Title: Downregulation of muscle cell Insr and insulin receptor signaling by hyperinsulinemia in vitro and in vivo

Authors: Haoning Howard Cen, José Diego Botezelli, Su Wang, Niloufar Noursadeghi, James D. Johnson*

Affiliation: Department of Cellular and Physiological Sciences, Life Science Institute, University of British Columbia, Vancouver, BC. Canada.

Running title: Hyperinsulinemia-induced insulin resistance

Keywords: insulin, insulin receptor, insulin resistance, skeletal muscle, FOXO

*Address correspondence to: James D. Johnson, Ph.D., Professor. Diabetes Research Group, Dept. of Cellular and Physiological Sciences & Dept. of Surgery, University of British Columbia, 5358 Life Sciences Building, 2350 Health Sciences Mall, Vancouver, BC, Canada, V6T 1Z3

E-mail: James.D.Johnson@ubc.ca; Fax: (604) 822-2316

Twitter: @JimJohnsonSci

Disclosure Statement: The authors have no relevant conflicts of interest to disclose.
Abstract

Hyperinsulinemia is often viewed as compensatory to insulin resistance, but studies have shown that high levels of insulin may also contribute to insulin resistance. The precise mechanisms by which hyperinsulinemia contributes to insulin resistance remain poorly defined.

To understand the direct effects of prolonged exposure to excess insulin in muscle cells, we incubated differentiated C2C12 mouse myotubes with elevated insulin for 16 hours, followed by 6 hours serum starvation, before examining key insulin signaling nodes. Using this model, we found that prolonged high insulin treatment significantly increased the phosphorylation of insulin receptor (INSR) and AKT, but not ERK. After serum starvation, acute AKT and ERK signaling stimulated by 0.2 - 20 nM insulin was attenuated. INSR protein levels were significantly downregulated by hyperinsulinemia in an insulin-dose-dependent manner. Surface INSR was reduced proportionally to total INSR levels. Mechanistically, we found that hyperinsulinemia strongly downregulated Insr mRNA which was correlated with increased threonine 24 phosphorylation of FOXO1. Interestingly, 6h serum starvation reversed the effects of high insulin on basal phosphorylation of INSR, AKT and FOXO1, and Insr transcription. Using RNA sequencing, bioinformatic analysis, and follow-up RNAi studies, we identified SIN3A as a negative regulator of Insr mRNA levels and JUND, MAX and MXI as positive regulators of Irs2 mRNA. We validated our in vitro results by determining that INSR levels in mouse skeletal muscle were negatively correlated with circulating insulin in vivo. Together, our findings shed new light on the mechanisms underlying hyperinsulinemia-induced insulin resistance in muscle cells, which are likely to be relevant in the pathogenesis of type 2 diabetes.
Introduction

Hyperinsulinemia and insulin resistance are cardinal features of type 2 diabetes (T2D) and highly associated with each other. It has been a widely held view that insulin resistance is the primary cause of type 2 diabetes and that hyperinsulinemia is a compensatory response (1,2). However, a growing body of evidence suggests the opposite may be true in many cases (3-5). Hyperinsulinemia can be observed prior to insulin resistance in obesity and T2D (6-8) and hyperinsulinemia is the strongest predictor of T2D in long-term studies (9,10). We recently showed that hyperinsulinemia contributes causally to age-dependent insulin resistance in the absence of hyperglycemia (11). Reducing hyperinsulinemia in partial insulin gene knockout mice prevents and reverses diet-induced obesity in adult mice (11-13). Rodents (14,15), healthy humans (16,17), and people with type 1 diabetes (18) subjected to prolonged insulin administration also have reduced insulin responsiveness independent of hyperglycemia. This strongly suggests that hyperinsulinemia is a self-perpetuating cause of insulin resistance.

The mechanisms by which hyperinsulinemia contributes to insulin resistance remain poorly understood. The insulin receptor (INSR) is a critical component as the starting point of insulin action. It has been reported that obese or diabetic rodent models with hyperinsulinemia and insulin resistance have reduced insulin binding to the liver (19,20), fat (21) and pancreatic acinar cells (22). The reduction in insulin binding may be due to INSR downregulation rather than immediate changes in binding affinity, since chronic (2-16 hours), but not acute, high insulin exposure directly reduced insulin binding and INSR protein levels in adipocytes (23) and lymphocytes (24) in vitro. However, the molecular mechanisms of INSR down-regulation and post-receptor insulin resistance remain poorly characterized in muscle cells.
In the present study, we employed an in vitro model of insulin resistance using a muscle cell line and confirmed our observations in vivo using mice with variable hyperinsulinemia. We found that hyperinsulinemia induced insulin resistance featuring blunted acute insulin signaling and transcriptional INSR downregulation associated with reduced FOXO1 activity. Transcriptome wide consequences of hyperinsulinemia were also characterized, revealing Sin3a as a novel transcription factor suppressing Insr expression. Our data illuminate new mechanisms by which hyperinsulinemia contributes to insulin resistance in muscle cells.

