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The Impact of Neutrophil Proteinase 3 on IGFBP-3 Proteolysis in Obesity

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Abstract

Obesity is a complex disorder and is a major risk factor associated with the incidence of insulin resistance (IR), diabetes, cardiovascular disease (CVD) and other metabolic disorders. The endocrine paradigm suggests that visceral fat in obesity, consisting primarily of adipocytes, secretes various pro-inflammatory adipokines such as tumor necrosis factor (TNF), leptin, visfatin, resistin, and IL-6 creating a state of local inflammation further resulting in chronic systemic inflammation and accelerating the events leading to systemic IR, diabetes and metabolic syndrome.

The insulin-like growth factor (IGF) system plays a major role in growth, development and maintenance of homeostasis in normal cells. IGF binding protein-3 (IGFBP-3), the major binding protein in circulation, has been shown to be associated with obesity, IR, type II diabetes mellitus (T2DM) and CVD. Recent studies have demonstrated the IGFBP-3-specific receptor (IGFBP-3R) is a novel protein mediating the anti-inflammatory function of IGFBP-3. IGFBP-3 inhibits adipokine-induced insulin resistance and early manifestations of atherosclerosis via inhibition of NF-κB signaling in adipocytes.

Furthermore, decreases in total IGFBP-3 levels and increases in proteolyzed IGFBP-3 in circulation have been documented in obese populations compared to their normal counterparts further establishing a positive correlation between IGFBP-3 proteolysis and adiposity parameters as well as IR. Conversely, our recent studies have identified that neutrophil serine protease (NSP) PR3, an IGFBP-3 specific protease in obesity, is positively correlated with IGFBP-3 proteolysis, IR, body mass index, TNF and IL-8. These findings strongly suggest that obesity-induced activation of PR3 abrogates the anti-inflammatory, insulin-sensitizing IGFBP-3/IGFBP-3R cascade, resulting in IR and its progression to T2DM. The complete characterization of the underlying mechanism and functional significance of the PR3-IGFBP-3/IGFBP-3R cascade in obesity will foster identification of the diagnostic and therapeutic potential of PR3 inhibition in insulin resistance and its sequelae.

Keywords: Diabetes; Cardiovascular disease; Insulin resistance; IGFBP-3; Neutrophil serine protease PR-3

Introduction

According to World Health Organization estimates, overweight and obesity now overshadow underweight and malnutrition as significant causes of premature death [1]. Nearly two-thirds of adults in the United States are overweight or obese [2], and obesity is a major risk factor for a myriad of serious comorbidities including hypertension, type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), and other metabolic disorders [3-5]. Additionally, rapidly increasing rates of obesity in children and young adults has been observed and is resulting in immediate and lifelong metabolic disease risk [6]. Lifestyle changes to counteract obesity and physical inactivity have been emphasized as the first line of defense against progression to T2DM, however there has been no significant decrease in the incidence of obesity. More effective preventive and therapeutic strategies are needed to thwart obesity and associated metabolic complications.

Obesity and Insulin Resistance

Insulin resistance (IR) represents a common metabolic derangement that contributes to the development of many obesity-related comorbidities including T2DM [7]. Although it is generally established that low-grade adipose tissue inflammation contributes substantially to the burden of IR, the pathophysiology underlying the development of IR is complex and multifactorial [8]. Thus, a clearer understanding of the mechanisms leading to obesity-associated IR is necessary to identify novel targets for the prevention and treatment of many IR-driven conditions such as T2DM. The endocrine paradigm suggests that visceral fat in obesity, consisting primarily of adipocytes, secretes various pro-inflammatory adipokines such as tumor necrosis factor (TNF), leptin, visfatin, resistin, and interleukin (IL)-6 creating a state of local thus accelerating events leading to systemic IR, T2DM and metabolic syndrome. Recent studies have further identified that obesity-induced inflammatory adipokines/cytokines interfere with insulin signaling in visceral adipocytes by decreasing the levels of insulin receptor substrate-1 (IRS-1), glucose transporter-4 (GLUT4) and adiponectin leading to a state of IR via autocrine/paracrine influences [4,8-12].

