IRW improves glucose tolerance in high fat diet fed C57BL/6 mice via activation of insulin signaling and AMPK pathways in skeletal muscle

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Declarations of interest: None
Abstract

IRW, an ovotransferrin-derived peptide, previously showed antihypertensive and anti-inflammatory activities in rodents and in cells. Moreover, IRW prevented angiotensin-II- and tumor necrosis factor (TNF)-α-induced IR in skeletal muscle cells. In this study, we investigated the effects of IRW on body composition, glucose homeostasis and insulin sensitivity in a high-fat diet (HFD) induced obese model. C57BL/6 mice were fed HFD for 6 weeks, after which IRW was incorporated into the diet (45 or 15 mg/kg body weight (BW)) for another 8 weeks. IRW45 reduced BW (p<0.05), fat mass gain (p<0.01), and preserved lean mass (p<0.01) of HFD mice. This was accompanied by enhanced glucose tolerance in the oral glucose tolerance test (OGTT) and reduced fasting blood glucose. In skeletal muscle, IRW45 up-regulated insulin signaling, increased protein kinase B (AKT) phosphorylation (p<0.05) and glucose transporter 4 (GLUT4) abundance in the plasma membrane (P<0.0001). 5’-AMP-activated protein kinase (AMPKα) (p<0.05) and peroxisome proliferator-activated receptor gamma (PPARγ) RNA expression (p<0.05) and protein abundance (p<0.001) were increased in skeletal muscle of IRW45-treated mice, as was the expression of genes involved in myogenesis. Angiotensin converting enzyme-2 (ACE2) activity in the plasma (p<0.01) and angiotensin 2 receptor (AT2R) abundance (P<0.01) in skeletal muscle were enhanced. The thermogenic protein uncoupling protein-1 in WAT was suppressed by HFD and partially restored after IRW supplementation. IRW improves glucose tolerance and body composition in HFD-fed mice. IRW promotes glucose uptake in skeletal muscle via multiple signaling pathways, including insulin signaling and the AMPK pathway, independent of ACE inhibition.

Keywords: bioactive peptides, IRW, insulin resistance, obesity, PPARγ, ACE2
1. Introduction

Metabolic syndrome (MetS) is a condition characterized by obesity, IR, hypertension, and dyslipidemia. The underlying pathophysiology of MetS depends on hyperinsulinemia accompanied by free fatty acid accumulation in peripheral tissues, but the role of obesity, reactive oxygen species, and inflammation cannot be underrated during the progression of the syndrome[11]. MetS also involves the RAS, the system responsible for regulating blood pressure. In the context of MetS pathophysiology, RAS overactivation is linked to obesity and IR[2]. Besides the systemic RAS, there is an independent local RAS in several tissues including skeletal muscle[3]. Local RAS modulation in skeletal muscle influences the tissue response to insulin[4] and ACE inhibitors or angiotensin II type 1 receptor (AT1R) blockers reduce IR in animal models and also improve insulin sensitivity in humans[5-7], supporting the link between RAS and IR. Also, RAS blockade reverses the deleterious effect of exogenous angiotensin II on skeletal muscle mitochondria and improves glycemic control in mice[8].

Food-derived bioactive peptides exert effects beyond their nutritional value; they modulate physiological parameters in different tissues and exert a plethora of health effects[9]. Previous research demonstrated the beneficial effects of egg-derived peptides and hydrolysates on glucose tolerance[10], adipogenic capacity in vitro[11], and osteoblast differentiation[12]. Interestingly, some egg-derived bioactive peptides have ACE inhibitory activity[13] and potentially exert beneficial effects in the management of IR and glucose intolerance[14]. Of particular interest is the ovotransferrin-derived tripeptide IRW (isoleucine-arginine-tryptophan). IRW is an ACE inhibitory peptide[15] that exhibits anti-inflammatory and antioxidant effects in endothelial cells[16, 17], reduces blood pressure in rodents[18, 19], and improves angiotensin II- or TNF-α-induced IR in skeletal muscle cells[20, 21].