Results

Hyperinsulinemia induces insulin resistance in muscle cells in vitro

Circulating insulin in humans oscillates in a range between 0.03 nM and 1.9 nM, and fasting insulin less than 0.17 nM is considered normal (25). To establish a muscle cell model of hyperinsulinemia-induced insulin resistance, we incubated differentiated mouse C2C12 myotubes for 16 hours in a physiologically high insulin dose of 2 nM or supraphysiologically high dose of 200 nM (Fig. 1A). Hyperinsulinemia was confirmed after treatment with high insulin (Fig. 1A'). After 6-hour serum starvation, insulin signaling was characterized by measuring the phosphorylation of AKT and ERK proteins, which represent two major insulin signaling nodes (26). Since alterations in the basal state of the insulin signal transduction network have also been reported in hyperinsulinemic humans and animals (27), we also measured the effects of hyperinsulinemia on AKT and ERK phosphorylation before and after serum starvation. These experiments showed that total AKT protein levels were significantly downregulated by prolonged 200 nM, but not 2 nM, insulin treatment, while ERK abundance was not significantly changed (Fig. 1B-D). After prolonged 200 nM insulin exposure and before serum starvation, AKT
phosphorylation at threonine (T) 308 and serine (S) 473 was significantly elevated, but ERK phosphorylation was unaffected (Fig. 1E,F). Of note, phosphorylation of ERK1/2 was increased by serum starvation alone, as previously reported in other cell types (Fig. 1F)(28). Acute AKT and ERK signaling in the context of 2 nM acute insulin was significantly reduced by hyperinsulinemia treatment in an insulin dose-dependent manner (Fig. 1E,F). We also characterized the insulin dose- and time-dependent signaling in our in vitro hyperinsulinemia model (200 nM insulin). Phosphorylation of AKT and ERK1/2 were significantly reduced under 0.2, 2, or 20 nM insulin stimulations (Fig. 2A,B). Together, these results establish a robust muscle cell insulin resistance model induced by hyperinsulinemia and characterized its effects on basal and acute insulin signaling.

**Hyperinsulinemia reduces INSR protein abundance but not its phosphorylation or internalization**

To better understand the effects of hyperinsulinemia on the earliest stages of insulin signalling, we examined on INSR abundance, phosphorylation, and internalization. Remarkably, we found the total INSR protein abundance was robustly decreased in both hyperinsulinemia groups in an insulin dose-dependent manner (Fig. 3A, B). Serum starvation slightly recovered the INSR downregulation in 200 nM insulin group (Fig. 3B). These results clearly demonstrated that hyperinsulinemia can modulate INSR abundance in this cell system.

We also examined INSR tyrosine 1150/1151 autophosphorylation, which is an early step of insulin signaling that recruits IRS and SHC, leading to PI3K-AKT or RAS-ERK activation (26). Before starvation, both hyperinsulinemia groups had increased INSR phosphorylation, suggesting that there was continuous insulin signaling during the high insulin treatments (Fig. 3C). Serum
starvation completely reversed INSR hyperphosphorylation (Fig. 3C). While INSR phosphorylation was not significantly different after 10 min of acute insulin stimulation (Fig. 3C), analysis of dose- and time-dependent insulin signaling revealed a tendency for increased phosphorylated-to-total INSR ratio in insulin-stimulated cells exposed overnight to 200 nM insulin (Fig. 3D). The increased INSR phosphorylation per receptor was offset by the reduced INSR number, leading to a decreased phospho-INSR-to-tubulin ratio (i.e. the overall INSR phosphorylation events per cell) (Fig. 3D). These data indicate that the were not defects in INSR phosphorylation upon acute insulin stimulation in our system.

Impaired INSR endocytosis has been implicated in insulin resistance (29,30). Therefore, basal surface INSR, as well as dose- and time-dependent INSR internalization were examined in our hyperinsulinemia model using a surface biotinylation assay (Fig. 4A). Serum starvation slightly decreased surface-to-total INSR ratio, while hyperinsulinemia had no significant effects (Fig. 4B). Upon acute insulin stimulation, the internalized INSR to total INSR ratio did not have evident differences except for a small increase when stimulated by 2 nM insulin (Fig. 4C). Therefore, hyperinsulinemia-induced insulin resistance may be mediated by a reduction in total INSR that results in a proportional reduction in INSR protein at the cell surface. The fraction of INSR internalized during acute insulin signaling was not drastically affected by hyperinsulinemia under these conditions. Collectively, our experiments suggest that hyperinsulinemia-induced insulin resistance in muscle cells is mediated in large part by a reduction in total INSR, and not by affecting its activity or internalization.

Circulating insulin negatively correlates with INSR level in vivo

To validate our in vitro studies, we examined the relationship between in vivo insulin
concentration and muscle INSR levels in mice. As in our previous studies (12), insulin gene dosage was manipulated (Ins1+/-;Ins2-/- and Ins1+/-;Ins2-/-) to generate variance in circulating insulin. The mice were fed with high fat diet (HFD) known to induced pronounced hyperinsulinemia (12,31) or low fat diet (LFD). These experiments showed that INSR protein abundance negatively correlated with both fasting insulin and fasting glucose in the HFD group (Fig. 5A-B). However, the LFD group only had a negative correlation between INSR level and insulin, with no correlation between INSR and glucose (Fig. 5C-D). These data support the concept that insulin, independent from glucose, can negatively regulate INSR levels in skeletal muscle. This is consistent with our in vitro hyperinsulinemia model and our previous in vivo data demonstrating improved insulin sensitivity over time in mice with genetically reduced insulin production (11). These data also suggest an interaction between insulin, glucose and INSR that is dependent on the conditions of the HFD.

