Modulation of *Insr* and insulin receptor signaling by hyperinsulinemia *in vitro* and *in vivo*

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ABSTRACT

Hyperinsulinemia is often viewed as a compensatory mechanism for insulin resistance, but recent 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 myotubes with 200 nM insulin for 16 hours, followed by a 6-hour period of serum starvation, before examining insulin signaling. Using this model, we found that prolonged high insulin treatment significantly increased the phosphorylation of insulin receptor and AKT but not ERK. After serum starvation, acute AKT and ERK signaling stimulated by 0.2 - 20 nM insulin were attenuated. Total and surface insulin receptor protein levels are significantly downregulated by hyperinsulinemia, which resulted in an inhibition of acute insulin signaling. Mechanistically, we found that both isoforms of insulin receptor mRNA were reduced by hyperinsulinemia and implicated the transcription factor FOXO1. Interestingly, 6h serum starvation reversed the effects of high insulin on basal phosphorylation of insulin receptor, AKT and FOXO1 and insulin receptor transcription. Finally, we validated our results *in vivo*, by determining that insulin receptor levels in mouse skeletal muscle were negatively correlated with circulating insulin. Together, our findings shed 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 and highly associated with each other. It is a widely held

view that insulin resistance is the primary cause of type 2 diabetes and that hyperinsulinemia is a compensatory response (1-4). However, a growing body of evidence suggests the opposite may be true (5-8). Hyperinsulinemia can be observed prior to insulin resistance in obesity and T2D (9-11). Indeed, hyperinsulinemia is the strongest predictor of T2D in long-term studies (12,13). Our group has recently shown that hyperinsulinemia can contribute causally to age-dependent insulin resistance in the absence of changes in glycemia (14). Reducing hyperinsulinemia using partial insulin gene knockout was also found to prevent and reverse diet-induced obesity in adult mice (14-16). Healthy humans (17,18) and rodents (19,20) subjected to prolonged insulin administration also have reduced insulin responsiveness independent of hyperglycemia, strongly suggesting that hyperinsulinemia is a self-perpetuating cause of insulin resistance.

The mechanisms by which hyperinsulinemia contributes to insulin resistance remains poorly understood. The insulin receptor (INSR) is a critical component as the starting point of insulin action. It has been reported that diabetic or obese rodent models with hyperinsulinemia and insulin resistance have reduced insulin binding to the liver (21-23), fat (24-26) and pancreatic acinar cells (27). The insulin binding defect appeared to 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 (28) and lymphocytes (29) *in vitro*. However, the molecular mechanisms of INSR down-regulation and post-receptor insulin resistance are poorly characterized.

In the present study, we employed a model of *in vitro* insulin resistance using a muscle cell line. We found that prolonged hyperinsulinemia induced insulin resistance featuring blunted acute AKT and ERK signaling and INSR downregulation. Reduced *Insr* transcription and FOXO1 activity contributed to the INSR downregulation. We confirmed the inverse relationship between hyperinsulinemia and INSR abundance *in vivo*.

Results

Hyperinsulinemia induces insulin resistance in muscle cells in vitro

To establish a muscle-cell model of hyperinsulinemia-induced insulin resistance, differentiated mouse C2C12 myotubes were cultured in 200 nM insulin for 16 hours (Fig. 1A). After 6-hour serum starvation, insulin signaling was characterized by measuring the phosphorylation of AKT and ERK proteins, which represent the two major insulin signaling pathways (30). For high-insulin treated cells, AKT phosphorylation at threonine (T) 308 and serine (S) 473 were significantly reduced at several time points under 0.2, 2, or 20 nM insulin stimulations (Fig. 1B). Similarly, ERK1/2 also had reduced phosphorylation (Fig. 1C). Total AKT and ERK1/2 levels were not significantly different after starvation (Fig. 1D).

Therefore, both AKT and ERK signaling branches of acute insulin signaling were blunted, confirming insulin resistance in the cells exposed to hyperinsulinemia.

Alterations in the basal state of the insulin signal transduction network have also been reported in hyperinsulinemic patients and animals (31). Therefore, we measured the effects of hyperinsulinemia on AKT and ERK phosphorylation before and after serum starvation. After prolonged hyperinsulinemia and before serum starvation, T308 and S473 phosphorylation of AKT was significantly elevated ~4 fold, but ERK phosphorylation was unaffected (Fig. 1D). Interestingly, basal p-AKT and p-ERK1/2 in the hyperinsulinemia group became lower relative to the controls after serum starvation (Fig. 1D), which may predispose the AKT and ERK signaling pathway to milder response to the following acute insulin stimulation. Of note, p-ERK1/2 was increased by serum starvation, as previously reported in other cell types (32,33), but not hyperinsulinemia (Fig. 1D). Total AKT and ERK were also downregulated by hyperinsulinemia, but this effect was completely rescued by serum starvation. Thus, the attenuated acute insulin signaling was not limited by total AKT and ERK. Together, these results establish a robust muscle cell insulin resistance model induced by hyperinsulinemia and characterized its effects on time and dose-dependent insulin signaling.