IGF System

The biology of the IGF system is complex, consisting of ligands (IGF-I and IGF-II) and the cognate receptors (IGF-IR and IGF-IIR) in addition to six IGF-binding proteins (IGFBPs), and plays an important role in cell growth and proliferation [13,14]. IGF-I shares structural homology with insulin and, like insulin, promotes the peripheral uptake of glucose and fatty acids [15]. IGFBP-3 is a glycoprotein that forms the 150 kDa ternary complex in circulation consisting of IGFBP-3, acid-labile subunit (ALS) and IGF-I. This ternary complex reduces the passage of IGF-I to the extravascular compartment, and extends its half-life [15]. Recent studies have shown that IGFBP-3 appears to be associated with IR, T2DM and CVD [15]. In addition to alterations in other metabolic pathways, perturbations in the growth hormone/ IGF-1 axis have been implicated in the process [16]. In addition to its role as a carrier protein, the results of our previous work point to an IGF receptor independent anti-inflammatory and anti-tumor action of IGFBP-3 in a variety of human diseases including asthma, other inflammatory diseases and cancer [13,17-19]. Initially, the IGF receptor independent actions of IGFBP-3 focuses on its anti-proliferative and pro-apoptotic functions in a variety of human cancer cells since IGFBP-3 is one of the genes transcriptionally activated by the tumor suppressor gene p53 [13]. Several lines of evidence reveal that these intrinsic anti-proliferative and pro-apoptotic function of IGFBP-3 in cancer cells are mediated through putative receptors, IGFBP-3 interacting proteins and nuclear association13. Recent studies further demonstrated that IGFBP-3 interferes with inflammatory NF-κB signaling pathway, thereby inhibiting NF-κB-regulated genes involved in pro-tumor and pro-inflammatory actions in cancer and normal cells, respectively [18-20]. Furthermore, we have identified a novel IGFBP-3 specific receptor (IGFBP-3R), which represents a novel gene composed of 915 base pairs and encoding a 240-amino acid polypeptide (GenBank^TM accession #FJ748884) and is expressed in all human tissues with high expression in pancreas, heart, prostate, ovary and spleen [17]. IGFBP-3R binds specifically to IGFBP-3 but no other IGFBP species and mediates IGFBP-3's intrinsic biological functions including its anti-inflammatory and anti-tumor functions in normal and cancer cells [13,17-19].

IGF System in Obesity and Insulin Resistance

Whereas elevated levels of IGF-I are associated with decreased IR, T2DM and CVD risk [21] in overweight and obese states, IGF-I levels are decreased, thus limiting its glucose regulating and anti-inflammatory actions and increasing IR. These biological effects are modulated by IGFBP-3, the most abundant circulating binding protein and transporter of as much as 90% of IGF [22]. In multiple animal as well as insect models, IGFBP-3 inhibits activation of the insulin receptor and demonstrates high affinity for binding to IGF-I and II, pro-insulin and mini-proinsulin [22]. IGFBP-3 is associated with hepatic IR and decreased peripheral glucose sensitivity whereas IGF-I produced the opposite effect indicating that the balance of IGF-I/IGFBP-3 is critical in glucose homeostasis [23,24]. Research has shown that independent of age and race lower IGF-I/IGFBP-3 ratio is significantly associated with metabolic syndrome [25]. Gender has been found to differentially affect the concentrations of IGF-I and IGFBP-3 in healthy older adults. It has been observed that while total and free IGF-I and IGFBP-3 were decreased in both obese men and women over decades of aging; the decreases were significantly higher in women [26]. With excessive gestational weight gain and obesity, IGFBP-3 expression is increased resulting in decreased IGF

bioavailability and subclinical IR [27]. In observational human studies, cancer mortality is increased in the presence of obesity as well as T2DM. One underlying mechanism is likely hyperinsulinemia given that individuals with low insulin, IGF-I and II levels lowers cancer risk [28]. In reviews of the effects of IGF-I and IGFBPs, exogenous administration of recombinant human IGF-I has been shown to improve insulin sensitivity in healthy and IR individuals and insulin sensitivity and hyperglycemia in diabetic individuals as well as improve systemic inflammationraj [14], further supporting the role of the IGF-I system in IR and T2DM.