Hyperinsulinemia reduces Insr mRNA alongside FOXO1 inhibition

One possible cause of INSR downregulation would be the reduced expression of the Insr gene (22). To test this hypothesis, we measured the mRNA of Insr isoforms A and B (Insr-A and Insr-B) by qPCR. In our hands, Insr-A is the predominant isoform in C2C12 myotubes (Fig. 6A). Both Insr-A and Insr-B mRNA were equally and robustly downregulated after hyperinsulinemia and partially recovered by serum starvation (Fig. 6A), consistent with the change of INSR protein abundance. Interestingly, insulin-like growth factor 1 receptor (Igfr1), which has similar structure and signaling as INSR, was also reduced by hyperinsulinemia at the transcriptional level (Fig. 6B). Alteration in Insr alternative splicing has been implicated in hyperinsulinemia (32). In our model, the ratio of Insr-A and Insr-B mRNA was not affected by hyperinsulinemia or serum starvation (Fig.
Our evidence suggests that hyperinsulinemia and serum starvation regulate INSR protein abundance by regulating *Insr* mRNA levels.

Forkhead box protein O1 (FOXO1) is a known transcriptional regulator of the *Insr* gene and is also a key mediator of insulin signaling (33-35). In *Drosophila* and mouse myoblasts, FOXO1 activity is necessary and sufficient to increase *Insr* transcription under serum fasting and reverse this effect in the presence of insulin (34). Therefore, we sought to determine the activity of FOXO1 in our hyperinsulinemic model. Indeed, high insulin increased FOXO1 phosphorylation on T24, which is an AKT-associated event known to exclude FOXO1 from the nucleus and decrease its transcriptional activity (36), but did not affect total FOXO1 abundance (Fig. 6D). T24 phosphorylation of FOXO1 significantly decreased after starvation (Fig. 6D), consistent with our observed effects on AKT phosphorylation and *Insr* transcription. Our data therefore support the work of other groups indicating a role for FOXO1 in *Insr* gene expression. However, knocking down *Foxo1* mRNA by 40% was insufficient to alter *Insr* mRNA level in C2C12 myoblast (n=5, data no shown), suggesting the possibility of additional redundancy associated with either the remaining 60% of FOXO1 expression or other proteins.

**Hyperinsulinemia and serum starvation remolds transcriptome**

To further investigate the mechanisms of hyperinsulinemia-induced insulin resistance and to identify new molecular targets, we conducted RNA sequencing (RNAseq) to illustrate the effects of prolonged insulin and serum starvation. We compared the transcriptomes of 5 cultures in each of 4 treatment groups (0 nM or 200 nM insulin, both before and after serum starvation). Principle component analysis (PCA) of RNAseq data showed distinct clustering by hyperinsulinemia or starvation after hyperinsulinemia, but not by starvation itself (Fig. 7A). Many interesting genes
Hyperinsulinemia-induced insulin resistance

Previously linked to metabolism and diabetes were differentially expressed between control and hyperinsulinemic groups both before and after serum starvation (Fig. 7B). Several genes that are altered by hyperinsulinemia before starvation changed in the opposite direction after serum starvation, including *Irs2* (Fig. 7B). Through KEGG pathway enrichment analysis, we found that the top pathways upregulated by hyperinsulinemia were related to transcription, translation and DNA repair (Fig. S1A), while the top downregulated pathways included FOXO signaling, RAS, and MAPK signaling (Fig. 7C). Many of these downregulated pathways were upregulated by starvation (200 nM, BS vs AS), but also remained downregulated after starvation (AS, 0 vs 200 nM) (Fig. 7C). We focused on the effects of hyperinsulinemia before starvation and the genes altered in the pathways closely related to insulin signaling by protein-protein interaction network, such as insulin signaling, FOXO signaling, PI3K-AKT signaling, and RAS and MAPK signaling pathways (Fig. 7D, E, Fig. S1B,C). Altered components of these signaling pathways of interest were also mapped onto KEGG pathways (Fig. S2A-E). Of note, many genes of key insulin signaling proteins were downregulated by hyperinsulinemia, including *Insr*, *Irs1*, *Irs2*, *Akt3*, *Pik3ca*, *Pik3cb*, and *Pik3r3*. Hyperinsulinemia has been previously shown to downregulate insulin signaling proteins such as IRS1 and IRS2 (37,38). Overall, our transcriptomics data revealed strong, reciprocal effects of prolonged hyperinsulinemia and insulin removal on insulin signaling genes.

**Novel transcription factors regulate Insr expression and transcriptomic remodeling by insulin**

Next, we bioinformatically predicted the upstream transcriptional regulators using a transcriptional factor-gene network built from the differentially expressed genes (Fig. 8A). The top
30 transcriptional factors with high degree of connections to altered genes (Fig. 8A), were compared to common transcription factor binding sites of several differentially expressed genes that encode key proteins in insulin signaling (Fig. 8B). The resulting 11 transcription factors were deemed candidates to affect the transcriptional changes in Insr and other insulin signaling genes during hyperinsulinemia (Fig. 8B). To investigate the role of these transcription factors on Insr expression, we conducted siRNA knockdown for each transcription factor, with knockdown efficiencies varying between 45% and 85% (Fig. 8C). Sin3a knockdown (~70%) resulted in a significant increase in Insr mRNA, indicating that this transcription factor has a repressive effect (Fig. 8D). In addition, we also assessed the expression of Irs2, which had larger fold change than Insr upon hyperinsulinemia and starvation. Knockdown of Jund, Max, and Mxi1 downregulated Irs2, which suggested that these transcription factors are involved in Irs2 transcription and may contribute to insulin resistance (Fig. 8D). In conclusion, we identified transcription factors for Insr and Irs2 genes among the predicted upstream transcriptional regulators.

