western blotting for protein RBP4, using full-length RBP4 antibody, has been suggested as a 'gold standard' for RBP4 level determination [17], we used this method to assess both serum and adipose tissue RBP4 protein levels in normal/overweight, obese and obese/diabetic subjects and correlated these to other markers of obesity and insulin resistance. In addition, whilst there is evidence to support that obesity and high-fat diet feeding leads to induction of ER stress response and induction of ER stress-response genes in adipose tissue in mice [13], to our knowledge, there is not much data available if this is also the case in humans. We therefore analyzed PTP1B expression levels and levels of BIP, ATF4 and GRP94 in adipose tissue of normal/overweight subjects and compared it to obese and obese/diabetic subjects resident in North East of Scotland.

Methods

Volunteers and protocols

Male, non-smoking subjects were included if they were not on any special-diet and had a normal medical examination excluding their diabetic status, where appropriate. Only those diabetic volunteers who were controlling their diabetes by diet or metformin (500 mg 4 times a day) were accepted. These inclusion criteria were also checked with the participant's primary-care physician. All patients provided informed written-consent before inclusion in the study, approved by North of Scotland Research Ethics Committee (NOSREC).

The study comprised of three groups (see Table 1): normal/overweight volunteers with a BMI <30 (n=21), obese volunteers with a BMI >30 (n=9) and obese/diabetic volunteers with a BMI >30 (n=14) controlling their diabetes by diet or metformin. All blood samples were taken overnight-fasted (10-12 hours). Each subject's height, weight, waist and hip were measured using standard protocols [18]. Percentage body fat was determined using air-displacement whole-body plethysmography (BodPod® Body Composition System, CT, USA).

Fat biopsies

A subcutaneous, abdominal region, adipose-tissue biopsy was taken after an overnight-fast (10-12 hours). The biopsy was performed in the periumbilical triangle with a 14G needle, after intradermal anaesthesia with 2% lignocaine. Adipose-tissue was drawn by successive suctions into a 50ml syringe. We routinely obtain 0.5-1 gram of adipose-tissue. Tissue was immediately frozen in liquid nitrogen. Samples were subsequently processed for RNA and protein extraction.

Oral-Glucose Tolerance Testing (OGTT)

Volunteers were fasted overnight (10-hours) to receive the OGTT. Blood samples were drawn at 0- and 120-minutes after consuming 75g of glucose. Plasma glucose levels (and triacylglycerol) were measured using an automated clinical analyzer (Kone Oyj, Espoo, Finland). Plasma insulin was measured using an enzyme-linked immunosorbent assay (Mercodia, Sweden). The inter- and intra-assay coefficients of variation (CVs) for insulin using this assay were 2.6%–3.6% and 2.8%–3.4%, respectively. Homeostasis-model-assessment of insulin resistance (HOMA-IR) was calculated using fasting glucose and insulin values. Total cholesterol, LDL- and HDL-cholesterol were analysed using commercial kits (Microgenics Gmbh, UK).

Plasma ELISAs

Plasma leptin and CRP were detected, in duplicate, using commercial kits according to the manufacturer's instructions (R&D Systems, UK). For leptin the minimum level of detection was less than 7.8pg/ml and the intra- and inter-assay CVs were 3.2 and 4.2%, respectively. For CRP the minimum level of detection 0.005ng/ml and the intra- and inter-assay CVs were 8.7% and 8.7%, respectively.

Biochemical analyses

Adipose-tissue lysates were prepared by extraction in RIPA buffer, as described previously [1,19]. 10µg of human adipose-tissue lysates were used for analysis. Serum RBP4 levels were measured by adding 1µl of patients' serum directly into 29 µl of 1% SDS loading-buffer and

boiling for 10 minutes [20]. Proteins were resolved by SDS-PAGE (10% gels in MES buffer), transferred to nitrocellulose membranes and immunoblots performed using polyclonal antibodies against anti-human RBP4 and TTR, following manufacturer's instructions. Proteins were visualized using enhanced-chemiluminescence (ECL), and quantified using a high-sensitivity imaging system (Fusion imaging system and Bio1D software, Vilber Lourmat) [1,10,19,21].

mRNA expression analysis

Total RNA was isolated from adipose tissue using TRI Reagent® (Ambion,Warrington, U.K.) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total RNA employing the BiorscriptTM Preamplification System (Bioline, London, U.K.) and an oligo(dT)12–18 primer as the reverse primer. Then, target genes were amplified by real-time PCR using GoTaqTM qPCR Master Mix (Promega, Southampton, U.K.) in Roche LightCycler® 480 System (Roche Diagnostics, Burgess Hill, U.K.). Relative gene expression was calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method as described previously [10].