Hyperinsulinemia reduces INSR protein abundance but not its phosphorylation

To understand how hyperinsulinemia induces insulin resistance, we started at the insulin receptor (INSR), the initial point of insulin signaling. Remarkably, we found the total INSR protein abundance was robustly decreased both before and after serum starvation (Fig. 2A). As insulin can only access surface INSR to initiate signaling, we employed surface biotinylation assay to measure the surface INSR (Fig. 2B). Surface INSR was indeed decreased after hyperinsulinemia both before and after starvation, while the surface-to-total INSR ratio was only slightly increased before starvation (Fig. 2C), indicating increased surface translocation, reduced internalization or increased degradation of intracellular INSR. Furthermore, serum starvation decreased surface-to-total INSR ratio in the control group (Fig. 2C), suggesting that INSR localization is tightly regulated by its ligand concentration. Therefore, hyperinsulinemia-induced insulin resistance is mediated by a reduction in total INSR that results in a proportional reduction in INSR protein at the cell surface, and the subcellular localization of INSR can adapt to the ambient insulin concentration.

INSR autophosphorylation upon insulin binding is a key initial step of insulin signaling, which recruits IRS and SHC that leads to the activation of PI3K-AKT or RAS-ERK signaling branches (34). Therefore, we examined the tyrosine(Y) 1150/1151 phosphorylation of INSR. Similar to AKT, hyperinsulinemia induced a ~9-fold increase in INSR phosphorylation, suggesting that there is continuous insulin

signaling during the high insulin treatment (Fig. 3A). Serum starvation completely reversed INSR hyperphosphorylation (Fig. 3A). The phosphorylated-to-total INSR ratio was slightly increased in the high-insulin treated group under acute insulin stimulations (Fig. 3B). However, p-INSR-to-tubulin ratio, which is proportional to the overall INSR phosphorylation events per cell, was decreased at several time points (Fig. 3C). The increased INSR phosphorylation per receptor could be compensation to reduced INSR number, indicating that INSR does not have defects in autophosphorylation upon acute insulin stimulation. Thus, the limited availability of surface and total INSR contributes to the reduced INSR signaling.

Hyperinsulinemia decreases Insr mRNA via FOXO1 inhibition

One possible cause of INSR downregulation would be the reduced expression of the *Insr* gene (27). 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. 4A). Both *Insr-A* and *Insr-B* mRNA were equally downregulated after hyperinsulinemia and partially recovered after serum starvation (Fig. 4A), consistent with the change of INSR protein abundance. Interestingly, insulin-like growth factor 1 receptor (IGF1R), which has high similarity in structure and signaling as INSR (35), was also reduced at the transcriptional level (Fig. 4B). Alteration in *Insr* alternative splicing has been implicated in insulin resistance and T2D (36-41). Here, the ratio of *Insr-A* and *Insr-B* mRNA was not affected by hyperinsulinemia or serum starvation (Fig. 4C), indicating that hyperinsulinemia and starvation did not affect the alternative splicing of *Insr* transcripts in our model. Our evidence suggests that hyperinsulinemia and serum starvation regulate INSR protein at the transcriptional level.

Forkhead box protein O1 (FOXO1) is a known transcriptional regulator of the *Insr* gene and is also a key mediator of insulin signaling (42-44). 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 (43). Therefore, we sought to determine the activity of FOXO1 in our hyperinsulinemic model. Indeed, high insulin inhibited FOXO1 via increased phosphorylation on T24, which is an Akt-associated event known to exclude this protein from the nucleus and decrease FOXO transcriptional activity (45), but did not affect total FOXO1 abundance (Fig. 4D). T24 phosphorylation of FOXO1 significantly decreased after starvation (Fig. 4D), consistent with our observed effects on AKT phosphorylation and *Insr* transcription. Together, these data strongly suggest that hyperinsulinemia downregulates *Insr* transcription via FOXO1 phosphorylation.

Circulating insulin negatively correlates with INSR level in vivo

To validate our *in vitro* studies, in a specific

in vivo model of hyperinsulinemia, we further examined the relationship of insulin concentration and INSR level in mice fed a high-fat diet. Mice with different insulin gene dosage (*Ins1*$^{+/+}$;*Ins2*$^{-/-}$ and *Ins1*$^{+/-}$;*Ins2*$^{-/-}$) previously studied by our group (15) were used to generate variance in circulating insulin. The mice were fed with high-fat diet (HFD) known to induced pronounced hyperinsulinemia (15,46) or low-fat diet (LFD). INSR protein abundance in skeletal muscle was measured by western blots. INSR levels negatively correlated with both fasting insulin and fasting glucose in the HFD group (Fig. 5A-B). However, the LFD group only had a significantly 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, 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 (14). These data also suggest an interaction between insulin, glucose and INSR that is dependent on the conditions of the HFD.

