**Title:** Modulation of muscle cell *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 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 a 6-hour period of serum starvation, before examining key insulin signaling nodes. Using our 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 were attenuated. INSR protein levels were significantly downregulated by hyperinsulinemia in an insulin-dose-dependent manner, which resulted in the comparative reduction of acute insulin signaling. Surface INSR was also reduced proportionally to total INSR levels. Mechanistically, we found that both isoforms of *Insr* 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 INSR, AKT and FOXO1, and *Insr* transcription. Finally, we validated our results *in vivo* by determining that INSR 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 (T2D) 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 (1,2). 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 using partial insulin gene knockout 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, strongly suggesting 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 diabetic or obese rodent models with hyperinsulinemia and insulin resistance have reduced insulin binding to the liver (19), fat (20) and pancreatic acinar cells (21). The insulin binding defect 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 (22) and lymphocytes (23) *in vitro*. However, the molecular mechanisms of INSR down-regulation and post-receptor insulin resistance remain poorly characterized, especially in muscle cells.

In the present study, we employed a model of *in vitro* insulin resistance using a muscle cell line and confirmed our observations *in vivo* using mice with variable hyperinsulinemia. We found that prolonged hyperinsulinemia induced insulin resistance featuring blunted acute AKT and ERK signaling and transcriptional INSR downregulation via FOXO1 activity. Our data illuminate the mechanism 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 (24). 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 superphysiologically high dose of

200 nM (Fig. 1A). Insulin levels at the end of this hyperinsulinemia period were confirmed to be hyperinsulinemic (Fig. 1A'). After 6-hour serum starvation, acute insulin signaling was characterized by measuring the phosphorylation of AKT and ERK proteins, which represent two major insulin signaling nodes (25). Since alterations in the basal state of the insulin signal transduction network have also been reported in hyperinsulinemic humans and animals (26), 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. 1C,D). After prolonged 200 nM insulin 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)(27). 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. 3B). Serum starvation also 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 (25). 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 the phosphorylation of INSR had no significant difference after 10 min acute insulin stimulations (Fig. 3C), the dose- and time-dependent insulin signaling revealed slightly increased phosphorylated-to-total INSR

ratio in the 200 nM insulin-treated group under acute insulin stimulations (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 INSR do not have defects in autophosphorylation upon acute insulin stimulation in our system.

Impaired INSR endocytosis has been implicated in insulin resistance (28,29). 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, internalized to total INSR ratio did not have evident differences except for a small increase when stimulated by 2 nM insulin (Fig. 4B). 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 is not drastically affected by hyperinsulinemia. Collectively, our experiments suggest that hyperinsulinemia-induced insulin resistance is mediated in large part by a reduction in total INSR, and not by affecting its activity or internalization.

***Hyperinsulinemia decreases Insr mRNA via FOXO1 inhibition***

One possible cause of INSR downregulation would be the reduced expression of the *Insr* gene (21). 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. 5A). Both *Insr-A* and *Insr-B* mRNA were equally downregulated after hyperinsulinemia and partially recovered after serum starvation (Fig. 5A), 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. 5B). Alteration in *Insr* alternative splicing has been implicated in hyperinsulinemia (30). In our model, the ratio of *Insr-A* and *Insr-B* mRNA was not affected by hyperinsulinemia or serum starvation (Fig. 5C). 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 (31-33). 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 (32). 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 (34), but did not affect total FOXO1 abundance (Fig. 5D). T24 phosphorylation of FOXO1 significantly decreased after starvation (Fig. 5D), 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, we examined the relationship between *in vivo* insulin concentration and muscle INSR level 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 (12,35) 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, 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.

## 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 superphysiological hyperinsulinemia induced a reduction of AKT and ERK signaling. Remarkably, while serum starvation partially reversed the effects of overnight hyperinsulinemia, much of the impaired acute insulin signaling we observed after 16 hours of hyperinsulinemia 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 dose-dependent from the physiological to the superphysiological range. We demonstrated that the impaired insulin response in our system can be accounted for by INSR downregulation at the transcription level via FOXO1 phosphorylation. We 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 multiple components of insulin signaling.