Statistical analyses

The statistical package GraphPad Prism 5 was used for analysis. Results are reported as mean ± SD. One way ANOVA was performed for analysis of more than 2 variables followed by post hoc test for detection of significance. Simple linear correlation (Pearson's correlation) was used for quantitative data. Spearman correlation coefficient was used for qualitative data to estimate the association of RBP4 levels and BMI, leptin, insulin, adiponectin, CRP, cholesterol, triglycerides, HOMA-IR, SLPI, waist/hip ratio, lean weight, fat mass and % fat mass. P value is significant if $p \leq 0.05$.

Results

Three groups of male subjects were recruited into the study (see Table 1). The study comprised of a group of normal/overweight subjects with a BMI<30 (n=21), an obese group with a BMI>30 (n=9) and a similar obese group, with a BMI>30, who in addition were diagnosed with diabetes (n=14) controlled by diet or metformin. Whilst there were no significant differences in age or height of the volunteers, they had significantly different waist circumference (1-way ANOVA; $p<0.001$), BMI ($p<0.001$), % body fat ($p<0.001$), waist/hip ratio ($p<0.001$) and leptin ($p<0.01$) (Table 1). In addition, markers of insulin resistance between the three groups were significantly different, namely oral glucose tolerance test (OGTT) at 0 and 2 hours ($p<0.001$), fasting plasma glucose and insulin ($p<0.001$), HOMA-IR ($p<0.001$) and serum triglycerides ($p<0.05$).

As the method of measuring RBP4 levels has been suggested to account for some of the discrepancies in findings between different studies and Western blotting for protein RBP4, using full-length RBP4 antibody, has been suggested as a 'gold standard' for RBP4 level determination [17], we used this method to assess both serum- and adipose-tissue RBP4 protein levels in normal/overweight, obese and obese/diabetic subjects and correlated these to other markers of adiposity and insulin resistance. By using anti-human RBP4 and TTR antibodies, we found that obese subjects had significantly higher serum RBP4 protein levels in comparison to the normal/overweight subjects (1-way ANOVA; $p<0.01$; treatment effect $p=0.005$, $F=6.046$) (Fig. 1A, B). Furthermore, in obese/diabetic subjects treated with either diet or metformin, serum RBP4 protein levels were completely normalized (1-way ANOVA; $p<0.05$) (normal/overweight $1.34x10^6 \pm 0.26x10^6$ vs obese $1.64x10^6 \pm 0.18x10^6$ vs ovese/diabetic $1.40x10^6 \pm 0.17x10^6$), whilst serum TTR protein levels remained unaltered (Fig. 1A, C). Interestingly, whilst serum RBP4 levels were up-regulated in human obesity and normalized by improving whole body insulin sensitivity in obese subjects, the adipose-tissue RBP4 protein levels were comparable between all three groups of subjects (Fig. 2A, B), as were the serum and adipose tissue TTR levels (Fig. 1A, C; Fig. 2A, C).

To assess if serum and/or adipose RBP4 protein levels correlated with any of the adiposity or insulin sensitivity markers, we performed additional correlation analysis using Speaman correlation coefficient (R) as done previously [5]. Whilst body mass index (BMI) correlated significantly, as expected, to serum leptin ($R=0.89$; $p<0.0001$), insulin ($R=0.72$; $p<0.0001$), adiponectin ($R=-0.48$; $p<0.01$), CRP ($R=0.46$; $p<0.01$), triglycerides ($R=0.36$; $p<0.05$) and HOMA-IR ($R=0.72$; $p<0.0001$), we found no correlation between serum and/or adipose RBP4 protein levels and any of the above mentioned adiposity- and insulin-sensitivity markers (Table 2).