In this study, we aimed to investigate the insulin sensitizing effects of IRW in vivo using a high fat diet (HFD)-induced obese C57BL/6 mouse model. Because of the intimate crosstalk between obesity, hypertension, and IR, we hypothesize that IRW supplementation improves glucose intolerance by inhibiting RAS locally in skeletal muscle. This, in turn, will improve muscle insulin signaling and consequently enhance GLUT4 translocation to the plasma membrane in skeletal muscle.
2. Materials and methods

2.1 Animals, diet, and body weight (BW) measurements

The animal experimental protocol was approved by the Animal Care and Use Committee of the University of Alberta (Protocol# 1402) in accordance with guidelines issued by the Canadian Council on Animal Care. This study followed the ARRIVE guidelines. Thirty-two male 4-week-old C57BL/6 mice were purchased from Charles River Canada (St. Constant, QC, Canada) and housed 2 per cage with ad libitum access to standard chow and water for 1 week. C57BL/6 mice is a well-established model of insulin resistance that mimics many features of human biology such as impaired glucose tolerance accompanied by molecular mechanisms that are conserved across species. The animals were in a 12:12-hour cycle of light: dark with controlled humidity and temperature (60% and 23 °C). Eight mice were fed with low fat diet (LFD, 10% kcal from fat, Envigo, Indianapolis, IN, USA, TD06415) and the remainder with HFD (45% kcal from fat, Envigo TD110675) for 6 weeks. After this period, animals were randomly assigned to 4 groups: low fat diet control (LFD), high fat diet control (HFD), high dose IRW (45mg/kg BW) + HFD (IRW45), low dose IRW (15mg/kg BW) + HFD (IRW15) (n=8 per group). These diets continued for another 8 weeks with ad libitum access to food and water. In total mice consumed HFD for 14 weeks to induce obesity and glucose intolerance[22]. IRW was synthesized by Genscript (Piscataway, NJ, USA). Food consumption was measured once every 3 days and BW twice weekly. Body composition was evaluated before and after IRW treatment using Echo MRI™ (Echo Medical Systems LLC, Houston, TX, USA).

2.2 Oral glucose tolerance and insulin tolerance tests

After 6 and 7 weeks of IRW feeding, respectively, ITT and OGTT were performed as previously described[23] and with the following modifications: For ITT, 1.5 IU/kg BW insulin was injected intraperitoneally. For OGTT, 70% glucose solution was used and 1g of glucose/kg BW was given via oral gavage. In both cases, blood glucose was measured after 0, 15, 30, 60, 90, and 120 minutes. Fasting glucose and fasting insulin measurements in the tissue collection day were used to calculate homeostatic model assessment insulin resistance (HOMA-IR) using the formula: ([fasting glucose (mmol/L)]* [fasting insulin (μU/mL)]/22.5). OGTT was the primary outcome assessed in this study.
2.3 Tissue collection

At the end of the study, all animals were fasted for 16 hours and injected with insulin (2 IU/kg BW) intraperitoneally to stimulate insulin signaling 10 min prior to euthanization. Animals were euthanized using CO₂ and blood was collected via cardiac puncture. Blood was centrifuged at 3000g for 15 min to obtain plasma, which was stored at -80°C. Gastrocnemius skeletal muscle and WAT from retroperitoneal and epidydimal depots were collected, snap frozen and stored at -80°C until further analysis.

2.4 Protein extraction and western blotting

Skeletal muscle total protein was extracted using a lysis buffer containing phosphatase and protease inhibitors (aprotinin, sodium fluoride, sodium orthovanadate, and protease inhibitor cocktail). WAT total protein extraction was performed using a commercial kit (Invent Biotechnologies Inc., Plymouth, MA, USA) following the manufacturer’s instructions. Plasma membrane protein was extracted as previously described using a commercial kit (Thermo Fisher Scientific, Waltham, MA, USA)[20]. Total protein content was measured using the bicinchoninic acid assay. Western blotting was performed as previously described[10] with the following modifications: a 9% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run and protein was transferred to a nitrocellulose membrane, which was incubated overnight with antibodies against p-AKT (cs9271, Cell Signaling Technology, Beverly, MA, USA), AKT (sc82434, Santa Cruz Biotechnologies Inc, Dallas, TX, USA), GLUT4 (ab654, Abcam, Toronto, ON, Canada), ACE (ab75762), ACE2 (ab15348), AT1R (ab18801), AT2R (ab19134), Mas receptor (sc-390453), PPAR-γ (cs2430), mammalian target of rapamycin (mTOR) (CS4517), p-mTOR (cs5536), AMPKα (cs2532), p-AMPKα (Thr172, cs2535), P70 S6 kinase (S6K) (cs2708), p-P70 S6K (Thr389, cs9206), uncoupling protein (UCP)-1 (U6382, Sigma, St. Louis, MO, United States), UCP-2 (ab203244), UCP-3 (ab180643), β-actin (Sigma A5441) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab181602). After incubating with appropriate fluorescent-conjugated secondary antibodies (Li-cor Biosciences, Lincoln, NE, United States) for 1 hour at room temperature, protein bands were quantified by densitometry using Image Studio Lite 5.2 (Li-Cor Biosciences).