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 (36,37). The molecular mechanisms mediating the down-regulation of signaling components are unclear, but we speculate that the proteosome may be involved because IGF1 treatment can cause proteasome-dependent downregulation of AKT protein (41). The mechanisms of sustained alterations in AKT and ERK phosphorylation are also not fully understood. In our experiments, there was 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 (40). Our observations verified the distinct responses to hyperinsulinemia of the bifurcate insulin signaling pathways. Chronic 200 nM insulin treatment preferentially increased basal AKT phosphorylation, but did not increase the basal ERK phosphorylation, which is a result that has also been reported in neurons (38). 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, has been reported to protect insulin resistance both *in vitro* and *in vivo* (38,39). Further work is required to understand the interplay between INSR expression and downstream signaling.

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 (21,42). 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 (43). While relative hyperglycemia can increase *Insr* expression in lymphocyte and cancer cell lines (44,45), high glucose inhibits β-cell *Insr* expression through autocrine insulin action and INSR-FOXO1 signaling (44,45). Interestingly, glucose can only induce insulin resistance in the presence of insulin in cultured hepatocytes, adipocytes and skeletal muscle (20,46,47). 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 (48-50). Several human trials suggest that fasting regimes can be more effective for reducing insulin and increasing insulin sensitivity than they are for reducing glucose

(51,52). 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 the restoration of protein phosphorylation in insulin signaling pathways and partial recovery of *Insr* transcription and INSR protein. 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 insulin incubation instead of more chronic hyperinsulin treatment. The main reason for this is that insulin promotes the further differentiation of C2C12 myotubes (53), meaning that chronic treatment during differentiation would likely introduce unmanageble variance in the muscle cell model. Another limitation to our study is that it was not comprehensive with regards to key insulin signaling nodes. Indeed, hyperinsulinemia has been shown to downregulate other insulin signaling proteins such as IRS1 and IRS2 (54,55). In future studies, 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. Besides FOXO1, other transcription factors such as SP1, HMGA1, C/EBPβ, and NUCKS have been shown to regulate *Insr* expression (56-58). For example, HMGA1 is downregulated in diabetes, which inhibits *Insr* as well as *Foxo1* transcription (57). 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 (21,22,59). 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 may be involved in modulating the protein abundance of INSR. In addition to the downregulation of INSR protein abundance, INSR was proportionally internalized with a subtle increase, yet the role and mechanism of this phenomenon remain unknown. The mechanisms of INSR and insulin signaling 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 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 1% penicillin-streptomycin (100 µg/ml; Gibco). 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. *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. 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' (60); *Igf1r*

forward 5'-GGCACAACTACTGCTCCAAAGAC-3' and reverse 5'-CTTTATCACCACCACACACT TCTG-3' (60); *Hprt* forward 5'-TCAGTCAACGGGGGACATAAA-3' and reverse 5'-GGGGCTGTACTGCTTAA CCAG-3' (61).

***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). Protein band intensities were quantified with Image Studio Lite software (LI-COR).

***Surface Protein Biotinylation Assay***

Biotinylation of surface proteins was performed as previously described(62) with modifications (Fig. 5A). 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. For isolating surface proteins, cells were washed using ice-cold PBS and lysed in complete RIPA buffer (supplemented with cOmplete mini protease inhibitor cocktail (Roche, Laval, QC) and $Na_3VO_4$). For detecting internalized proteins, cells were washed with PBS and incubated in serum-free medium supplemented with 0.2, 2 or 20 nM insulin at 37°C to stimulate INSR internalization. After certain time periods, cells were placed on ice, washed with ice-cold PBS, incubated with Glutathione solution (50 mM glutathione, 75 mM NaCl, 1 mM EDTA, 1% BSA, 75 mM NaOH) for 20 min to strip remaining surface biotin, washed with excess PBS, and lysed in complete RIPA buffer. Lysates were quantitated and incubated with NeutrAvidin beads (Pierce) overnight at 4 °C to isolate biotinylated surface or internalized proteins. Biotinylated proteins were eluted from the 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.

***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 (63). Linear mixed effect models (R package – lme4) were fitted using restricted maximum likelihood (63,64). 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 (63). Where the random effect was not significant, linear fixed effect modeling was used. Heteroscedasticity and normality of residuals were analyzed used 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.