In addition to its role as a binding protein, mounting evidence suggests that IGFBP-3 acts independently of IGF. In cellular and animal models, IGFBP-3 inhibited tumor growth independent of IGF [13,17,22,29]. In vitro, IGFBP-3 reduces cell proliferation in cancer cells as well as insulin-stimulated glucose utilization in adipocytes [22]. Additionally, IGFBP-3 (as well as IGFBP-2) may be responsible for improvements in metabolic status following calorie restriction in obese women with T2DM [30].
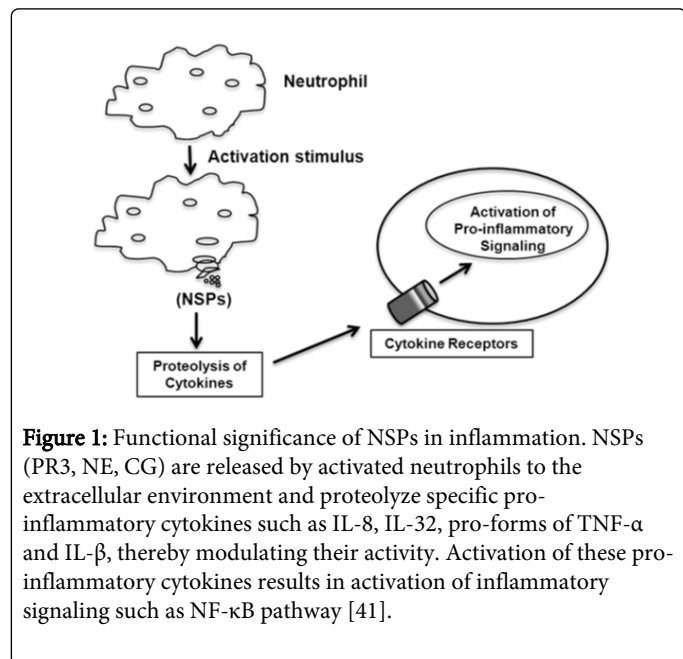
Visceral fat obesity and not subcutaneous fat obesity, correlates significantly with insulin resistance [31], hypertension [32] and cardiac dysfunction [33]. In obesity, fat depots increase the levels of pro-inflammatory cytokines/adipokines such as TNF-α, C-reactive protein (CRP), -α, IL-6, leptin, visfatin, resistin, angiotensin II, and plasminogen activator inhibitor creating a state of local inflammation further resulting in chronic systemic inflammation and accelerating the events leading to metabolic disorders [34-36]. With respect to insulin resistance, those pro-inflammatory cytokines/adipokines contribute to insulin resistance in adipocyte in an autocrine/paracrine manner by impairing insulin signaling through inhibition of expression and posttranslational (phosphorylation) modification of genes involved in insulin signaling such as insulin receptor substrate-1 (IRS-1), glucose transporter type 4 (GLUT4) [5]. In addition, they propagate the inflammatory diathesis systemically and further impair insulin action in peripheral tissues including liver and skeletal muscle [37].

Interestingly, as outlined in our recent publication, IGFBP-3 inhibits TNF-α-induced NF-κB activity through IGFBP-3R, thereby restoring insulin signaling and negating TNF-α-induced inhibition of glucose uptake in human primary adipocytes [20]. These results suggest that the IGFBP-3/IGFBP-3R system plays an important role in cytokine-induced IR in visceral adipocytes. Furthermore, we demonstrated a decrease in total IGFBP-3 levels and an increase in proteolyzed IGFBP-3 in the circulation of overweight and obese adolescents when compared with their non-obese counterpart [20]. Moreover, we observed significant positive correlations between IGFBP-3 proteolysis and adiposity parameters such as waist circumference, body mass index and homeostasis model assessment of insulin resistance (HOMA-IR) [20]. These findings suggest that increased IGFBP-3 proteolysis in overweight and obese adolescent likely results in reduced levels of intact IGFBP-3 in circulation, effectively blunting the anti-inflammatory and insulin-sensitizing functions of IGBPB-3 in adipose tissue.