Discussion

The goal of this study was to explore the mechanisms of hyperinsulinemia-induced insulin resistance in skeletal muscle cells. We demonstrated that prolonged physiological and supraphysiological hyperinsulinemia induced a reduction of AKT and ERK signaling (Fig. 8E). Remarkably, while serum starvation partially reversed the effects of overnight hyperinsulinemia, much of the impaired acute insulin signaling was sustained after 6 hours of insulin withdrawal and serum starvation, suggesting that long-term molecular changes underlie these differences. The effects of prolonged hyperinsulinemia were insulin dose-dependent from the physiological to the supraphysiological range. We demonstrated that the impaired insulin response in our system can
be partially accounted for by INSR downregulation at the transcription level via FOXO1 phosphorylation, and also used transcriptomic profiling to discover new factors that regulate insulin signalling in our system including SIN3A, JUND, MAX, and MXI1.

Our in vitro cell culture model provided a robust and controlled system for examining the direct effects of excess insulin, and insulin withdrawal, on multiple components of insulin signaling in muscle cells. Our results are consistent with other in vitro cell culture systems designed to examine the effects of hyperinsulinemia. For example, reduced AKT and ERK signaling and INSR abundance were also reported in hyperinsulinemia-treated β-cells (INS1E cell line and rat islets) and enteroendocrine L cells (39,40). Insulin exposure over 3 days blunted IRS1 and AKT phosphorylation in human muscle derived cells (41). Nevertheless, the mechanisms of sustained alterations in AKT and ERK phosphorylation were not fully understood. In our experiments, there were suppressed AKT and ERK phosphorylation at all time points, suggesting that the insulin resistance we observed was impaired responsiveness consistent with signaling deficiencies at both the receptor level and in post-receptor components (42). Indeed, multiple components of insulin signaling were reduced at the transcriptional level revealed by RNAseq, including Akt3 which is consistent with the reduced AKT protein abundance. We speculate that the proteosome may also be involved because IGF1 treatment can cause proteasome-dependent downregulation of AKT protein (43). Our observations verified the distinct responses to hyperinsulinemia on the bifurcate insulin signaling pathways. Chronic 200 nM insulin treatment preferentially increased basal AKT phosphorylation as a sign of sustained activation, but did not increase the basal ERK phosphorylation, possibly due to desensitization, as reported in neurons (44). Diet- and hyperinsulinemia-induced insulin resistance is generally considered to be related specifically to AKT phosphorylation. Interestingly, chemical inhibition of AKT pathway, but not ERK pathway,
Hyperinsulinemia-induced insulin resistance

has been reported to protect insulin resistance both in vitro and in vivo (44,45). Further work is required to understand the interplay between INSR expression and both major branches of downstream signaling.

A major observation of our work is that hyperinsulinemia directly reduced Insr mRNA in cultured cells, consistent with reports from other cell culture systems (22,46). In a previous study, we observed a ~20% increase in Insr mRNA from skeletal muscle samples of Ins1+/--;Ins2−/− mice compared to Ins1+/+--;Ins2−/− mice, as well as a trend of a ~50% increase in Foxo1 mRNA (12). Our in vivo data in mice are also consistent with the limited data from human studies. For example, T2D patients with higher fasting insulin were found to have lower Insr mRNA expression in skeletal muscle biopsies (47). While relative hyperglycemia can increase Insr expression in lymphocyte and cancer cell lines (48,49), high glucose inhibits β-cell Insr expression through autocrine insulin action and INSR-FOXO1 signaling (48,49). Interestingly, glucose could only induce insulin resistance in the presence of insulin in cultured hepatocytes, adipocytes and skeletal muscle (21,50,51). Therefore, reduced Insr expression by hyperinsulinemia may be a key, independent factor of INSR downregulation and insulin resistance.

Intermittent fasting, time-restricted feeding, caloric restriction, and/or carbohydrate restriction have been shown to have health benefits in diabetes, including reducing hyperinsulinemia, increasing insulin sensitivity, improving β-cell responsiveness, and lowering the levels of circulating glucose (52-54). Several human trials suggest that fasting regimes can be more effective for reducing insulin and increasing insulin sensitivity than they are for reducing glucose (55,56). By mimicking the low-insulin state, the serum starvation phase of our studies revealed some possible molecular mechanisms of the beneficial effects of fasting on muscle cells, which includes the restoration of protein phosphorylation in insulin signaling pathways and partial
recovery of \textit{Insr} transcription and INSR protein. These data hint that some deleterious effects of hyperinsulinemia are reversible but may require a long enough low-insulin period. RNAseq results revealed broad effects of hyperinsulinemia and serum starvation on insulin signaling and FOXO signaling pathways, and highlighted potentional upstream transcription factors. Besides FOXO1, other transcription factors such as SP1, HMGA1, C/EBP\(\beta\), and NUCKS have been reported to regulate \textit{Insr} expression (57-59), but their role in hyperinsulinemia will require further investigation. We identified at least one novel transcriptional repressor of the \textit{Insr} gene, SIN3A, which was upregulated by hyperinsulinemia. SIN3A interacts with histone deacetylases to inhibit transcription and interacts with other transcription factors that were found in our transcriptomics/bioinformatics analyses (60). For example, SIN3A and MYC inhibits each other and form a negative feedback loop (61). MAX dimerizes with either MYC or MXI1 (MAD family protein) in a competing manner to activate or repress target genes (62), and MXI1 recruits SIN3A for gene inhibition (62). Interestingly, the knockdown level we achieved for MYC, MAX, MXI1 did not have significant effects on transcription of \textit{Insr}. One possibility is that the roles of these transcription factors on \textit{Insr} are indirect and rely on the action of SIN3A, while another possibility is that SIN3A acts through alternative pathways. A recent study identified SIN3A as a FOXO1 corepressor of the glucokinase gene in liver (63). Additional studies will be required to elucidate possible interactions between SIN3A and FOXO1 on the downregulation INSR in our system.