Discussion

The goal of this study was to explore the mechanisms of hyperinsulinemia-induced insulin resistance in skeletal muscle cells. We confirmed that prolonged hyperinsulinemia induced sustained reduction of AKT and ERK signaling, which was characterized over time after acute stimulation with insulin at multiple doses. We demonstrated that the impaired insulin response originates with INSR downregulation at the transcription level downstream of FOXO1 phosphorylation. Serum starvation partially reversed the effects of hyperinsulinemia. We also validated our *in vitro* system *in vivo* in mice with varying degrees of diet-induced hyperinsulinemia.

Our *in vitro* cell culture model provided a robust and controlled system for examining the direct effects of excess insulin, and insulin withdrawal, on insulin signaling. Our results are consistent with other *in vitro* cell culture systems designed to examine the effects of hyperinsulinemia. For example, recent studies also reported reduced AKT and ERK signaling and INSR abundance in high-insulin treated β-cells and enteroendocrine L cells (47,48). Increased basal AKT phosphorylation and unaltered ERK phosphorylation have also been reported in neurons treated with elevated insulin for 24 hours (49). It has been suggested that increased basal AKT mediates insulin resistance because an AKT inhibitor, but not ERK inhibitor, improved insulin resistance (49,50). Our observations verified the independent responses of the bifurcate insulin signaling pathways during hyperinsulinemia. It is worth noting that the AKT and ERK phosphorylation appeared to be suppressed at all time points in our studies, suggesting that the insulin resistance we observed was impaired responsiveness instead of a change in sensitivity; this is consistent with

signaling deficiencies at both the receptor level and in post-receptor components (51). Remarkably, the impaired acute insulin signaling due to 16 hours of hyperinsulinemia we observed was sustained after 6 hours of insulin withdrawal, suggesting that long-term molecular changes underlie these differences.

The main finding of our study was that that hyperinsulinemia directly reduced *Insr* mRNA in cultured cells, consistent with reports from other cell culture systems (27,52). 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* (15). 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 (53). While relative hyperglycemia can increase *Insr* expression in lymphocyte and cancer cell lines (54,55), high glucose inhibits β-cells *Insr* expression through autocrine insulin action and Insr-FoxO1 signaling (54,55). Interestingly, glucose can only induce insulin resistance in the presence of insulin in cultured hepatocytes, adipocytes and skeletal muscle (25,56,57). Therefore, reduced *Insr* expression by hyperinsulinemia may be a key, independent factor of INSR downregulation and insulin resistance.

Hyperinsulinemia promoted the surface localizaiton of INSR in the present study, while serum starvation had the opposite effect. The mechanisms by which INSR localization is regulated remain poorly understood. A recent study demonstrated that the E3 ligase, MARCH1, can specifically ubiquitinate surface INSR, leading to their internalization and degradation (58). Interestingly, FOXO1 is also a transcription factor for *March1* gene, and insulin-induced FOXO1 inhibition downregulates MARCH1, resulting in increased surface INSR (58). Therefore, the INSR localzation regulated by ambient insulin in our model may associate with MARCH1 function.

Intermittent fasting, time restricted feeding, caloric restriction, and/or carbohydrate restriction have been shown to have health benefits in diabetes, including lowering insulin and glucose levels, increasing insulin sensitivity, and improving β cell responsiveness (59-63). Several human trials suggest that these fasting regimes are more effective on reducing insulin and increasing insulin sensitivity than on reducing glucose (62,64-67). By mimicking the low-insulin state, the serum starvation phase of our studies revealed some possible molecular mechanisms of the beneficial effects of fasting, which includes restoration of AKT and ERK protein levels, protein phosphorylation in insulin signaling pathways, and partial recovery of *Insr* transcription. These data hint that many deleterious effects of hyperinsulinemia are reversible but may require a long enough low-insulin period.

The present study has limitations and unanswered questions. We employed a 16-hour incubation with a super-physiological insulin

dosage (200 nM) instead of more chronic treatment with lower insulin. The main reason is that insulin promotes the further differentiation of C2C12 myotubes (68), thus chronic treatment during differentiation introduces variance in the muscle cell model. Hyperinsulinemia is also known to downregulate proteins such as IRS1, IRS2, GLUT4 (69-72), and our study was not comprehensive in this regard. Thus, it would be interesting to examine the effect of hyperinsulinemia and fasting on other signaling molecules in our model to further dissect the cause of insulin resistance. In addition, besides FOXO1, other transcription factors such as SP1, HMGA1, C/EBPβ and NUCKS have been shown to regulate *Insr* expression (73-77). For example, HMGA1 is downregulated in diabetes, which inhibits *Insr* as well as *Foxo1* transcription (76). The roles of these other transcriptional regulators in hyperinsulinemia could be investigated in future studies using our system. INSR degradation and proteolysis are also increased by high insulin in lymphocyte and adipocytes (27,28,78). Although *Insr* mRNA and protein cannot be directly compared, the fold reduction of *Insr* mRNA was smaller than fold reduction of INSR protein in our cell model, suggesting that additional mechanisms are involved. Therefore, the mechanisms of INSR and insulin signalling down-regulation in hyperinsulinemia-induced insulin resistance will require more detailed future investigations.