Neutrophil Serine Proteases and Inflammation

Obesity is also associated with activation of neutrophils and the innate immune system [38]. In addition to directly secreting pro-inflammatory cytokines, adipocytes further enhance the inflammatory milieu in obesity by recruiting in situ inflammatory cells including

macrophages and lymphocytes [39]. Neutrophil serine proteases (NSPs) represent one third of all identified proteases and are released at sites of inflammation where they activate pro-inflammatory cytokines (Figure 1) [40]. Although classically associated with innate immunity and pathogen destruction, NSPs are also involved in the regulation of inflammation and the pathogenesis of many chronic inflammatory conditions.

Figure 1: Functional significance of NSPs in inflammation. NSPs (PR3, NE, CG) are released by activated neutrophils to the extracellular environment and proteolyze specific pro-inflammatory cytokines such as IL-8, IL-32, pro-forms of TNF-α and IL-β, thereby modulating their activity. Activation of these pro-inflammatory cytokines results in activation of inflammatory signaling such as NF-κB pathway [41].
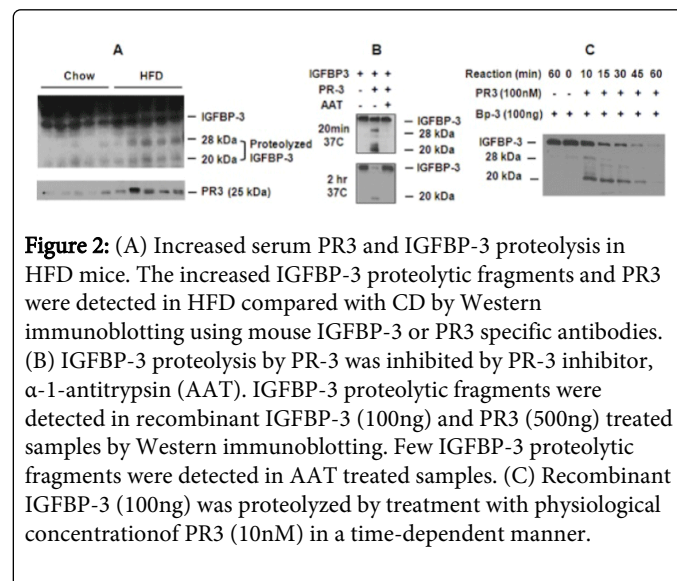
Proteinase 3 (PR3) is secreted from activated neutrophils and is critically involved in bacterial defense but also regulates non-infectious inflammatory processes by inducing endothelial cell apoptosis and modulating the activities of cytokines including TNF-α, IL-1β, IL-8, IL-18 and IL-32 [42-48] Recent studies further suggest that PR3, as well as other NSPs, namely neutrophil elastase (NE) and cathepsin G (CG) contribute to neutrophil-dependent inflammation and progression of chronic inflammatory diseases including T2DM, cystic fibrosis and glomerulonephritis [49-52]. Conversely, NSP inhibitors such as α-1-antitrypsin (AAT) have been proposed as treatments in patients with chronic inflammatory diseases including diabetes, cystic fibrosis and ischemic heart disease [28,53-56].

PR3 and IGFBP-3 proteolysis in Obesity

Evidence of increased IGFBP-3 proteolysis in overweight individuals, suggests its potential role as a biomarker for predisposition to IR [20]. Since it has been reported that PR3 appears to contribute to progression of chronic inflammatory diseases, we characterized PR3 as a specific IGFBP-3 protease in obese conditions. As shown in Figure 2A, serum PR3 and IGFBP-3 proteolytic fragments were significantly increased in mice fed a high fat diet compared to those fed a chow diet. Furthermore, recombinant IGFBP-3 was proteolyzed by PR3 while AAT completely inhibited PR3-induced IGFBP-3 proteolysis (Figure 2B). In fact, treatment with PR3 at a physiological concentration (10nM) resulted in significant IGFBP-3 proteolysis following 10 minutes of incubation at $37^{\circ}C$ (Figure 2C). Taken together, these findings suggest that systemic inflammation in obesity increases PR3 activity, which causes a significant increase of IGFBP-3 proteolysis and abrogates the insulin-

sensitizing actions of the IGFBP-3/IGFBP-3R cascade in visceral fat, thereby resulting in IR and further progression to T2DM.