The present study has limitations and leaves many questions unanswered. We employed a 16-hour insulin incubation instead of a more chronic, multiday hyperinsulinemia treatment. The main reason for this is that insulin promotes the further differentiation of C2C12 myotubes (64), meaning that chronic treatment during differentiation would likely introduce unmanageable variance in the muscle cell model. Our study cannot rule out effects of hyperinsulinemia on INSR
protein degradation and overall proteolysis, which can be increased by high insulin in lymphocytes and adipocytes (22,23,65). However, the fold reduction of *Insr* mRNA was smaller than fold reduction of INSR protein in our cell model, suggesting that additional mechanisms may be involved in modulating the protein abundance of INSR. In addition to the downregulation of INSR protein abundance, we found a subtle increase in INSR internalization with, but future studies will be required to determine the role and mechanism of this phenomenon. Interestingly, chick liver cells exposed to 50 nM insulin for 18 h had decreased surface, but unchanged total, INSR with no alteration in INSR protein synthesis and degradation rates, suggesting that the regulation of INSR by hyperinsulinemia may have distinct control mechanisms in certain cell types (20).

Despite its inherent reductionism, our in vitro model reemphasized the critical role of hyperinsulinemia in the development of insulin resistance and T2D. We demonstrated that in vitro hyperinsulinemia and serum fasting have profound effects on AKT and ERK signaling and protein levels, INSR abundance and localization, and transcriptional activities, which provided valuable insights on the molecular mechanisms of insulin resistance. Future additional characterization of the effect of hyperinsulinemism on INSR trafficking, degradation, and detailed post-receptor alterations will provide more insight in the molecular mechanisms of diabetes progression and further highlights dysregulated basal insulin as a direct cause and hallmark of diabetes.

**Experimental Procedures**

**Cell culture**

The C2C12 mouse myoblast (ATCC cell line provided by Dr. Brain Rodrigues, University of British Columbia, Vancouver, Canada) was maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), and
For downstream analysis, $8 \times 10^5$ cells/well of cells were seeded in 6-well plates and cultured at 37 °C under 5% CO$_2$. Confluent (90%) myoblasts were differentiated into myotubes by culturing the cells in differentiation medium (DMEM supplemented with 2% horse serum and 1% penicillin-streptomycin) for 10 days. To induce insulin resistance by hyperinsulinemia in vitro, C2C12 myotubes were cultured in differentiation medium containing 2 or 200 nM human insulin (Cat.# I9278, Sigma) for 16 hours prior to reaching day 10 (Fig.1A). Insulin concentrations after the 16 h hyperinsulinemia treatment were measured using human insulin RIA kit (Millipore). For serum starvation, myotubes were maintained in serum-free medium (DMEM supplemented with 1% penicillin-streptomycin) for 6 hours. All experiments were repeated with biological replicates using cells in different passages.

**Experimental Animals**

Animal protocols were approved by the University of British Columbia Animal Care Committee. $\text{Ins}1^{+/+};\text{Ins}2^{-/-}$ and $\text{Ins}1^{+/+};\text{Ins}2^{-/-}$ mice were randomly assigned to be fed ad libitum either a high fat diet (Research Diets D12492, 20% protein, 60% fat, 20% carbohydrate content, energy density 5.21Kcal/g, Brunswick, NJ, US) or low fat diet (Research Diets D12450B, 20% protein 10% fat, 70% carbohydrate content, energy density 3.82Kcal/g, Brunswick, NJ, US) for 4 weeks starting from 8 weeks old. Blood fasting glucose was measured using OneTouch Ultra2 glucose meters (LifeScan Canada Ltd, BC, Canada), and serum fasting insulin were assessed using mouse insulin ELISA kit (Alpco Diagnostics, Salem, NH, USA), following 4-hour fasting.

**Western blot analyses**
Hyperinsulinemia-induced insulin resistance

C2C12 myotubes or mice skeletal muscle (gastrocnemius) tissues were sonicated in RIPA buffer (50 mM β-glycerol phosphate, 10 mM HEPES, 1% Triton X-100, 70 mM NaCl, 2 mM EGTA, 1 mM Na3VO4, and 1 mM NaF) supplemented with complete mini protease inhibitor cocktail (Roche, Laval, QC), and lysates were resolved by SDS-PAGE. Proteins were then transferred to PVDF membranes (BioRad, CA) and probed with antibodies against p-ERK1/2 (Thr202/Tyr204) (1:1000, Cat. #4370), ERK1/2 (1:1000, Cat. #4695), p-AKT (Ser473) (1:1000, Cat. #9271), p-AKT (Thr308) (1:1000, Cat. #9272), AKT (1:1000, Cat. #9272), INSR-β subunit (1:1000, Cat. #3020S), p-INSRβ (Tyr1150/1151) (1:1000, Cat. #3024), FOXO1 (1:1000, Cat. #2880), p-FOXO1 (Thr24) (1:1000, Cat. #9464), all from Cell Signalling (CST), and β-tubulin (1:2000, Cat. #T0198, Sigma). The signals were detected by secondary HRP-conjugated antibodies (Anti-mouse, Cat. #7076; Anti-rabbit, Cat. #7074; CST) and Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Protein band intensities were quantified with Image Studio Lite software (LI-COR).

siRNA knockdown in C2C12 myoblasts

All siRNAs are from Thermo Fisher Scientific with the specific Assay IDs as follows: Foxo1 (MSS226201), Sin3a (151684), Elf1 (157302), Mxi1 (68202), Myc (68302), Ets1 (101877), Hcfc1 (158001), Nrf1 (68266), JunD (67635), Ctcf (60925), Max (155266), Maz (501159), Silencer Cy3-labeled Negative Control No.1 siRNA (AM4621). siRNAs were transfected into C2C12 myoblasts using the Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions with 50 pmol of siRNA and 4 µl of transfection reagent per well of a 12-well plate.