Despite its inherent reductionism, our *in vitro* model reemphasized the critical and detrimental role of hyperinsulinemia in the development of insulin resistance and T2D. We demonstrated that *in vitro* hyperinsulinemia and serum fasting had profound effects and interactions in regulating AKT and ERK signaling and protein levels, INSR levels and surface 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 cell line (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 1% penicillin-streptomycin (100 µg/ml; Gibco). For downstream analysis, 8×10^5 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 200 nM insulin (Cat.# I9278, Sigma) for 16 hours prior to reaching day 10 (Fig.1). For serum starvation, myotubes are 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. *Ins1*$^{+/+}$;*Ins2*$^{-/-}$ and *Ins1*$^{+/-}$;*Ins2*$^{-/-}$ 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.

RNA isolation and quantitative real-time PCR analysis

Before and after serum starvation, total RNA was isolated from both control and high insulin-treated C2C12 myotubes 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 $2^{-\Delta Ct}$ method (79). The following primers are used in qPCR: *Insr-A/B* forward 5'-TCCTGAAGGAGCTGGAGGAGT-3', *Insr-A* reverse 5'-CTTTCGGGATGGCCTGG-3', *Insr-B* reverse 5'-TTCGGGATGGCCTACTGTC-3' (80); *Igf1r* forward 5'-GGCACAACTACTGCTCCAAAG AC-3' and reverse 5'-CTTTATCACCACCACA CACTTCTG-3' (80); *Hprt* forward 5'-TCAGTCAACGGGGGACATAAA-3' and reverse 5'-GGGGCTGTACTGCTTAACCAG-3' (81).

Western blot analyses

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 Na_3VO_4, 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. #9275), 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).

Surface Protein Biotinylation Assay

Biotinylation of surface proteins was performed as previously described(82) with modifications (Fig. 2B). In brief, cells were incubated with cell-impermeable EZ-Link-NHS-SS-biotin (300 μg/ml in PBS; Pierce) at 37 °C for 2 min. Cells were then immediately placed on ice and washed with ice-cold 50 mM Tris-buffered saline (TBS) to remove excess biotin. Next, cells were washed using ice-cold PBS and lysed in RIPA buffer supplemented with cOmplete mini protease inhibitor cocktail (Roche, Laval, QC). Lysates were quantitated and incubated with NeutrAvidin beads (Pierce) overnight at 4 °C to isolate biotinylated surface proteins. Surface proteins were eluted from the NeutrAvidin beads by boiling in Blue Loading Buffer (CST) containing 50 mM DTT for 5 min. Surface INSR in eluent and total INSR in lysates were detected in Western blot analysis.

Statistics

Data were presented as mean ± SEM unless otherwise indicated. 2-way ANOVA or Student t-test were used as indicated (Prism; GraphPad). Specifically, western blot data in control and high-insulin groups at each acute insulin or starvation time point were tested by paired t-test because each replicate used the same batch of cells. $p < 0.05$ was considered significant.

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Contributions: HC designed the study, performed experiments, analyzed data and wrote the manuscript. JDB performed experiments and analyzed data. JDJ designed the study, supervised the research and edited the manuscript.

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Figure Legends

Figure 1. Acute and basal insulin signaling in an *in vitro* hyperinsulinemia-induced insulin resistance model. (A) The workflow of C2C12 myotube differentiation, high insulin treatment and serum starvation. **(B)** phospho-AKT (T308, S473) and **(C)** phospho-ERK1/2 stimulated by acute insulin after serum starvation. Myotubes cultured in control (0 nM insulin) or hyperinsulinemic (200 nM insulin) medium were stimulated with 0.2, 2 or 20 nM insulin for 1, 5, 10, 15 or 30 min (n = 4). **(D)** Basal levels of AKT and ERK phosphorylation before starvation (BS) and after serum starvation (AS)(n = 3 in BS groups; n = 10 in AS groups). * $p < 0.05$, student t-test (paired t-test between insulin groups at each time points). $^{\#}$ effect of hyperinsulinemia; $^{\&}$ effect of starvation; $^{\%}$ interaction between the two factors, 2-ANOVA.

Figure 2. Effects of *in vitro* hyperinsulinemia and serum starvation on total and surface INSR. (A) The level of total INSR protein before or after serum starvation. **(B)** Scheme of surface biotinylation to measure surface INSR. Biotins label all surface proteins which are then isolated by NeutrAvidin beads from lysates. Surface INSR in isolated proteins is detected by western blots. **(C)** The surface INSR abundance and the ratio of surface-to-total INSR (n = 3-4 in BS groups; n = 10 in AS groups). * $p<0.05$, student t-test (paired t-test between insulin groups at each time points). $^{\#}$ effect of hyperinsulinemia; $^{\&}$ effect of starvation; $^{\%}$ interaction between the two factors, 2-ANOVA.

Figure 3. Effects of prolonged hyperinsulinemia on INSR phosphorylation *in vitro*. (A) Basal phospho-INSR Y1150/1151 before or after serum starvation. (n = 3-4 in BS groups; n = 10 in AS groups). (B) Insulin-stimulated INSR phosphorylation after serum starvation (n=4). * $p<0.05$, student t-test (paired t-test between insulin groups at each time points). $^{\#}$ effect of hyperinsulinemia, 2-ANOVA.