2.5 Plasma RAS components and insulin
Plasma concentrations of RAS components were quantified by mouse-specific commercial ELISAs according to the manufacturers’ instructions as follows: ACE (Aviva System, San Diego, CA, USA), ACE2 (Abcam), Angiotensin II (Enzo Life Sciences, Burlington, ON, Canada), Angiotensin (1-7) (Aviva System). Insulin was measured using an ELISA from (Abcam, Toronto, ON, Canada).

2.6 RNA sequencing

Total RNA was extracted from skeletal muscle using TRIzol reagent (Invitrogen, Life Technologies Inc., Burlington, Canada) and quantified by measuring the absorbance at 260 nm and purity assessment of RNA by the A260/280 ratio. The RNA integrity number (RIN) for all samples used in RNAseq was >8. Total RNA (500 ng) was used for the preparation of RNAseq libraries with the NEBNext Ultra II Directional RNA Library Prep Kit from Illumina (NEB, Mississauga, ON, Canada). Following enrichment of mRNAs, they were reverse-transcribed and second-strand cDNA synthesis was performed. Double-stranded cDNAs were A-tailed to enable adapter ligation and, finally, libraries were indexed by 15 PCR cycles. Libraries were sequenced on a NextSeq 500 instrument (Illumina), following a paired-end 150 cycle protocol. Deregulated transcripts were annotated using the BioMart database from Ensembl (EMBL-EBI Hinxton, Cambridgeshire, UK).

2.7 RT-PCR

Total RNA was isolated from mouse gastrocnemius muscle with TRIzol reagent. cDNA was synthesized from 1 μg total RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). mRNA expression of target genes was determined by real-time qPCR using GAPDH as the endogenous control. All of the qPCR (quantitative PCR) experiments and analyses were conducted using the MIQE guidelines for qPCR. The primers were designed based on the genomic sequence deposited in GenBank are described in table S1.

2.8 Statistics

All data presented were expressed as mean ± SEM of 5-8 mice from each treatment group as indicated in the figure and table legends. Statistical analysis was performed using GraphPad Prism 7.0. Outliers indicated by the statistical software were removed prior to data evaluation.
Data were evaluated by one-way ANOVA, Kruskal-Wallis test, or two-way ANOVA when appropriate as indicated in the figures. For RNA sequencing, transcripts were considered differentially expressed when they had a corrected p-value <0.05. Post hoc analysis was done using Bonferroni’s or Dunn’s test. P value was considered significant if <0.05.

3. Results

3.1 Food intake and body composition

Food intake throughout the study indicated no sustained differences between the groups (Supplementary figure S1). As expected, at the end of the trial the LFD group had lower absolute BW than the HFD group (p<0.001). Of the peptide treatments, only the high dose of IRW (IRW45) reduced absolute final BW (p<0.05) and BW gain (p<0.001). Moreover, the IRW45 group presented lower absolute (p<0.0001) and relative fat mass gain (p<0.01), and less relative lean mass loss (p<0.01) compared to the HFD group (Table 1). However, BW and composition changes were not seen with IRW15.

3.2 Glucose homeostasis and plasma insulin

The IRW45 group had lower fasting blood glucose beginning at the 5th week of treatment compared to the HFD group (Figure 1A). Two-way ANOVA analysis of fasting blood glucose over time showed significant interaction (p<0.0001) and diet effects (p<0.0001). After 8 weeks of supplementation, the LFD and IRW45 groups had lower fasting plasma insulin concentration (p<0.001) and fasting blood glucose (p<0.0001) compared to the HFD (Table 1). HOMA-IR was lower in LFD (p<0.0001) and IRW45 (p<0.0001) groups compared to HFD. However, HOMA-IR from the IRW15 did not differ from the HFD group (Table 1). Two-way ANOVA showed a diet effect on OGTT (p=0.001). HFD worsened glucose tolerance compared with LFD at most of the time points measured, while both doses of IRW lowered circulating glucose at 15 and 30 minutes compared with HFD (Figure 1B). The area under the curve (AUC) analysis indicated significant differences between LFD (p<0.001) and IRW45 (p<0.05) compared to the HFD group (Figure 1C). Despite changes in ITT when expressed as absolute glucose concentrations (Supplementary figure S2), no significant differences between the groups were observed when data were adjusted to baseline blood glucose concentration (Figure 1D).
3.3 Insulin signaling and PPARγ abundance

Insulin-stimulated phosphorylation of AKT (Ser473) in gastrocnemius muscle was higher in LFD and IRW45 compared to the HFD group (p<0.05), whereas IRW15 was non-significantly increased (Figure 2A). Consistent with this result, GLUT 4 translocation to the plasma membrane in skeletal muscle was ~4-fold higher in the IRW45 group (p<0.0001) compared to the HFD group, as shown by the ratio of membrane GLUT4/cytosol GLUT4 (Figure 2B). IRW15 treatment in HFD fed mice increased GLUT4 translocation by 2-fold (p<0.05). In skeletal muscle, PPARγ abundance was 15-17-fold higher in LFD (p<0.01) and IRW45 (p<0.001) compared to the HFD group (Figure 2C).

