Short Communication

Journal of Nutritional Biology

Insulin Receptor Levels Regulated by the Receptor-Associated Protein Progesterone Receptor Membrane Component 1 (PGRMC1)

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Rec date: Apr 17, 2018; Acc date: May 17, 2018; Pub date: May 22, 2018

Abstract

As of 2014, 29.1 million Americans suffer from diabetes, creating a severe socioeconomic and medical burden on society. Impaired insulin signaling is key to the development of type 2 diabetes, presenting a unique therapeutic challenge. Obese individuals demonstrate decreased insulin binding due to a reduction in IR levels, without an alteration in ligand-receptor binding affinity. The progesterone receptor membrane component 1 (PGRMC1) is an endosomal protein that promotes cellular signaling via altered receptor trafficking. A recent translational study determined that PGRMC1 was decreased in patients with insulin-resistant disease, suggesting a role in insulin signaling. In the present study, we hypothesized that PGRMC1 affects the levels of IR β (insulin receptor- β sub-unit) in adipocytes. Indeed, we show that treatment with PGRMC1 ligands significantly increase IR β protein levels in fully differentiated human subcutaneous adipocytes. Protein levels are likely affected through the direct interaction of PGRMC1 and IR β , as we demonstrate their co-immunoprecipitation in differentiated 3T3-L1 cells. Notably, PGRMC1 ligand treatment significantly reduced IR β protein levels in two rodent model systems, indicating a pharmacological difference across species.

Keywords: Signaling; Insulin receptor; Adipocyte

Abbreviations: EGFR: Epidermal Growth Factor Receptor; IR β : Insulin Receptor β ; PGRMC1: Progesterone Receptor Membrane Component 1

Introduction

Rates of diabetes are expected to increase due to rampant obesity and will become a worldwide health crisis in the future [1]. The manifestations include hypertension, hyperlipidemia, hyperglucocorticoidemia and type 2 diabetes. The latter is associated with insulin resistance, a heterogeneous disease characterized by defects in insulin signaling [2]. The insulin receptor (IR) is a receptor tyrosine kinase that initiates IR signaling through

multiple cascades [3]. Binding of insulin to its receptor (IR) triggers downstream signaling that includes IRS1/2 (insulin receptor substrate), AKT and ERK/extracellular signal-regulated kinase [4]. IR is a tetramer comprised of 2 α (ligand binding) and 2 β (kinase domain) chains that is expressed in numerous tissues. Signaling from the IR through the IRS-1/PI3K (phosphatidylinositol-3 kinase)/AKT pathway results in a rapid translocation of the GLUT-4 (glucose transporter 4) from intracellular vesicles to the plasma membrane, increasing the cellular uptake of

glucose [5], a step that is critical in preventing diabetes complications.

Adipose tissue performs a key function in glucose homeostasis, because it acts as a reservoir for circulating glucose [6]. When there is an excess of adipose tissue, such as in high BMI individuals, glucose catabolism is disrupted, and diminished insulin receptor function is thought to contribute [7]. Indeed, obese individuals show decreased insulin binding in skeletal muscle, adipose tissue and liver [8]. Mouse models of skeletal muscle-specific vs fat-specific IR-knockout (MIRKO and FIRKO respectively) suggest that glucose uptake by muscle is more significant for diabetes, which was anticipated since the muscle is believed to account for 80% of glucose absorption [3,9]. However, other studies suggest that adipose is a critical site for glucose metabolism, and the development of obesity and its associated abnormalities [10].

PGRMC1 (progesterone receptor membrane component 1 [11]) plays an important role in signaling by transporting transmembrane receptors to the plasma membrane [12,13], including receptor tyrosine kinases [13,14]. PGRMC1 localizes to endosomes, the endoplasmic reticulum [15,16] and plasma membrane [17]. In cancer, PGRMC1 associates with the EGFR (epidermal growth factor receptor) tyrosine kinase [16,18-20], increases plasma membrane levels of EGFR [16] and increases cellular signaling [21-23]. In contrast, the EGFR-related protein HER2/neu was not PGRMC1-dependent [16], suggesting a trafficking specificity.