Figure 2: (A) Increased serum PR3 and IGFBP-3 proteolysis in HFD mice. The increased IGFBP-3 proteolytic fragments and PR3 were detected in HFD compared with CD by Western immunoblotting using mouse IGFBP-3 or PR3 specific antibodies. (B) IGFBP-3 proteolysis by PR-3 was inhibited by PR-3 inhibitor, α-1-antitrypsin (AAT). IGFBP-3 proteolytic fragments were detected in recombinant IGFBP-3 (100ng) and PR3 (500ng) treated samples by Western immunoblotting. Few IGFBP-3 proteolytic fragments were detected in AAT treated samples. (C) Recombinant IGFBP-3 (100ng) was proteolyzed by treatment with physiological concentrationof PR3 (10nM) in a time-dependent manner.

In order to identify the functional significance of PR3 in obesity-induced IGFBP-3 proteolysis in human and its correlation with obesity, we have analyzed 34 serum samples of lean (n=14), overweight (n=14) and obese (n=6) premenopausal women aged 35-50 years (Tables 1-3). Comparisons of clinical features in all groups show that the overweight and obese groups had significantly higher BMI, waist circumference, systolic BP and diastolic BP than lean group (Table 1). Triglycerides levels were markedly increased in the obese group. The obese group had much higher levels of TNF-α and IL-8 than the controls (Table 2). Levels of HMW adiponectin were significantly decreased in overweight and obese groups whereas total adipnectin was decreased only in overweight group compared to the controls. The levels of fasting insulin, 2 hours fasting insulin, 2 hours fasting glucose and HOMA-IR were increased in the obese group compared to the controls whereas fasting glucose was not significantly different among the groups (Table 3).

	Lean	Overweight	Obesity	P value
Subjects	14	14	6	NS
Age (years)	42.7 ± 1.1	45.14 ± 1.2	43.5 ± 2.0	NS
BMI (kg/m 2)	22.1 ± 0.4	26.9 ± 0.3	31.5 ± 0.4	a
Waist Circumference (cm)	29.8 ± 0.5	34.3 ±0.5	38.2 ± 0.9	b
Systolic BP (mmHg)	101.4± 2.0	112.7 ± 3.2	115.2 ± 5.7	c
Diastolic BP (mmHg)	68.0 ± 0.5	73.6 ± 2.3	76.8 ± 3.2	d

Table 1: Clinical and anthropometric characteristics of subjects.

NS: Not Significant; BMI: Body Mass Index.

Each group was classified by the BMI percentile according to Growth Charts.

[a]lean vs. overweight (P < 0.001), and lean vs. obesity (p < 0.001).

[b]lean vs. overweight (P < 0.01), and lean vs. obesity (p < 0.001).

[c]lean vs. overweight (P < 0.05), and lean vs. obesity (p< 0.05).

[d]lean vs. obesity (p < 0.05).

Values are expressed as mean ± SE.

P values were calculated by one-way ANOVA.

	Lean	Overweight	Obesity	*P* value
Total cholesterol (mg/dL)	187.6 ± 6.5	201.9 ± 9.5	202.3 ± 21.0	NS
HDL-cholesterol (mg/dL)	59.4 ± 2.7	52.2 ± 2.9	47.8 ± 4.1	NS
Triglycerides (mg/dL)	62.0 ± 4.2	99.4 ± 16.2	120.0 ± 19.5	a
LDL-cholesterol (mg/dL)	106.9 ± 5.1	123.3 ± 9.0	128.2 ± 15.1	NS
TNF-alpha	9.9 ± 4.8	11.5 ± 3.4	34.1 ± 22.4	a
IL-8	2.8 ± 0.3	2.9 ± 0.4	5 ± 0.9	a
HMW Adiponectin	2886 ± 320.8	1562 ± 242.6	1809 ± 324.9	a,b
Total Adiponectin	9255 ± 653.7	6894 ± 565.5	8009 ± 1022	c
hs-CRP (mg/dL)	1.6 ± 0.7	1.4 ± 0.3	6.1 ± 2.6	NS

Table 2: Biochemical characteristics of subjects.