Figure 4. 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)** The ratio of *Insr-A* to *Insr-B* mRNA. **(C)** *Igf1r* mRNA level. n=5. **(D)** Total and T24 phosphorylation of FOXO1 (n = 3 in BS groups, n = 10 in AS groups). * $p<0.05$, student t-test (paired t-test between insulin groups at each time points).

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 glucose in LFD-fed mice. **(C)** INSR and fasting insulin in HFD-fed mice. **(D)** INSR and glucose in HFD-fed mice. (n = 7-11)

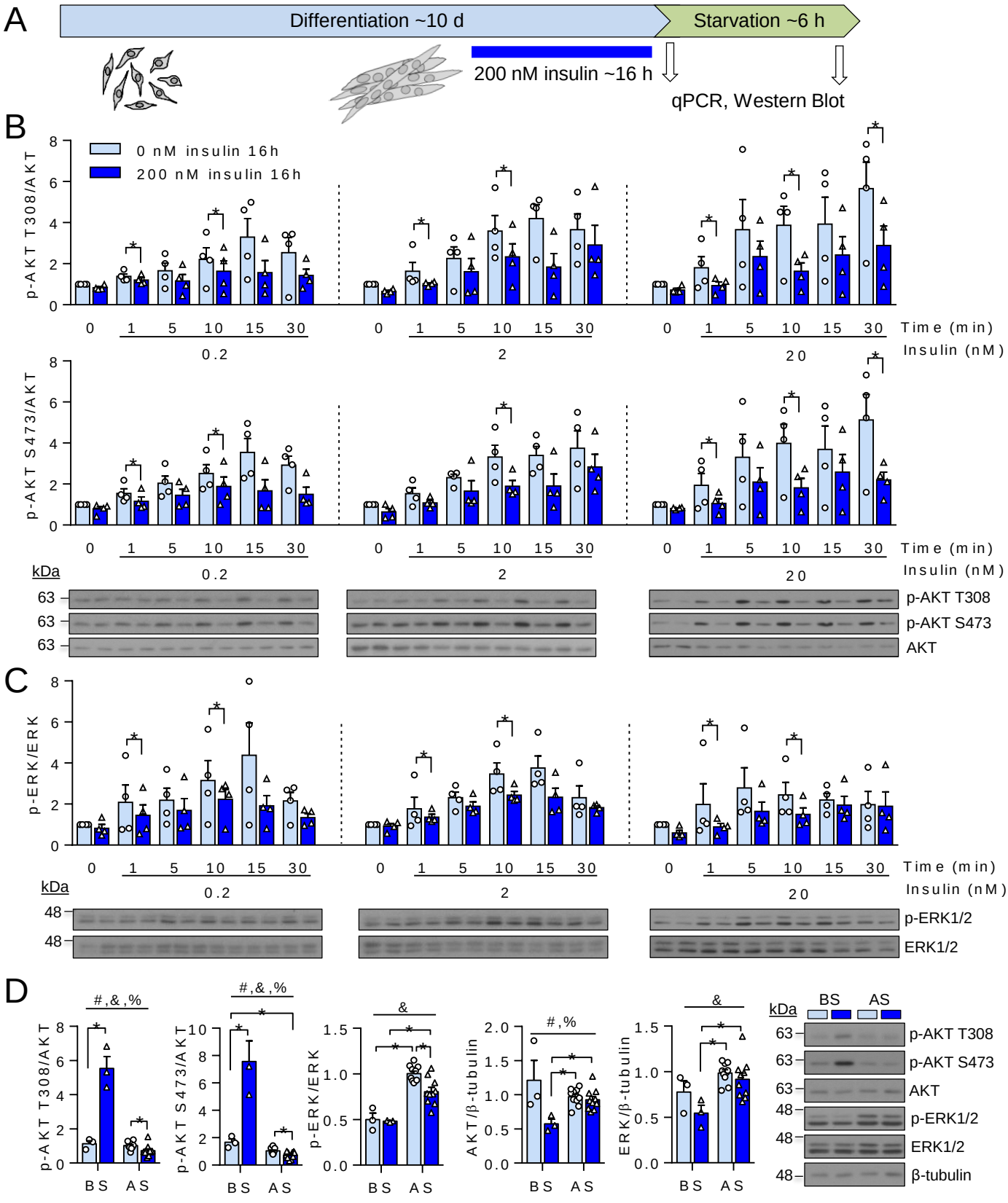


Figure 1. Acute and basal insulin signaling in an *in vitro* hyperinsulinemia-induced insulin resistance model.