In both retroperitoneal and epididymal adipose tissues, no changes in AKT phosphorylation (Supplementary figure S3 A-D) or PPARγ abundance were seen following IRW supplementation (Supplementary figure S3 E-F).

3.4 RAS components

In skeletal muscle, there were no significant differences in ACE2, AT1R, and Mas receptor between the groups (Figures 3B, C, F). However, a lower ACE abundance in LFD, IRW15, and IRW45 (P<0.05) groups was observed compared to the HFD control (Figure 3A). In addition, AT2R abundance increased almost 3-fold in the IRW45 group compared to the HFD group (p<0.01) (Figure 3D). The plasma ACE and angiotensin II activities were similar between groups (Figure 4A, C). On the other hand, plasma ACE2 activity was highest in the IRW45 group (p<0.01) (Figure 4B), while plasma Ang (1-7) plasma concentration was not increased in IRW treated groups compared to the HFD control group (Figure 4D).

3.5 Skeletal muscle gene expression

RNA sequencing of gastrocnemius muscle genes revealed that IRW45 had a generally higher abundance of transcripts related to muscle synthesis (Figure 5A). qPCR validation showed that IRW45 upregulated the expression of *Rbm5*, *Mdm2*, *Dlg1*, and *Myom1* genes and downregulated the expression of *Aspn* compared to the HFD group in vivo (Figure 5B). For PPARγ related genes, IRW45 enhanced the expression of *Pparg* and *Lpl*, while no IRW45-induced changes regarding *Plin2* and *Cebpa* were seen (Figure 5C). However, it should be noted that the expression of *Plin2* and *Cebpa* were 15 and ~50-fold higher in HFD and IRW45 than LFD.

3.6 AMPKα abundance and mTOR signaling
A ~10-fold increase in AMPKα phosphorylation (Thr172) was seen in skeletal muscle of the IRW45 group compared to HFD (p<0.05) (Figure 6A), while no changes were seen between groups in total AMPKα (Figure 6B). No changes in total or phosphorylated AMPKα abundance in retroperitoneal (Supplementary figure S4 A-B) or epididymal (Supplementary material figure S5 A-B) WAT were observed after IRW supplementation.

Phosphorylated (Ser2448) mTOR, although increased in skeletal muscle after IRW45 supplementation, was not statistically significant (p=0.2332), while in IRW15 p-mTOR (Ser2448) was increased (p<0.05) (Figure 6C). The downstream p70 S6K protein phosphorylation was enhanced in IRW45 but lacked statistical significance (p=0.2075) (Figure 6E); however, total p70 S6K protein was significantly increased (p<0.05) in IRW45 compared to HFD (Figure 6F). In adipose tissue, no changes were seen in total and phosphorylated mTOR or p70 S6K in both retroperitoneal and epididymal WAT (Supplementary figure S4 C-F and figure S5 C-F, respectively).

3.7 Adipose tissue UCP-1 abundance

The investigation of UCP-1 abundance in retroperitoneal and epididymal WAT is shown in Figure 7. UCP-1 was decreased by HFD compared with LFD and this was significant in epididymal WAT (p<0.05) (Figure 7B). IRW15 and IRW45 exhibited intermediate abundance in epididymal WAT.

4. Discussion

Metabolic diseases such as obesity, MetS, and type 2 diabetes (T2D) are a global epidemic. Diabetes prevalence is expected to rise up to 10.2% globally by 2030, affecting approximately 578.4 million adults[25]. Natural health products[26] are used by a wide range of the population but their efficacy in managing the complexity metabolic disease is still debatable. Despite that, food-derived bioactive peptides exhibit positive physiological effects related to metabolic disease and its complications[14, 27, 28]. IRW is an ovotransferrin-derived bioactive peptide previously shown to cause beneficial effects in cells and rodents in the context of hypertension and inflammation[17, 19]. In addition, in vitro, antioxidant and insulin signaling effects of IRW were shown by our group[20, 21]. In the current study, using an obese, insulin resistant rodent model we demonstrated that IRW supplementation at 45mg/kg BW: 1) prevented BW and fat mass gain during HFD treatment while
protecting lean body mass; 2) improved glucose tolerance and fasting blood glucose and insulin concentrations, which generated a significant decrease in HOMA-IR index; and, 3) enhanced insulin-dependent and -independent signaling governing glucose uptake in skeletal muscle of HFD fed mice. IRW15 was not as effective as IRW45, illustrating dose dependence. Contrary to our hypothesis, these activities of IRW did not appear to involve the inhibition of local RAS.