To assess if obesity led to an increase in ER stress-response genes in the adipose tissue of our subjects, and if this could be reversed by insulin sensitization, we analyzed adipose tissue gene expression of PTP1B, BIP, ATF4 and GRP94. In agreement with mouse studies, adipose tissue PTP1B gene expression levels were increased in obese subjects in comparison to the normal/overweight group (Fig. 3A) [1]. However, this was not reversed with insulin sensitization in obese/diabetic subjects treated with diet or metformin, suggesting that in humans, like in mice, adipose tissue PTP1B does not regulate insulin sensitivity [1]. Gene expression levels of other ER stress response genes were not regulated by obesity or insulin sensitivity (Fig. 3B-D).

Discussion

RBP4 is an adipokine proposed to be a marker of human insulin resistance, type 2 diabetes [5] and gestational diabetes. RBP4 levels have been found to be normalized by certain anti-diabetic drug treatments such as rosiglitazone or pioglitazone [7]and lowering RBP4 levels has been suggested as a potential anti-diabetic therapy. However, many studies have not been able to show this association between human obesity and insulin resistance and found no correlation between BMI and serum and/or adipose tissue mRNA RBP4 levels [22].

In our volunteer study, we demonstrate that serum- but not adipose-tissue RBP4 protein levels are elevated in human obesity. We also show that this can be completely reversed in obese/diabetic subjects treated with diet or metformin, in agreement with studies from other patient cohorts [5]. Thus, improved insulin sensitivity in this small cohort study may be at least partly due to the lowering effects of diet or metformin on serum, but not adipose tissue, RBP4 levels. However, in agreement with other studies which found no correlation between the actual body mass index [22] and serum RBP4 levels, we also show that serum and subcutaneous adipose-tissue RBP4 protein levels do not correlate with BMI, nor do they correlate to any other known investigated adiposity- and insulin-sensitivity markers. This suggests that there may be other factor(s) responsible for the observed rise in serum RBP4 protein levels during the progression of human obesity. Some studies, however, have found an association between serum RBP4 levels and visceral fat (VF) [23,24]and it is possible that serum RBP4 levels would correlate directly to those; since we did not have accurate data on VF for each volunteer, normally obtained by MRI, we cannot exclude the possibility that these would show a correlation.

We also demonstrate that in human subcutaneous adipose tissue, PTP1B expression levels are regulated by obesity, in agreement with the mouse studies which demonstrate that adipose tissue PTP1B levels are up-regulated upon high-fat diet feeding [1]. In agreement with the mouse studies, furthermore, we also observe that an increase in adipose tissue PTP1B levels in human obesity cannot be reversed by improvements in insulin sensitivity in obese/diabetic subjects treated with diet or metformin.

On the other hand, the levels of the other ER stress response genes remain unaltered in the adipose tissue of the three study groups, suggesting that perhaps ER stress response pathway may not play a major role in this tissue in human obesity. We should however be cautious at interpreting these data due to a relatively low sample size that we had available for this specific study and perhaps a larger study size is required to quantitatively examine the role of ER stress response pathway in human obesity.

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Author contributions: NH collected the data and analysed data in Table 1, AA collected the data in Figure 3, MD and NM performed experiments in Figures 1 and 2, and analysed the data; MD wrote the manuscript.

Figure Legends

Figure 1. Serum RBP4 protein levels in normal/overweight, obese and obese/diabetic subjects treated with diet or metformin.

A) Serum RBP4 and TTR protein levels. 1 microliter of serum was denatured in SDS loading buffer and subjected to SDS-PAGE analysis per individual. Quantification of serum B) RBP4 and C) TTR protein levels using Bio1D quantitative software.

Figure 2. Adipose-tissue RBP4 protein levels in normal/overweight, obese and obese/diabetic subjects treated with diet or metformin.

A) Adipose tissue RBP4 and TTR protein levels. 10 micrograms of adipose tissue lysate was denatured in SDS loading buffer and subjected to SDS-PAGE analysis per individual. Quantification of adipose tissue B) RBP4 and C) TTR protein levels using Bio1D quantitative software.

Figure 3. Adipose tissue relative mRNA levels of PTP1B, ATF4, GRP94 and BiP.

Graphs A to D show relative mRNA levels of the indicated genes in adipose tissue, measured by quantitative real-time PCR and normalized against HPRT mRNA. n=9 for Normal/Overweight group; n=7 for Obese group and n=8 for Obese/Diabetic/Treated group. Data are represented as mean +/- SEM and analyzed using 1-way ANOVA with post-hoc analysis.