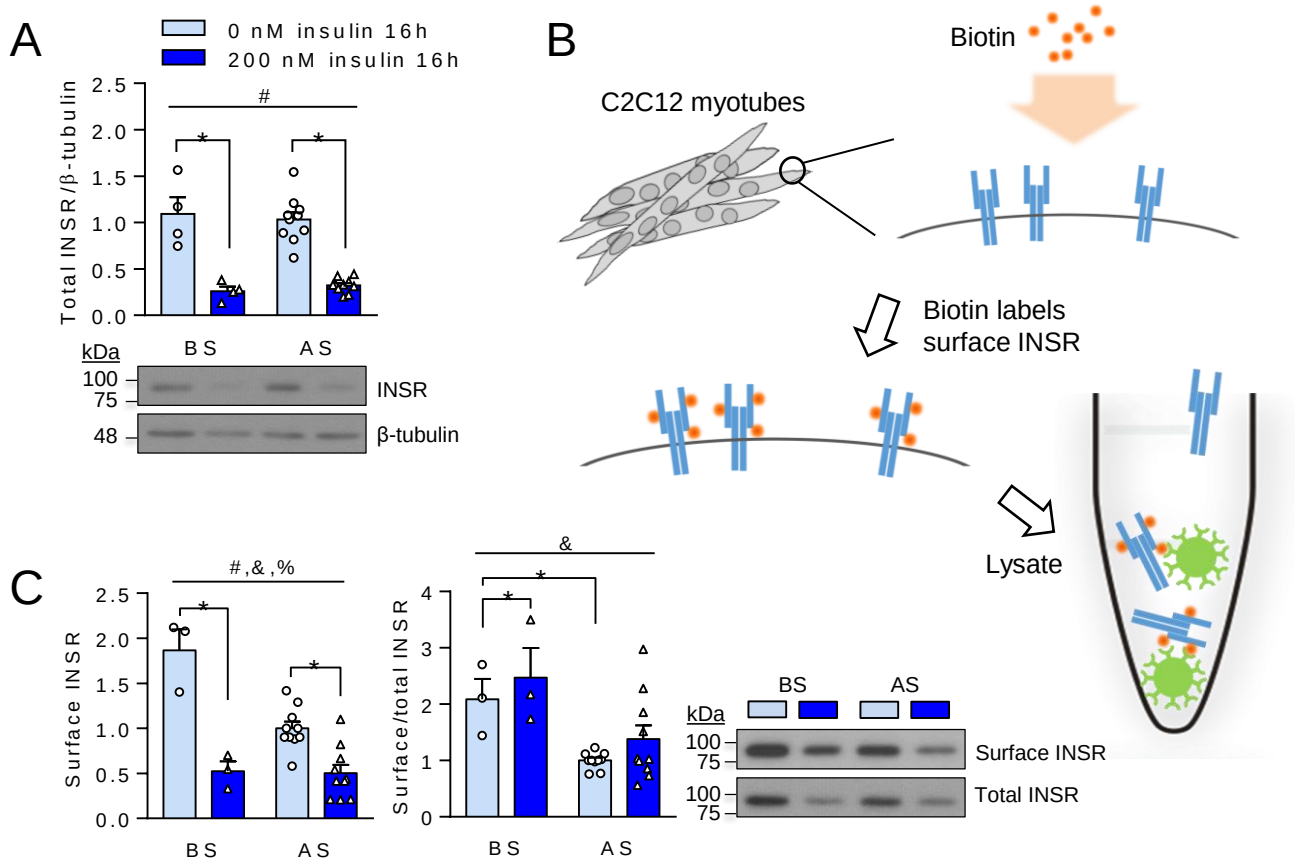


Figure 2. Effects of *in vitro* hyperinsulinemia and serum starvation on total and surface INSR.