NS: Not Significant;

[a]Lean vs. obesity (P < 0.05).

[b]Lean vs. overweight (P < 0.01).

[c]Lean vs. overweight (P < 0.05).

[d]Overweight vs. obesity (P < 0.05).

Values are expressed as mean ± SE.

P values were calculated by one-way ANOVA.

	Lean	Overweight	Obesity	*P* value
Fasting glucose (mg/dL)	85.4 ± 2.0	89.9 ± 1.7	92.0 ± 4.2	NS
2 hours fasting glucose (mg/dL)	89.0± 5.9	94.1 ± 4.1	96.8 ± 2.5	a
Fasting insulin (µIU/Ml)	3.9 ± 1.5	4.9 ± 0.6	13.6 ± 5.3	b
2 hours fasting insulin (uIU/MI)	15.1 ± 2.2	26.3 ± 5.3	50.2 ± 16.1	c
HOMA-IR	0.8 ± 0.3	1.1 ± 0.1	3.4 ± 1.5	a

Table 3: Insulin resistance of subjects

NS, not significant;

[a]Lean vs. obesity (*P* < 0.05).

[b]Lean vs. obesity (*P* < 0.01).

[c]Lean vs. obesity (*P* < 0.001).

Values are expressed as mean ± SE.

P values were calculated by one-way ANOVA.

Furthermore, as shown in Figure 3, an increase in PR3 and proteolyzed IGFBP-3 was observed in circulation in overweight and obese women when compared to their lean counterparts. Additionally, IGFBP-3 proteolysis was inversely correlated with adiponectin (r=-0.490, p<0.01) and positively correlated with PR3 (r=0.8377, p<0.001), HOMA-IR (r=0.4579, p<0.01), BMI (r=0.725, p<0.001), TNF-α (r=0.413, p<0.05) and IL-8 (r=0.402, p<0.05). Although sample numbers are limited, our data clearly demonstrate correlation of PR3 with IGFBP-3 proteolysis and adiposity parameters in overweight and obese population and it is statistically significant. We are currently initiating further investigation of functional significance of PR3 as well as NE and CG in a larger population. We anticipate that all three NSPs are involved in obesity-induced IGFBP-3 proteolysis and insulin resistance.
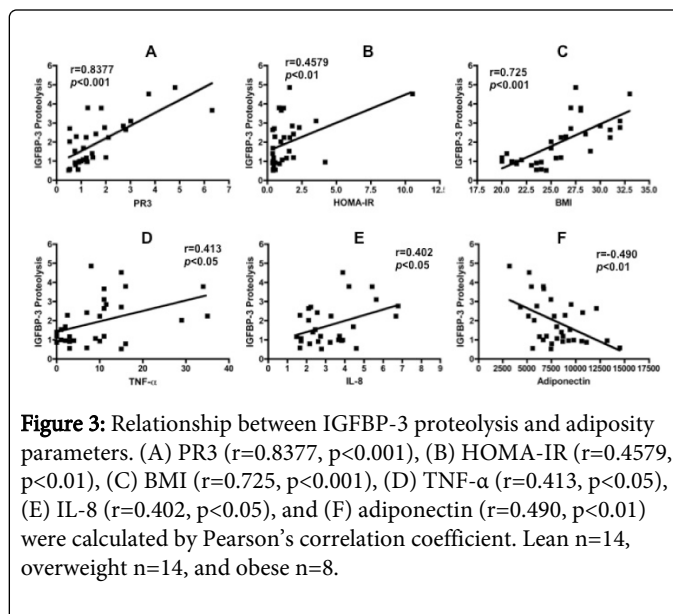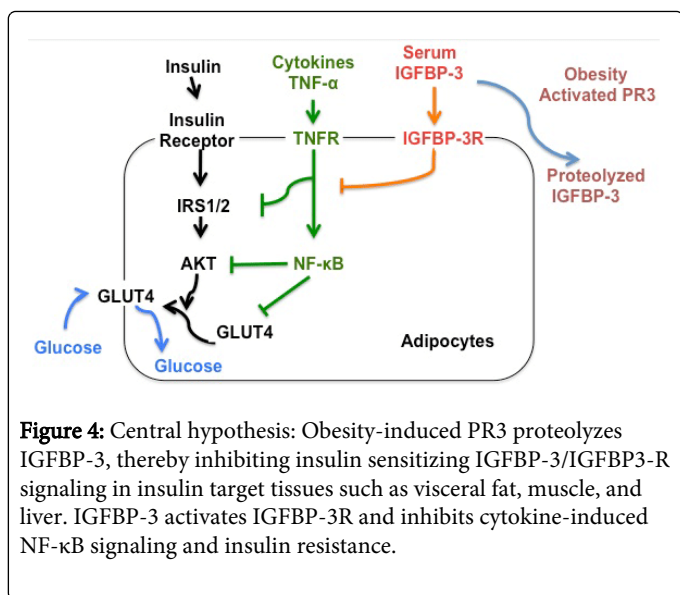