In addition to tyrosine kinases, PGRMC1 also increases plasma membrane pools of GLP-1R (glucagon-like peptide 1 receptor) and mPR α 1, a plasma membrane progesterone receptor [13,14]. The latter likely contributes to progesterone binding activity by the PGRMC1 complex, which was how PGRMC1 was originally identified [24-27]. Binding to the liganded GLP-1R complex likely contributes to glucose homeostasis, as PGRMC1 has been found to modulate glucose-induced insulin stimulation in beta cells [14]. PGRMC1 has numerous other binding partners, including cytochrome P450 proteins, PAIR-BP1 (plasminogen activator inhibitor RNA binding protein 1) and β -tubulin [28]. PGRMC1 is an appealing therapeutic target because it has a small molecule ligand, called AG205 [22], that was identified by our group and has been verified by others [22,29]. Based on photoaffinity cross-linking studies with a labelled sigma-2 receptor ligand called WC-21, PGRMC1 was also identified as a

component of the sigma-2 receptor complex [29]. Sigma receptors are low molecular weight receptors for multiple endogenous and exogenous ligands [30]. It is notable that PGRMC1 was originally thought to be a sigma receptor [27], but recent evidence indicates that they are separate proteins [31-33].

In the present study, we hypothesized that PGRMC1 affects the levels of IR β (insulin receptor- β sub-unit) in adipocytes. We have investigated the role of PGRMC1 in adipose specific IR regulation. There is a precedent for PGRMC1 being associated with insulin signaling, because a clinical study of insulin-resistant, high BMI (body mass index) subjects demonstrated decreased PGRMC1 RNA levels compared to insulin-sensitive subjects [34]. However, this study did not examine mechanistic link between IR and PGRMC1.

The down-regulation of PGRMC1 RNA in insulin-resistant diabetes suggests a role for PGRMC1 in regulating IR in adipocytes. To test this model, human tissue-derived adipocytes were treated with PGRMC1 ligand AG205 [22]. PGRMC1 has been identified as a sigma-2 receptor-associated protein in binding studies [29], so we also tested the activity of the sigma-2 receptor ligand PB28 [35]. The cellular morphology of the adipocytes was unchanged upon treatment with either ligand. AG205 significantly increased IR β protein levels in adipocytes derived from pooled BMI (body-mass index, median 26.8, range of [25.3-28.6]) donors (Figure 1A and B, $p=0.004$, t -test) and the high BMI donor (BMI 38, $p=0.013$, t -test). Treatment with PB28 did not affect IR β protein levels in adipocytes derived from high BMI donors, but increased IR β in adipocytes derived from pooled BMI donors (Figure 1A and B, $p=0.027$, t -test). There was no significant change in IR β protein levels after ligand treatments in adipocytes derived from the low BMI 23 donor. Protein analysis was performed by western blot using an antibody to the IR β sub-unit. Total basal IR β protein levels were lower in adipocytes derived from pooled BMI donors and the high BMI donor (Figure 1A and B, $p=0.001$ and 0.006 , respectively, t -test), indicative of reduced insulin sensitivity. In adipose tissue from subjects with varying BMI, both IR and PGRMC1 protein levels decreased in subjects with high BMI (Figure 1C), consistent with RNA levels detected by microarray.

Although human tissue-derived adipocytes are an accurate model system for studying human metabolic processes, they vary between individuals and populations. In order to further characterize the interaction of

Figure 1: PGRMC1 ligand treatment in human adipocytes.

Insulin Receptor β (IR β) levels increase significantly after 24 hour treatment with 10 μ M AG205 and 1 μ M PB28 in human subcutaneous cultured adipocytes. (A) Western blot analysis of human subcutaneous adipocytes derived from 10 separate donors. GAPDH served as a loading control. (B) IR β protein levels were determined by western blot in fully differentiated human adipocytes sourced from multiple donors with a median BMI of 26.8 (Pooled), a donor with a 23 BMI (Low BMI), and donor with a 38 BMI (High BMI). IR β protein levels were significantly elevated after treatment with AG205 and PB28 ($p=0.004$ and $p=0.027$ respectively) in pooled BMI adipocytes. Treatment with AG205 significantly increased IR β levels in adipocytes derived from a high BMI donor ($p=0.013$). (C) Adipose samples taken from human patients matched with BMI. Protein analysis via western blot show a reduction of both PGRMC1 and IR β levels with increasing BMI.

Figure 2: PGRMC1 ligand treatment and co-immunoprecipitation with IR in rodent model systems.