As previously demonstrated,[18, 19] IRW retained biological activity when administered in vivo. This could be attributed to the practice of mixing IRW into the HFD, which may protect peptides from degradation by digestive enzymes and increase their bioactivity.[29]. However, IRW can be degraded into the dipeptide IR and the free amino acid W in simulated gastrointestinal digestion, which decreases its ACE inhibitory activity drastically in vitro.[15]. We did not calculate the concentration or characterize the bioactive form reaching the bloodstream in the current study and we cannot exclude the possibility of the dipeptide IR being bioactive in vivo. Nevertheless, IRW at a dosage of 45mg/kg BW was effective in overcoming the bioavailability challenge in vivo and in promoting beneficial physiological effects.

T2D is characterized by IR and hyperglycemia,[30], the latter being the major cause of several macro- and microvascular complications such as cardiovascular disease (CVD), nephropathy, and retinopathy.[31, 32]. In this study, IRW45 improved both fasting and glucose-stimulated glucose indices and decreased fasting insulin in HFD fed, glucose-intolerant mice. This is consistent with reduced HOMA-IR in IRW45 animals, which is interpreted as representing hepatic insulin sensitivity during fasting, thus, suggesting a beneficial effect of IRW in managing glucose homeostasis in the fasting state. Similarly, an egg white hydrolysate that does not contain IRW has been shown to improve glucose tolerance and insulin sensitivity measured via ITT in Sprague Dawley rats,[10], and to reduced HOMA-IR and HOMA-β indexes in Zucker Fatty rats.[33]. In the latter case, treated animals had similar fasting blood glucose concentration to the control group, but presented lower fasting plasma insulin concentration.[33]. Although our ITT study shows that IRW45 treatment improved insulin sensitivity compared to the HFD group, the significance was lost after adjustment to baseline blood glucose concentration, suggesting that the effects observed were dependent on differences in fasting blood glucose concentration.

Glucose uptake in skeletal muscle may occur via insulin-dependent and -independent pathways. Insulin activates the PI3K-AKT cascade leading to the final translocation of GLUT4 to the plasma membrane, which increases glucose uptake.[34]. Skeletal muscle of IRW45 treated
animals revealed increased AKT phosphorylation and translocation of GLUT4 to skeletal muscle plasma membrane compared to the HFD control. However, because ITT was not different between groups, we cannot reject the possibility of other insulin-independent pathways playing a role in the observed effects. AMPK activation, such as in muscle contraction, enhances GLUT4 translocation to the plasma membrane of skeletal muscle\cite{35} and increases glucose uptake in skeletal muscle of rats independently of insulin\cite{36}. Indeed, AMPKα phosphorylation in skeletal muscle of IRW45 treated animals was significantly increased. Both AKT and AMPK pathways are consistent with improved glucose tolerance observed in IRW45 treated animals, suggesting a role for insulin-dependent and -independent enhanced glucose uptake in treated animals. We also acknowledge the possibility of enhanced basal AKT phosphorylation by IRW directly. In this study, only insulin stimulated animals were included, which does not allow for this latter analysis.

RAS modulation has also been linked to insulin sensitivity. We initially hypothesized that improvements in insulin signaling would be associated with reduced local RAS activity, based on results in SHR hypertensive rats and in vitro assays\cite{15, 18, 19, 37}. Despite IRW being identified as an ACE inhibitory peptide in vitro\cite{15}, we found no effect of IRW on systemic ACE activity in this IR model, similar to previous results using IRW in SHR rats\cite{18}. However, IRW reduced ACE protein abundance in skeletal muscle, which might contribute to lower local ACE activity. Moreover, plasma ACE2 activity was increased in our study, consistent with previous studies showing that oral IRW supplementation enhanced ACE2 circulating levels and activity\cite{18}, and ACE2 protein expression in the aorta of SHR rats\cite{37}. ACE2 is known to antagonize the actions of angiotensin II, therefore reducing blood pressure, and reducing CVD risk through angiotensin (1-7)/Mas receptor axis as recently reviewed\cite{38}. Interestingly, in this study Angiotensin (1-7) was not increased after IRW treatment.