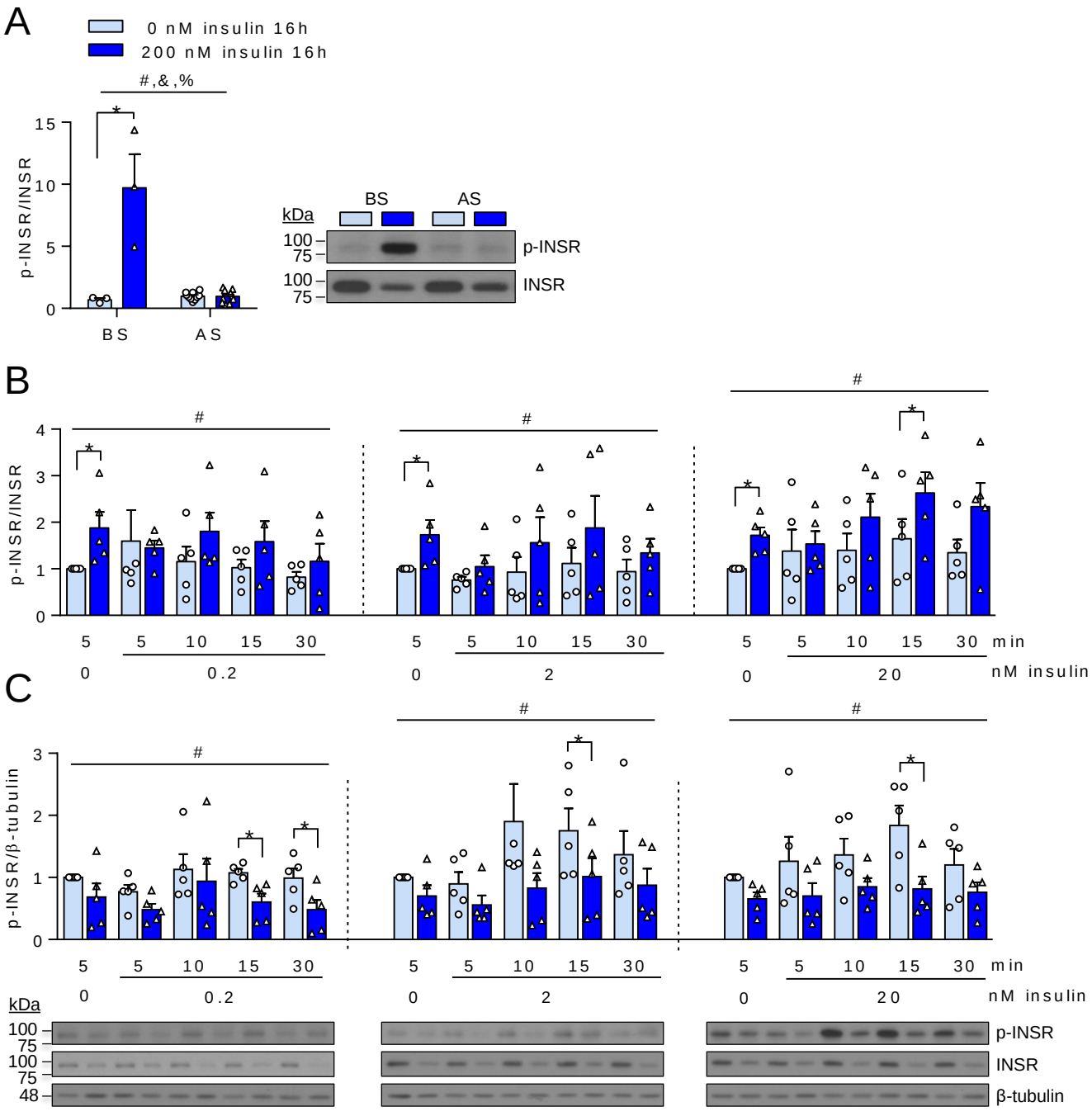


Figure 3. Effects of prolonged hyperinsulinemia on INSR phosphorylation *in vitro*.