Western blot analysis of total IR β protein levels in differentiated 3T3-L1 cells and differentiated rat SVF-isolated cells after AG205 (20 μ M) treatment. Total IR β protein levels were significantly reduced after treatment with AG205 (lane 2) in both differentiated 3T3-L1 (A) and rat SVF-isolated (B) cells ($p<0.05$ and $p<0.01$ respectively). (C) IR β and (D) PGRMC1 were immunoprecipitated (IP) from differentiated 3T3-L1 cells. Immunoprecipitation with an irrelevant antibody (con Ab, lane 1) is a control. (C) IR β was immunoprecipitated (lane 2) and probed (western-blot) for IR β (top panel) and PGRMC1 (bottom panel). (D) PGRMC1 was immunoprecipitated (lane 2) and probed for IR (top panel) and PGRMC1 (bottom panel).

PGRMC1 with IR, we utilized two rodent model systems- the 3T3-L1 murine cell line and cells isolated from stromal vascular fractions derived from the subcutaneous adipose of Sprague Dawley rats. To determine the effect of the PGRMC1 ligands on IR levels in these rodent model systems, we treated differentiated 3T3-L1 cells and differentiated rat SVF-isolated cells with AG205. Interestingly, the drug treatment significantly reduced total IR protein levels in both rodent model systems (Figure 2A and B, lanes 2. $p < 0.05$ and $p < 0.01$ respectively). These results were opposite of the pharmacological effects of AG205 in human adipocytes.

Next, we investigated the potential interaction of PGRMC1 with IR. PGRMC1 interacts directly with the EGFR receptor tyrosine kinase and the membrane progesterone receptor mPR α . Furthermore, we have detected a direct interaction between PGRMC1 and IR in human cancer cells (Hampton, et al. Molecular Pharmacology, in press). Both PGRMC1 and IR were immuno-precipitated from differentiated 3T3-L1 cells and analyzed by western blot. IR β was efficiently precipitated (Figure 2C, upper panel lane 2), and PGRMC1 co-precipitated with IR β (Figure 2C, lower panel lane 2). Similarly, PGRMC1 was efficiently precipitated (Figure 2D, upper panel lane 2), and IR β co-precipitated with PGRMC1 (Figure 2D, lower panel lane 2). The same lysates were precipitated with a control antibody that matched the antibodies for IR β and PGRMC1 (Figure 2C-D, lanes 1). These results suggest a direct interaction of PGRMC1 with IR in adipocytes.

Supplemental Methods

Cell culture

Differentiated human subcutaneous adipocytes from multiple donors were purchased from Zenbio, Inc. (Research Triangle Park, NC) and maintained in proprietary media according to the manufacturer's instructions. Lot#SL0055 contained differentiated human subcutaneous adipocytes derived from 10 female donors with a median age of 46.7 and median BMI of 26.8. Lot #L100610B contained differentiated human subcutaneous adipocytes from a female Caucasian with a BMI of 23.3 and age of 40. Lot#L072709 contained differentiated human subcutaneous adipocytes from a female of unknown ethnicity with a BMI of 38.0 and age of 39. All donors were non-smokers.

3T3-L1 cells were obtained from ATCC (Manassas, VA), verified by Genetica LLC (Cincinnati, OH) and cultured under the suggested conditions. 3T3-L1 cells were maintained in media (Zenbio, Inc., Research Triangle

Park, NC) and differentiated according to manufacturer's protocol. Medium included: Preadipocyte Medium (cat# PM-1-L1), Differentiation Medium (cat# DM-2-1) and Adipocyte Medium (cat# AM-1-L1).

The collection of stromal vascular fraction was obtained from the visceral fat of male Sprague-Dawley rats (Taconic Biosciences, Hudson, NY) after CO₂ asphyxiation. Harvested fat was washed in ice-cold HBSS (VWR, Radnar, PA), finely minced, and digested in 1 mg/ml type II collagenase solution prepared in HBSS (Sigma Aldrich, St. Louis, MO) for 45 min at 37°C with vigorous shaking. The mixture was then filtered through 250 μ m gauze mesh into a 50 mL tube and let stand 2-3 minutes. Infranant containing the collagenase solution was removed, and the floating layer of adipocytes were washed 3 $\times$ with 10 mL phosphate-buffered saline (VWR, Radnar, PA). The adipocytes were incubated with collagenase II solution (2 mL), vortexed and left shaking for 10 minutes at 37°C. After incubation, 2 mL of high-glucose DMEM (Corning, Manassas, VA) containing 10% BCS (Sigma Aldrich, St. Louis, MO) was added, mixed and the solution was filtered through 70 μ m nylon mesh cell strainer (VWR, Radnar, PA) and plated for cell culture. After cells were approximately 100% confluent, cells were differentiated according to the Zenbio, Inc., manufacturer's protocol (Research Triangle Park, NC).