In skeletal muscle, modulation of AT1R and AT2R regulates insulin action locally, with systemic AT2R blockade impairing insulin-stimulated Akt phosphorylation, whole body glucose uptake, and muscular microvascular function, while systemic AT1R blockade restored muscle insulin signaling\cite{4}. AT2R is believed to cause opposing effects to AT1R activation in blood vessels with their interplay regulating blood flow and glucose utilization in skeletal muscle\cite{39}. In our study, IRW45 increased AT2R abundance in skeletal muscle. Similarly, we previously showed that egg hydrolysate enhanced AT2R abundance in WAT and liver of treated animals, accompanied by improvement in glucose tolerance\cite{10}. As a possible explanation, increased AT2R
abundance in skeletal muscle may contribute to improved insulin sensitivity by increasing the binding of Angiotensin II to AT2R in the capillary endothelium, therefore improving blood flow to the tissue, facilitating insulin access to muscle cells, and enhancing glucose uptake. Moreover, it may directly facilitate AT2R activation in muscle cells, which we speculate may improve glucose transport via AMPK and PPARγ activation. Despite no clear direct link between AT2R and AMPK yet being demonstrated, RAS modulation improves glucose tolerance and insulin sensitivity via AMPK activation. For example, the AT1R blocker telmisartan improves glucose tolerance and insulin sensitivity in WAT while enhancing AMPK phosphorylation\[^{40}\], and 5-aminomidazole-4-carboxamide ribonucleotide, an AMPK activator, rescued angiotensin II induced IR in rats skeletal muscle\[^{41}\]. Moreover, resveratrol increased both AT2R abundance and AMPK phosphorylation in the aorta of treated mice\[^{42}\].

In some tissues, AT2R may also be linked to PPARγ, a transcription factor that, when activated, has insulin-sensitizing effects. For example, in WAT, PPARγ mRNA and activation were enhanced by AT2R agonists\[^{43}\] and egg white hydrolysate concomitantly increased AT2R and PPARγ abundance\[^{10}\]. Thiazolidinediones (TZDs) are antidiabetic drugs known to be PPARγ agonists and cause insulin sensitizing effects. Although their exact mechanism of action and main target organ remains to be elucidated, TZDs enhance skeletal muscle glucose uptake, reduce liver glucose output, and affect WAT physiology as reviewed elsewhere\[^{44}\]. Despite controversies about the benefits of PPARγ activation in non-adipose tissues, PPARγ agonists potentiate AKT phosphorylation in WAT and skeletal muscle\[^{45}\], and increase FA mitochondrial oxidation in human skeletal muscle cells\[^{46}\]. Besides, specific deletion of PPARγ in skeletal muscle of mice induced IR, hyperinsulinemia, and enhanced glucose uptake locally\[^{47}\]. In IR hamsters, PPARγ RNA expression in skeletal muscle was downregulated, along with other genes regulated by PPARγ such as Ppargc1a, Lpl, and adiponectin (Adipoq) genes\[^{48}\]. In this study, IRW45 treatment upregulated Pparγ and Lpl in skeletal muscle while increasing PPARγ protein abundance, suggesting that IRW may activate a cassette of PPARγ-related genes as part of its metabolic activity.

An important observation was that, despite similar caloric intake, IRW45 improved the body composition of treated animals by reducing BW and fat mass gain, while protecting lean mass. In humans, the reduction of whole-body fat mass after an exercise intervention was associated with increased insulin sensitivity index\[^{49}\]. Recently, an extract from rice hulls was
shown to decrease fat mass by suppressing adipogenic genes in epididymal WAT and liver while enhancing AMPKα protein, which may lead to increased fatty acid oxidation. In addition, PPARγ mRNA and protein abundance were reduced in WAT by the rice hull extract supplementation\textsuperscript{[50]}. However, neither AMPKα nor PPARγ abundance changed in visceral WAT after IRW treatment. We also investigated the possibility of enhanced thermogenesis in WAT (so-called “browning”) induced by IRW as an explanation for reduced fat mass because AT2R activation was previously shown to enhance UCP-1 in epididymal WAT\textsuperscript{[51]} and brown adipose tissue\textsuperscript{[52]}. Moreover, AT2R activation was shown to modulate lipid metabolism in WAT, preventing HFD-induced adiposity\textsuperscript{[52]}. We found that compared with LFD, UCP-1 was lower in abundance after HFD in epididymal WAT and not different in retroperitoneal WAT, similar to summarized findings\textsuperscript{[53]}. IRW45 treatment tended to increase UCP1 but was not as strongly induced as would be expected if thermogenesis was the main route eliciting fat mass loss. The mechanism by which IRW promoted reduced fat mass is still unclear and other pathways deserve further investigation, such as the lipolytic and lipogenic pathways.