RNA isolation and quantitative real-time PCR analysis
Total RNA was isolated from both control and high insulin-treated C2C12 myotubes before and after serum starvation or C2C12 myoblasts post siRNA transfection using the RNEasy mini kit (Qiagen). cDNA was generated by reverse transcription using qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). Transcript levels of target genes in the equal amount of total cDNA were quantified with SYBR green chemistry (Quanta Biosciences) on a StepOnePlus Real-time PCR System (Applied Biosystems). All data were normalized to Hprt by the $2^{-\Delta\Delta Ct}$ method. The following primers are used in qPCR: Insr-A/B forward 5'-TCCTGAAGGAGCTGGAGGAGT-3', Insr-A reverse 5'-CTTTCGGGATGGCCTGG-3', Insr-B reverse 5'-TTTGGATGAGGCTACTGTC-3' (66); Insr (in siRNA experiments) forward 5'-TTTGGATGAGGCTACTGTC-3' and reverse 5'-CCTCATCTTGGGGTTGAACT-3' (67) Igf1r forward 5'-GGCACAATCCTGGCTCAAGAC-3' and reverse 5'-CTTTATCACCACACACACTTCTG-3' (66); Hprt forward 5'-TCAGTCAACGGGGACATAAA-3' and reverse 5'-GGGGCTGTACTGCTTAACCAG-3' (68); Foxo1 forward 5'-CCCAGGCGGAGTTTAACC-3' and reverse 5'-GTTGCTCATAAGTCGGTGCT-3', Tbp forward 5'-AGAACAATCCAGACTAGCAGCA-3' and reverse 5'-GGGAACTTCACATCACAGCTC-3', Nrf1 forward 5'-TATGGCGGAAGTAATGAAAGACG-3' and reverse 5'-CAACGTAAGCTCGCTTGTG-3', JunD forward 5'-GAGAGGCGCCCTCTCTCTA-3' and reverse 5'-TGCGTGAGTAGCTGTCAG-3', Jund forward 5'-GAGGATAGCTGTCAG-3' and reverse 5'-GGGAACTTCACATCACACACT-3', Ctcf forward 5'-GATCCTACCCTTCATGAA-3' and reverse 5'-GTACCGTCACAGGAACGAAAG-3', Elf1 forward 5'-TGCCATGAGCTCGCTTGTG-3' and reverse 5'-CTTTGATGGCAAGAAG-3', Ets1 forward 5'-TCCTATCAGCTCGGAAGA-3' and reverse 5'-GCCCGAGGCGGATGCTTT-3', Mxi1 forward 5'-CACACAGCTGGAGGACCAAG-3', Mxa forward 5'-ACCATAATGCACGCAACGAAAG-3' and reverse 5'-GTCCCGCGAGGCAAGAAG-3'.
Hyperinsulinemia-induced insulin resistance

390 AAACTGTGAAAGC-3', Myc forward 5'-ATGCCCTCAACGTGAACCTTC-3' and reverse 5'-
391 CGCAACATAGGAGAGCA-3', Hcfc1 forward 5'-CGGCAACCGAGGGGATAGTG-3' and
392 reverse 5'-TAGGGCAAGTTACACACAC-3', Sin3a forward 5'-GCCTGTGAGATTTAATCAT
393 GCC-3' and reverse 5'-CCTCTTGCTCAGTCAAAGCTG-3', Irs2 forward 5'-CTCGGTCCCTCTCC
394 CAAAGTG-3' and reverse 5'-GGGGTCATGGGCATGTAGC-3' (PrimerBank https://pga.mgh.
395 harvard.edu/primerbank/index.html).

397 Surface Protein Biotinylation Assay

Biotinylation of surface proteins was performed as previously described (69) with
398 modifications (Fig. 4A). In brief, cells were incubated with cell-impermeable EZ-Link-NHS-SS-
399 biotin (300 μg/ml in PBS; Pierce) at 37°C for 2 min. Cells were then immediately placed on ice
400 and washed with ice-cold 50 mM Tris-buffered saline (TBS) to remove excess biotin. For isolating
401 surface proteins, cells were washed using ice-cold PBS and lysed in complete RIPA buffer
402 (supplemented with cOmplete mini protease inhibitor cocktail (Roche, Laval, QC) and Na₃VO₄).
403 For detecting internalized proteins, cells were washed with PBS and incubated in serum-free
404 medium supplemented with 0.2, 2 or 20 nM insulin at 37°C to stimulate INSR internalization. After
405 certain time periods, cells were placed on ice, washed with ice-cold PBS, incubated with
406 Glutathione solution (50 mM glutathione, 75 mM NaCl, 1 mM EDTA, 1% BSA, 75 mM NaOH) for
407 20 min to strip remaining surface biotin, washed with excess PBS, and lysed in complete RIPA
408 buffer. Lysates were quantitated and incubated with NeutrAvidin beads (Pierce) overnight at 4 °C
409 to isolate biotinylated surface or internalized proteins. Biotinylated proteins were eluted from the
410 NeutrAvidin beads by boiling in Blue Loading Buffer (CST) containing 50 mM DTT for 5 min.
Surface or internalized INSR in eluent and total INSR in lysates were detected in Western blot analysis.