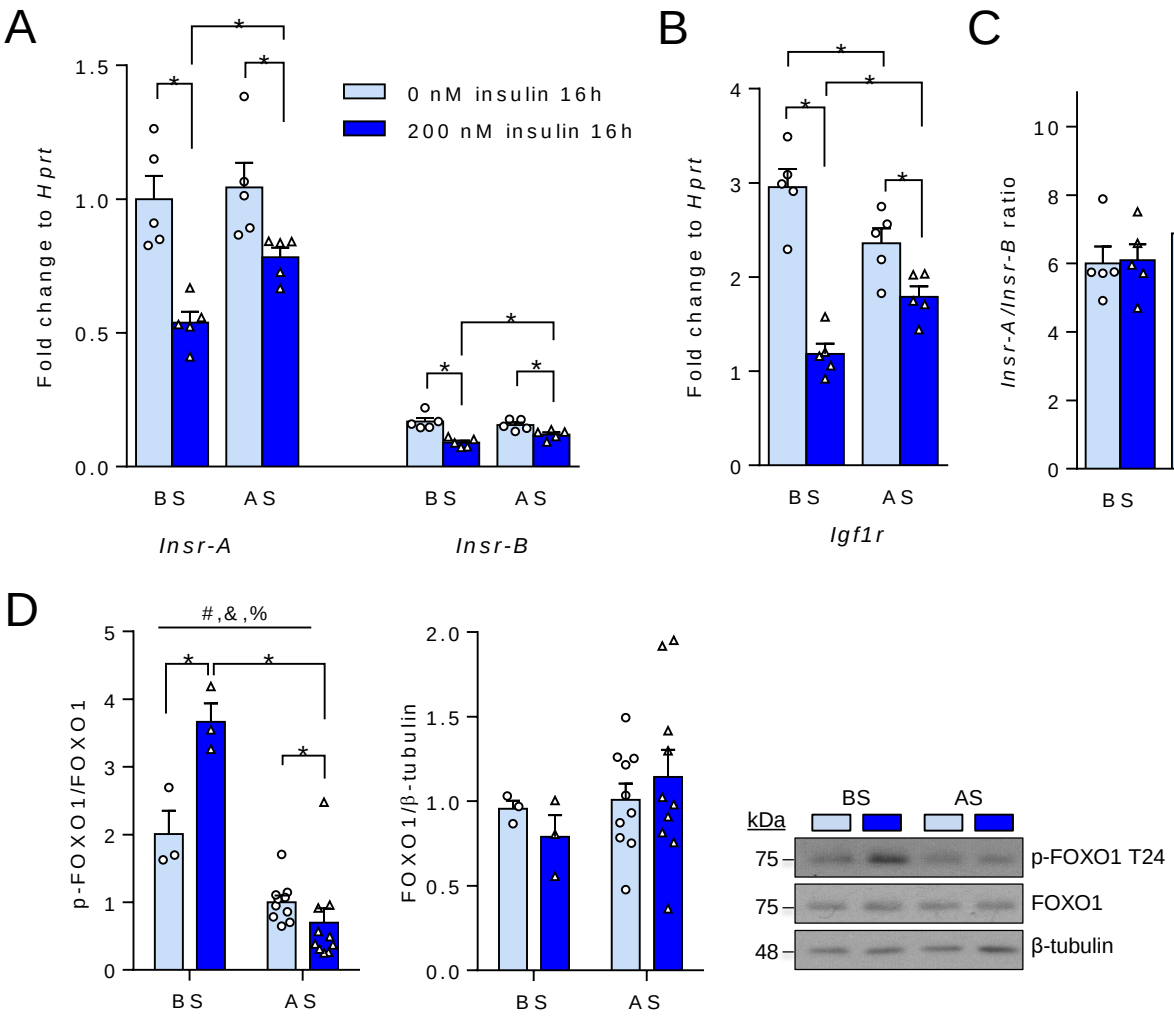
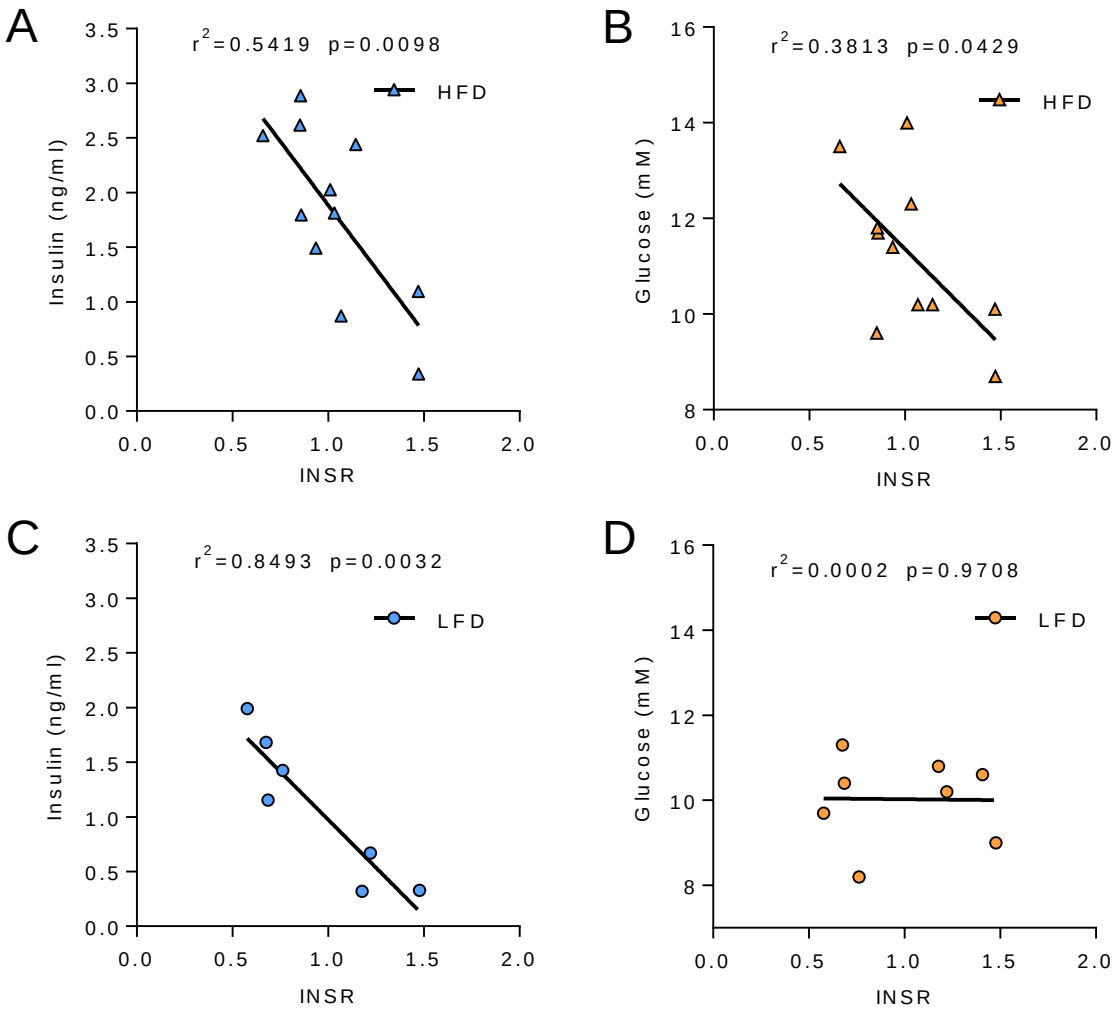


Figure 4. Effects of prolonged hyperinsulinemia and starvation on *Insr* transcription and FOXO1 phosphorylation *in vitro*.



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