Skeletal muscle is a highly plastic tissue that adapts to cope with changes in diet, metabolism, physical activity, and associated factors. Skeletal muscle synthesis is a key indicator of metabolic health and is regulated by insulin signaling\textsuperscript{[54]}. Upregulation of genes involved in muscle synthesis was induced by IRW supplementation. Primarily, mTOR activation is modulated by nutrients and, once activated, is involved in protein and lipid synthesis, and mitochondrial biogenesis\textsuperscript{[55]}. In addition, recent pieces of evidence highlight the importance of mTOR in thermogenesis\textsuperscript{[56]}. However, despite increased AMPK phosphorylation and muscle synthesis mRNA expression in skeletal muscle, changes in the phosphorylation of mTOR (Ser2448) were not IRW dose-dependent nor correlated with phosphorylation of the downstream S6K P70. Nevertheless, these results indicate the ability of IRW to trigger myogenesis pathways, which may be related to our observation that lean body mass was protected by IRW. It is also possible that IRW may be acting independently of mTOR to promote these effects.

5. Conclusion

In summary, IRW reduced BW and fat mass gain while improving glucose tolerance and insulin sensitivity in HFD fed mice. We identified several possible mechanisms of action for IRW
in skeletal muscle and, to a lesser extent, WAT (Figure 8) that were independent of ACE inhibition. Pathways influenced by IRW include the AKT/GLUT4 pathway and AMPKα/GLUT4, which would enhance glucose uptake in skeletal muscle, while activation of the AT2R/PPARγ pathway could improve insulin sensitivity. Furthermore, IRW may reduce inflammation as observed *in vivo* and *in vitro*[^18,^21], contributing to insulin sensitization. Because the liver regulates fasting glucose homeostasis, we cannot reject the possibility that IRW improves liver insulin sensitivity during fasting, leading to better glucose tolerance. Thus, IRW has the potential to exert beneficial effects on glucose homeostasis, making it a strong candidate to be further studied in the context of metabolic diseases.

6. Acknowledgement

We sincerely thank Nicole Coursen for the help with the animal trial and Dr. Juan Jovel and the genomics core facility for their assistance with the RNA sequencing.

7. Funding

None of the following sources had other involvement except providing funding for the study presented. This work was supported by Natural Sciences and Engineering Research Council of Canada [grant numbers CRDPJ 532150-18], Egg Farmers of Canada, and Global Egg Corporation.

8. Author contributions

Stepheny Carneiro de Campos Zani: investigation, formal data analysis, writing-original draft preparation, visualization, writing- review and editing. Myoungjin Son: conceptualization, methodology, investigation, formal data analysis, project management. Khushwant S. Bhullar: investigation, formal data analysis, visualization, writing- review and editing. Catherine Chan: conceptualization, writing- review and editing, supervision. Jianping Wu: conceptualization, supervision, writing- review and editing, project management, funding acquisition.
9. References