**RNA sequencing and bioinformatic analysis**

Total RNA isolated from both control and 200 nM insulin-treated C2C12 myotubes before and after serum starvation (4 groups, n = 5 each group) were sequenced by BRC Sequencing Core at the University of British Columbia. Sample quality control was performed using the Agilent 2100 Bioanalyzer. Qualifying samples were then prepped following the standard protocol for the NEBNext Ultra II Stranded mRNA (New England Biolabs). Sequencing was performed on the Illumina NextSeq 500 with Paired End 42bp × 42bp reads. Sequencing data was demultiplexed using Illumina's bcl2fastq2. De-multiplexed read sequences were then aligned to the Mus Musculus mm10 reference sequence using STAR aligner (https://www.ncbi.nlm.nih.gov/pubmed/23104886). Assembly was estimated using Cufflinks (http://cole-trapnell-lab.github.io/cufflinks/) through bioinformatics apps available on Illumina Sequence Hub using default settings. A visual analytic platform NetworkAnalyst 3.0 (http://www.networkanalyst.ca/) was used for PCA, differential expression (DESeq2), PPI network, and TF-gene network analysis. For the transcription factor and gene network, data are derived from the ENCODE ChIP-seq data. PPI network data are based on STRING interactome with high (900-1000) confidence score.

**Statistics**

Data were presented as mean ± SEM in addition to the individual data points. All data were analyzed using R Studio 3.4.1. A significance level of adjusted p < 0.05 was used throughout. All western blot quantifications (protein band intensity) were analyzed using linear regression.
modeling (70). Linear mixed effect models (R package – lme4) were fitted using restricted maximum likelihood (70,71). Predictor variables were included as fixed effects and sample IDs were included as random effects. Mixed effect modeling was used to account for repeated sample measurements and missing data (70). Where the random effect was not significant, linear fixed effect modeling was used. Heteroscedasticity and normality of residuals were analyzed using Levene’s test and the Shapiro–Wilk test, respectively. Predictor variables, insulin treatment (overnight and acute) and time, were treated as ordinal factors and continuous factor, respectively. The outcome variable, protein band intensity, was treated as a continuous factor and log-transformed when residuals are not homoscedastic and/or normally distributed. Multiple comparison p-values were adjusted using the Tukey method.

Acknowledgments: We acknowledge Dr. Brain Rodrigues for providing the C2C12 cells. We thank our colleagues Xiaoke Hu and Leanne Beet for performing the insulin radioimmunoassays. We thank those who provided helpful comments on the drafts of this manuscript posted on Biorxiv.

Contributions: HC designed the study, performed all experiments except the high-fat feeding, analyzed data and wrote the manuscript. JDB performed in vivo high fat feeding and analyzed the related data. SW performed statistical analysis and edited the manuscript. NN performed siRNA knockdown experiments. JDJ designed the study, supervised the research and edited the manuscript. JDJ is the ultimate guarantor of this work.

Funding: This study was funded by a CIHR Operating Grant to JDJ.
Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

References


Hyperinsulinemia-induced insulin resistance


Hyperinsulinemia-induced insulin resistance


Hyperinsulinemia-induced insulin resistance


Hyperinsulinemia-induced insulin resistance


Figure Legends

Figure 1. Basal and acute insulin signaling in an in vitro model of hyperinsulinemia-induced insulin resistance. (A) The workflow of C2C12 myotube differentiation, high insulin treatment and serum starvation. Differentiated myotubes were cultured in control (0 nM insulin) or hyperinsulinemic (2 or 200 nM insulin) medium for 16 hours and were analyzed before and after serum starvation. (A') The insulin concentration in the medium at the end of 16-hour high insulin treatment (n=3). (B) Representative western blot images of phospho-AKT (T308, S473), total AKT, phospho-ERK1/2, and total ERK1/2. (C) Total AKT and (D) total ERK abundance under high insulin treatments before starvation (BS) and after starvation (AS). (E) phospho-AKT (T308, S473) and (F) phospho-ERK1/2 measurements before starvation (BS), after starvation (AS), and stimulated by 0.2 or 2 nM insulin for 10 min after serum starvation. (n=6-9; # effect of hyperinsulinemia, $ effect of starvation, & effect of acute insulin, × interaction between two factors, Mixed Effect Model.)

Figure 2. Insulin dose- and time-depend acute insulin signaling in a hyperinsulinemia-induced insulin resistance model. Myotubes cultured in control (0 nM insulin) or hyperinsulinemic (200 nM insulin) medium were stimulated with acute 0.2, 2 or 20 nM insulin for 1, 5, 10, 15 or 30 min after serum starvation. (A) phospho-AKT (T308, S473) and (B) phospho-ERK1/2 was measured. (n=4; # effect of hyperinsulinemia, & effect of acute insulin, × interaction between two factors, Mixed Effect Model.)
Figure 3. Effects of hyperinsulinemia and serum starvation on INSR protein abundance and phosphorylation. (A) Representative western blot images of phospho-INSR (Y1150/1151) and total INSR. (B) The level of total INSR protein before or after serum starvation (n=4-6). (C) Phospho-INSR (T308, S473) measurements before starvation (BS), after starvation (AS), and stimulated by 0.2 or 2 nM insulin for 10 min after serum starvation (n=4-6). (D) Insulin dose- and time-dependent INSR phosphorylation after serum starvation (n=4). (# effect of hyperinsulinemia, $ effect of starvation, & effect of acute insulin, × interaction between two factors, mixed effect model.)