Differentiated human subcutaneous adipocytes were treated with the PGRMC1 ligands, AG205 (10 μ M, TimTec, Inc., Newark, NJ) and PB28 (1 μ M, Sigma Aldrich, St. Louis, MO), for 24 hours and analyzed via western blot. Dose response and time courses were performed previously (data not shown) to establish the most effective concentrations and times. Differentiated 3T3-L1 cells and differentiated rat SVF-isolated cells were incubated with AG205 (20 μ M) for 90 minutes and underwent protein analysis.

Immunological techniques

Western blots and immunoprecipitations were performed as previously described [16,36]. The antibodies used in this study were anti-insulin receptor β (Novus Biologicals, Littleton, CO, Cell Signaling, Danvers, MA), PGRMC1 (Abcam, Cambridge, MA), and anti-GAPDH (Santa Cruz). Western blots for PGRMC1 were performed with the PGR-UK1 polyclonal anti-body [22]. Western blots were performed at least in triplicate. Full-sized western blots and a table of statistics for figure 1A are on the web: <http://pharmns.med.uky.edu/users/rjcrav2#profileTab4>.

Conclusions

IR signaling is key in the progression of metabolic disease, and we have shown that IR levels are elevated in adipocytes treated with two different ligands for the PGRMC1 protein. This stands in stark contrast to the behavior of the PGRMC1 ligand AG205 in cancer cells, where it has no effect on overall IR levels (Hampton, et al. *Molecular Pharmacology*, in press) and decreases EGFR levels [16]. There is compelling evidence for an interaction between PGRMC1 and sigma-2 receptor [29], AG205 and PB28 behaved similarly in a set of pooled adipocytes from 10 donors, but the ligands were not alike in adipocytes from individual donors.

The mechanisms through which PGRMC1 regulates IR protein levels is unclear. Potential mechanisms include modulation of INSR RNA levels and changes in the translation and stability of the IR protein. We were unable to obtain both RNA and protein from the primary human cultures in this study, so the effects of AG205 on RNA levels are unknown. However, we note that AG205 decreased EGFR protein levels in human breast cancer cells with no effect on RNA levels [16]. There is no precedent for PGRMC1 affecting translation, although kinases affecting translation are regulated by PGRMC1 [36]. In contrast, PGRMC1 exerts a potent effect on autophagy and protease activation [20,36]. Thus, one potential mechanism for AG205 is that it acts through PGRMC1 to decrease IR- β proteolytic degradation in humans or promote degradation in rodents, possibly by modulating autophagy, matrix metalloproteinases and/or cathepsins. The results suggest an intriguing species-specific function for PGRMC1 in adipose tissues. Nagy and colleagues recently reviewed the physiological and metabolic profiles of rodent white adipose fat pads with white adipose fat depots in humans and urged researchers to carefully consider experimental designs given some of the stark differences in adipose tissue location and function among species [37]. Furthermore, Barnard and colleagues argued that dietary modification in rodent models has limited translatable benefit for understanding the pathogenesis of human obesity and diabetes based on the constraints of the high fat diet [38]. The effects of the PGRMC1 ligand AG205 may reflect differences in protease activation in humans and rodents. Further research is needed to refine these possibilities.

Finally, Elbein et al. showed that PGRMC1 RNA levels decrease in adipose tissue from insulin-resistant subjects compared to insulin-sensitive subjects [31]. Our findings extend the observation in a small cohort of adipose

tissues, revealing diminished PGRMC1 protein levels in two high BMI subjects. Thus, there is potentially a strong translational impact because the research could lead to new small molecule therapeutics that will enhance the treatment of insulin-sensitive diabetes and reverse the course of insulin-resistant diabetes. With millions of people worldwide expecting to develop diabetes in the future, PGRMC1-directed therapeutics could become an important approach to treating the disease. There are a number of diabetes treatments currently available, including drugs that lower blood sugar [39], activate IR and alter the production of insulin. Because of the long-term course of the disease and the vast numbers of people becoming diabetic, new therapeutics are needed for diabetes.

Acknowledgements

This work was supported by the Washington University Diabetes Research Center grant P30 DK020579-40, the University of Kentucky CTSA grant UL1 TR001998 and the Research Investment Program from the Department of Pharmacology and Nutritional Sciences and the University of Kentucky Office of the Vice President for Research. K.H. was a trainee of the NIH T32 DK007778 grant.

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