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	Normal/Overweight	Obese	Obese/ Diabetic	P value (ANOVA)
n	21	9	14	
Age	46.1 (9.2)	54.8 (13.8)	52.8 (13.2)	0.2
Waist circumference (cm)	89.6 (5.7)	113 (6.5)	120.2 (11.5)	<0.001
Body Mass Index (Kg/m^2)	25.5 (2.10)	34.3 (5.02)	36.7 (6.3)	<0.001
Height (m)	1.76 (0.64)	1.78 (0.08)	1.74 (0.66)	0.351
Body Weight (Kg)	79.2 (7.6)	109 (13.8)	111 (18.5)	<0.001
Body Weight/Height Ratio	0.51 (0.04)	0.64 (0.05)	0.69 (0.07)	<0.001
Percentage Body Fat	22.8 (6.8)	38.3 (6.1)	38.2 (7.0)	<0.001
Waist/Hip Ratio	0.88 (0.05)	0.96 (0.07)	1.05 (0.14)	<0.001
Plasma Cholesterol (mmol/l)	4.73 (1.32)	5.24 (1.06)	4.38 (0.49)	0.17
Plasma HDL Cholesterol (mmol/l)	1.28 (0.33)	1.15 (0.34)	1.19 (0.27)	0.54
Plasma LDL Cholesterol (mmol/l)	3.08 (1.11)	3.38 (0.80)	2.54 (0.42)	0.082
Triglycerides (pmol/l)	1.09 (0.52)	1.42 (0.53)	1.80 (1.08)	0.04
OGTT Plasma Glucose 0 hour (mmol/l)	5.42 (0.74)	5.88 (0.68)	8.92 (2.82)	<0.001
OGTT Plasma Glucose 2 hour (mmol/l)	5.22 (2.13)	4.75 (1.3)	13.8 (3.80)	<0.001
Fasting Plasma Insulin (mU/I)	4.74 (2.77)	9.81 (4.03)	16.3 (10.6)	<0.001
Fasting Plasma Leptin (ng/ml)	4.08 (1.88)	15.60 (12.34)	19.44 (8.84)	0.006
C-reactive Peptide (μg/ml)	1.22 (1.82)	2.39 (1.50)	2.78 (2.29)	0.127
HOMA-IR	1.19 (0.91)	2.66 (1.25)	6.39 (4.24)	<0.001
HOMA beta cell	52.6 (26.7)	80.9 (33.2)	84.6 (60.3)	0.032
QUICKI	1.12 (0.22)	1.44 (0.21)	1.66 (0.24)	<0.001

Table 1. Baseline characteristics of the lean/overweight, obese and diabetic study volunteers. Lean/overweight volunteers have a waist circumference <40 inches (101.6 cm). Data are mean (standard deviation) for all parameters. P values shown on the right are One-Way ANOVA analysis of variance.

Clinical Features:	**Normal/Overweight** N=12		**Obese** N=9		**Obese/Diabetic** N=11	
Variables:	r	p	r	p	r	p
BMI	-0.01	0.487	-0.45	0.114	-0.17	0.307
Leptin	-0.43	0.107	-0.2	0.307	0.23	0.251
Insulin	-0.03	0.457	0.23	0.276	-0.31	0.177
Adiponectin	-0.16	0.309	0.27	0.247	**0.52**	**0.051***
CRP	-0.315	0.159	-0.53	0.074	-0.12	0.364
Cholesterol	0.15	0.324	-0.07	0.44	0.43	0.09
Triglycerides	0.21	0.252	0.12	0.388	-0.06	0.426
HOMA-IR	-0.06	0.431	0.37	0.168	-0.24	0.242
SLPI	-0.23	0.235	0.32	0.205	-0.32	0.170
Waist/Hip	-0.08	0.406	0.30	0.218	0.03	0.462
Lean Weight	-0.17	0.301	-0.02	0.491	-0.47	0.071
Fat mass	-0.07	0.414	0.07	0.44	0.07	0.416
% fat	-0.01	0.483	-0.19	0.307	0.19	0.290

Table 2. Correlations between plasma RBP4 and BMI, leptin, insulin, adiponectin, CRP, cholesterol, triglycerides, HOMA-IR, SLPI, waist/hip ratio, lean weight, fat mass and % fat mass. P value is significant if $p \leq 0.05$.