Figure 3: Relationship between IGFBP-3 proteolysis and adiposity parameters. (A) PR3 (r=0.8377, p<0.001), (B) HOMA-IR (r=0.4579, p<0.01), (C) BMI (r=0.725, p<0.001), (D) TNF-α (r=0.413, p<0.05), (E) IL-8 (r=0.402, p<0.05), and (F) adiponectin (r=0.490, p<0.01) were calculated by Pearson's correlation coefficient. Lean n=14, overweight n=14, and obese n=8.

In obesity, fat depots secrete pro-inflammatory adipokines/cytokines, leading to a state of IR via autocrine/paracrine influences [4]. Recent in vitro studies have demonstrated that inflammatory cytokines interfere with insulin signaling in visceral adipocytes by decreasing the levels of insulin receptor substrate-1 (IRS-1), glucose transporter-4 (GLUT4) and adiponectin. As a result, we propose the novel hypothesis that obesity-induced activation of NSPs, particularly PR3, leads to reductions in levels of intact, biologically-active IGFBP-3, which, in turn, abrogates the insulin-sensitizing IGFBP-3/IGFBP-3R cascade in target tissues and contributes to development of systemic IR (Figure 4). Moreover, we speculate that interventions such as Aralast, Prolastin, and sivelestat that inhibit NSP activity, thereby reducing NSP-induced IGFBP-3 proteolysis would reduce inflammation and improve insulin sensitivity and further progression to T2DM in overweight and obese individuals.

Figure 4: Central hypothesis: Obesity-induced PR3 proteolyzes IGFBP-3, thereby inhibiting insulin sensitizing IGFBP-3/IGFBP3-R signaling in insulin target tissues such as visceral fat, muscle, and liver. IGFBP-3 activates IGFBP-3R and inhibits cytokine-induced NF-κB signaling and insulin resistance.

Conclusion

Given the prevalence and associated morbidity and mortality of obesity and the limited success of lifestyle changes in reducing its incidence, more effective preventive and therapeutic strategies are needed. Thus, a clearer understanding of the mechanisms leading to obesity-associated IR is necessary to identify novel targets for the prevention and treatment of many IR-driven conditions such as T2DM. To this end, in recent studies of overweight and obese individuals, we have identified strong positive correlations between IGFBP-3 proteolysis and adiposity parameters and PR3 as a specific IGFBP-3 protease in serum, suggesting that NSPs may also play a role in the development of IR. These findings strongly suggest that obesity-induced activation of PR3 abrogates the anti-inflammatory, insulin-sensitizing IGFBP-3/IGFBP-3R cascade, resulting in IR and its progression to T2DM. The complete characterization of the underlying mechanism and functional significance of the PR3-IGFBP-3/IGFBP-3R cascade in obesity will foster identification of the diagnostic and therapeutic potential of PR3 inhibition in insulin resistance and its sequelae.

Acknowledgement

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