Figure 4. Effects of in vitro hyperinsulinemia on INSR internalization. (A) Scheme of surface biotinylation assay to measure surface or internalized INSR. Internalized INSR in isolated proteins is detected by western blots. (B) The ratio of surface to total INSR ((n = 3 in BS group, n = 10 in AS group). (C) The ratio of internalized to total INSR (n = 4). (# effect of hyperinsulinemia, $ effect of starvation, × interaction between two factors, mixed effect model).

Figure 5. In vivo correlation between INSR abundance and fasting insulin or glucose in mouse skeletal muscle. (A) INSR and fasting insulin in LFD-fed mice. (B) INSR and fasting glucose in LFD-fed mice. (C) INSR and fasting insulin in HFD-fed mice. (D) INSR and fasting glucose in HFD-fed mice. (n = 7-11)

Figure 6. Effects of prolonged hyperinsulinemia and starvation on Insr transcription and FOXO1 phosphorylation in vitro. (A) The mRNA levels of Insr isoform A or B (Insr-A or B) before and after starvation (BS and AS) assessed by qPCR. (B) Igf1r mRNA level. (C) The ratio of Insr-
Hyperinsulinemia-induced insulin resistance

A to Insr-B mRNA (n=5). (D) Total and T24 phosphorylation of FOXO1 (n = 3 in BS group, n = 10 in AS group). (# effect of hyperinsulinemia, $ effect of starvation, × interaction between two factors, mixed effect model).

Figure 7. Transcriptomic analysis of hyperinsulinemia and serum starvation. (A) Principle component analysis (PCA) of RNA-seq data from 4 groups of treatments, including 0 nM or 200 nM prolonged insulin both before and after serum starvation. (B) Top 50 differentially expressed genes that have highest adjusted p value and more than 2-fold changes in nested comparisons for the effects of hyperinsulinemia before and after serum starvation. (C) Top 20 KEGG pathways enriched among downregulated genes by hyperinsulinemia before starvation (BS, 0 vs 200 nM insulin). The direction of change of the same pathways affected by starvation (200 nM, BS vs AS) or after starvation (AS, 0 vs 200 nM) are also shown. (D) Insulin signaling, and (E) FOXO signaling pathways enriched in the PPI network of deferentially expressed genes.

Figure 8. Identification of upstream transcription factors mediating the overall transcriptomic changes and insulin receptor expression. (A) TF-gene network predicted upstream transcriptional regulators of differentially expressed genes by hyperinsulinemia before starvation. (B) Common TFs between the top 30 TF nodes from (A) and key genes in insulin signaling pathways that are downregulated by hyperinsulinemia. TFs upregulated by hyperinsulinemia are in red. (C) mRNA levels of the common TFs in (B) after siRNA knockdown. (D) The effects of each siRNA knockdown on Insr and Irs2 mRNA levels. (n=4; * p<0.05, 1-ANOVA followed by Dunnett multiple comparison test against siControl). (E) Graphic summary of our current model. Hyperinsulinemia induced sustained phosphorylation of INSR and AKT, which
resulted in the inhibition of FOXO1 leading to reduced $\text{Insr}$ transcription. Dowregulated INSR and post receptor components resulted in reduced insulin signaling upon acute insulin stimulation. SIN3A, which was upregulated by hyperinsulinemia, might repress $\text{Insr}$ transcription.
Differentiation – 10 days
Starvation - 6 h

Figure 1. Basal and acute insulin signaling in an in vitro hyperinsulinemia-induced insulin resistance model.
Figure 2. Insulin dose- and time-depend acute insulin signaling in the hyperinsulinemia-induced insulin resistance model.
Figure 3. Effects of hyperinsulinemia and serum starvation on INSR abundance and phosphorylation.
Figure 4. Effects of *in vitro* hyperinsulinemia on INSR internalization.
Figure 5. *In vivo* correlation between INSR abundance and fasting insulin or glucose in mouse skeletal muscle.
Figure 6. Effects of prolonged hyperinsulinemia and starvation on *Insr* transcription and FOXO1 phosphorylation *in vitro*.
Figure 7. Transcriptomic analysis of hyperinsulinemia and serum starvation.
Figure 8. Identification of upstream transcription factors mediating the overall transcriptomic changes and insulin receptor expression.
Supplementary Figures

Figure S1. Transcriptomic analysis of hyperinsulinemia and serum starvation. (A) Top 20 KEGG pathways enriched from up or down regulated genes in all 4 comparisons of insulin and starvation treatments. (B) RAS and MAPK signaling, and (C) PI3K-AKT signaling pathways enriched in the PPI network of deferentially expressed genes by hyperinsulinemia before starvation (BS, 0 vs 200 nM insulin).

Figure S2. Alterations in KEGG pathways. Differentially expressed genes altered by hyperinsulinemia before starvation (BS, 0 vs 200 nM insulin) are mapped to corresponding proteins in (A) Insulin signaling, (B) PI3K-AKT signaling, (C) RAS and (D) MAPK, and (E) FOXO signaling pathways.
Figure S1. Transcriptomic analysis of hyperinsulinemia and serum starvation.
Figure S2. Alterations in KEGG pathways.
Figure S2. Alterations in KEGG pathways.
Figure S2. Alterations in KEGG pathways.