Figure 1: Glucose homeostasis after 8 weeks of IRW supplementation. Fasting glucose over time (n= 6-8) (B) Oral glucose tolerance test (OGTT) after 7 weeks of treatment (n= 6-8). (C) Area under the curve (AUC) for OGTT (n= 5-8). (D) Insulin tolerance test (ITT) after 6 weeks of treatment as percentage of the baseline glucose values (n= 8). Data expressed as mean ± SEM and analyzed by two-way ANOVA (A, C and D) or one-way ANOVA (B) followed by Bonferroni’s post-hoc comparison test. * p<0.05, ** p<0.01 and **** p<0.0001 between IRW45 and HFD. # p<0.05 between IRW15 and HFD. + p<0.05, ++ p<0.01, +++ p<0.001 and ++++ p<0.0001 between LFD and HFD.
Figure 2: Skeletal muscle insulin signaling and PPARγ protein abundance. (A) p-AKT, (B) GLUT4 membrane/cytosol, (C) PPARγ, and (D) representative blots. p-AKT protein band was normalized to total AKT. GLUT4 is expressed as a ratio of membrane to cytosolic GLUT4. PPARγ protein band was normalized to GAPDH as the loading control. Data expressed as mean ± SEM of n=6 mice. Analysis by one-way ANOVA followed by Bonferroni’s post-hoc test. * p<0.05, ** p<0.01 *** p<0.001 and **** p<0.0001 versus HFD. AKT, Protein kinase B; PPARγ, Peroxisome proliferator-activated receptor gamma; GLUT4, glucose transporter 4.
Figure 3: Skeletal muscle protein abundance of renin angiotensin system components. (A) ACE, (B) ACE2, (C) AT1R, (D) AT2R, (E) AT2R/AT1R ratio, (F) Mas receptor, and (G) representative blot. ACE, ACE2, AT1R, AT2R, and Mas protein bands were normalized to GAPDH as the loading control. Data expressed as mean ± SEM of n=5-6 mice. Analysis by one-way ANOVA followed by Bonferroni’s post-hoc test or Kruskal-Wallis test followed by Dunn’s post hoc test when appropriate. * p<0.05 and ** p<0.01 versus HFD. ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; AT1R, angiotensin receptor type 1; AT2R, angiotensin receptor type 2.
Figure 4: Plasma renin angiotensin system (RAS) components. (A) Plasma angiotensin converting enzyme (ACE), (B) Plasma angiotensin converting enzyme 2 (ACE2), (C) Plasma angiotensin II, and (D) Plasma angiotensin (1-7). Data expressed as mean ± SEM of n=4-7 mice. Analysis by one-way ANOVA followed by Bonferroni’s post-hoc comparison test or Kruskal-Wallis test followed by Dunn’s post hoc test when appropriate. ** p<0.01 versus HFD.
Figure 5: Skeletal muscle genes expression of mice fed IRW for 8 weeks. (A) Heatmap showing the abundance of major genes involved in muscle synthesis modulated by IRW45 (45mg/Kg BW) treatment determined by RNA sequencing. (B) Rbm5, Mdm2, Dlg1, Myom1, and Aspn qPCR validation of IRW in vivo using gastrocnemius skeletal muscle of C57BL/6 mice. Data expressed as mean ± SEM of n=5 mice. (C) Pparg, Plin2, Cebpa and Lpl qPCR validation using gastrocnemius skeletal muscle of C57BL/6 mice. Data expressed as mean ± SEM of n=5 mice. Analysis by one-way ANOVA followed by Bonferroni’s post-hoc comparison test or Kruskal-Wallis test followed by Dunn’s post hoc test when appropriate. * p<0.05, ** p<0.01 and **** P<0.0001 versus HFD.
Figure 6: Skeletal muscle AMPKα, mTOR and P70 S6K protein abundance. (A) p-AMPKα (B) AMPKα (C) p-mTOR (D) mTOR (E) p-P70 S6K (F) P70 S6K and (G) representative blots. Phospho proteins were normalized to their respective total protein. Total proteins were normalized to GAPDH as the loading control. Data expressed as mean ± SEM of n= 5-6 mice. Analysis by one-way ANOVA followed by Bonferroni’s post-hoc comparison test or Kruskal-Wallis test followed by Dunn’s post hoc test when appropriate. * p<0.05 versus HFD. AMPK, 5' AMP-activated protein kinase, mTOR, mammalian target of rapamycin; P70 S6K, Ribosomal protein S6 kinase beta-1.
Figure 7: White Adipose tissue (WAT) UCP-1 protein abundance. Retroperitoneal WAT UCP-1 (A) and representative blot (C). Epidydimal WAT UCP-1 (B) and representative blot (D). UCP-1 bands were normalized to β-actin as the loading control. Data expressed as mean ± SEM of n=5-6 mice. Analysis by one-way ANOVA followed by Bonferroni’s post-hoc test or Kruskal-Wallis test followed by Dunn’s post hoc test when appropriate. * p<0.05 versus HFD. UCP, uncoupling protein.
Figure 8: Potential mechanism of action of IRW to improve glucose tolerance and insulin signaling. A high fat diet activates the classical pathological RAS arm to increase ACE, and this activation is counteracted by IRW via elevation of the RAS protection arm AT2R. Mechanistically, in blood, IRW stimulates ACE2, which can use Angiotensin I (Ang I) as its substrate, to produce Angiotensin (1-9) (Ang (1-9)), which leads to direct activation of AT2R. This increase in AT2R may induce AMPK phosphorylation and neutralize the increased abundance of ACE. In addition, AMPK facilitates insulin-independent glucose uptake by skeletal muscle cells. AT2R may also trigger PPARγ upregulation as a downstream target, leading to transcription of myogenesis genes. Further, IRW-stimulated p-Akt increases glucose uptake via GLUT4. p-Akt plays a significant physiological role in insulin-stimulated glucose uptake in skeletal muscle and activates mTOR and p70-S6K as the downstream targets, although IRW involvement in this pathway requires further clarification.
Table 1: Body composition and metabolic profile of mice supplemented with IRW. Initial measurements were taken after 6 weeks of LFD or HFD feeding. Final measurements were taken 8 weeks after initiating IRW treatment. Analysis by one-way ANOVA followed by Bonferroni’s post hoc test (lean mass change, fasting glucose initial and HOMA-IR) or Kruskal-Wallis test followed by Dunn’s post-hoc test. Data expressed as mean ± SEM of n=7-8 mice. Values in the same row represented by different letters are statistically different (p<0.05 or lower) compared to HFD control.
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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