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**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. JDJ designed the study, supervised the research and edited the manuscript. JDJ is the ultimate guarantor of this work.

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**Figure Legends**

**Figure 1. Basal and acute insulin signaling in an *in vitro* hyperinsulinemia-induced insulin resistance model. (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 the 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 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. 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-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 6. *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)

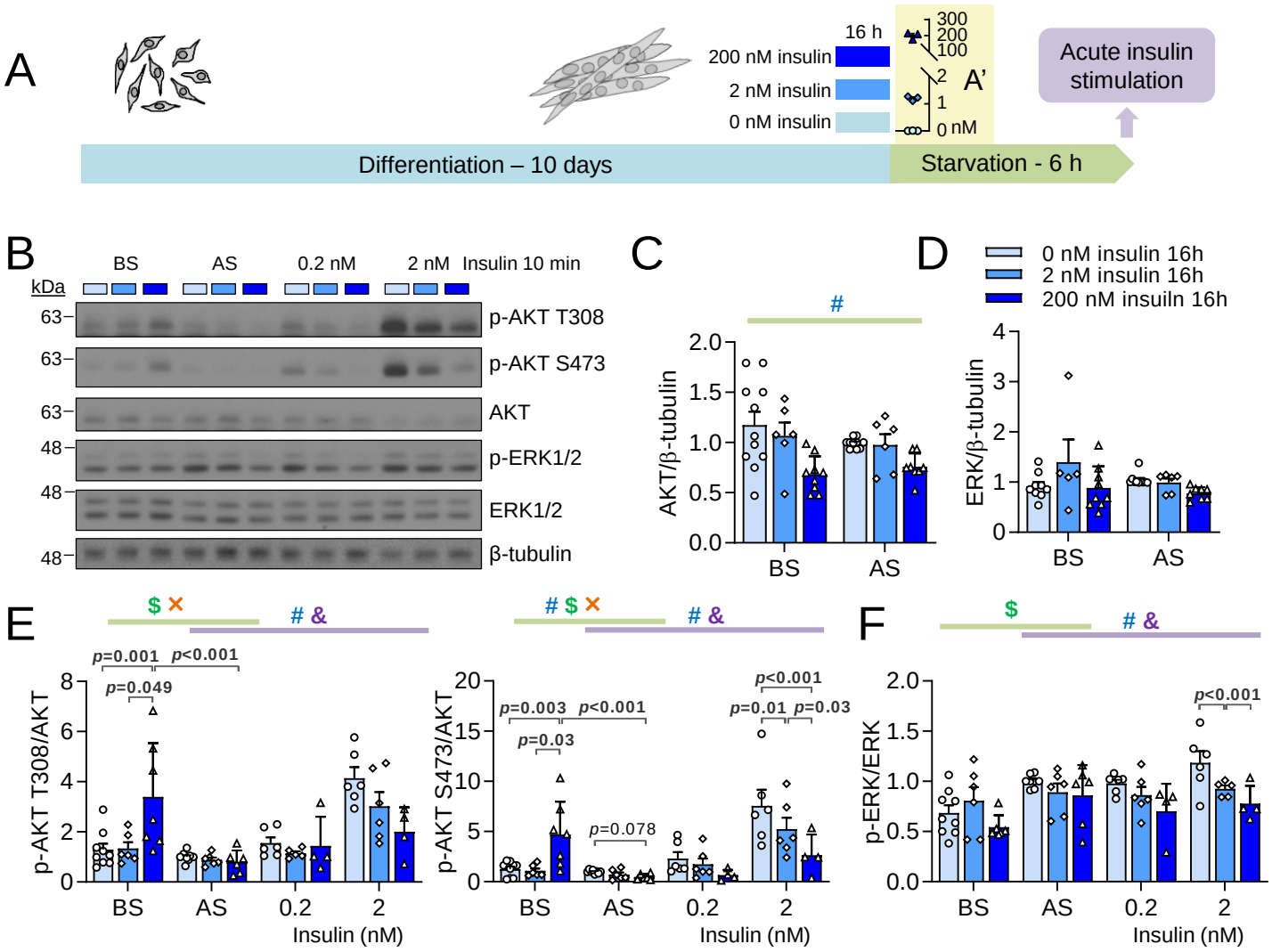


**Figure 1. Basal and acute insulin signaling in an *in vitro* hyperinsulinemia-induced insulin resistance model.**

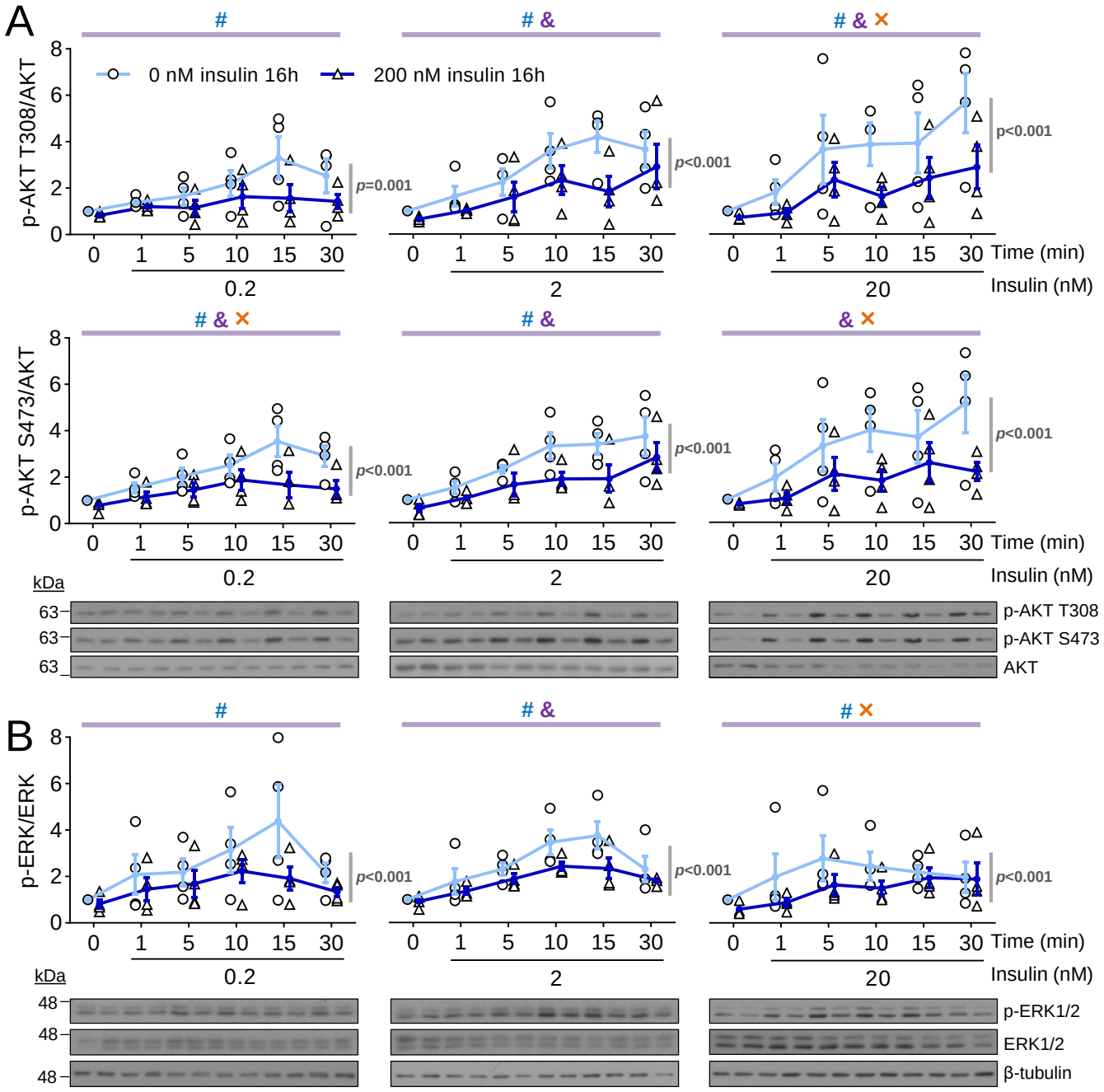


**Figure 2. Insulin dose- and time-depend acute insulin signaling in the hyperinsulinemia-induced insulin resistance model.**

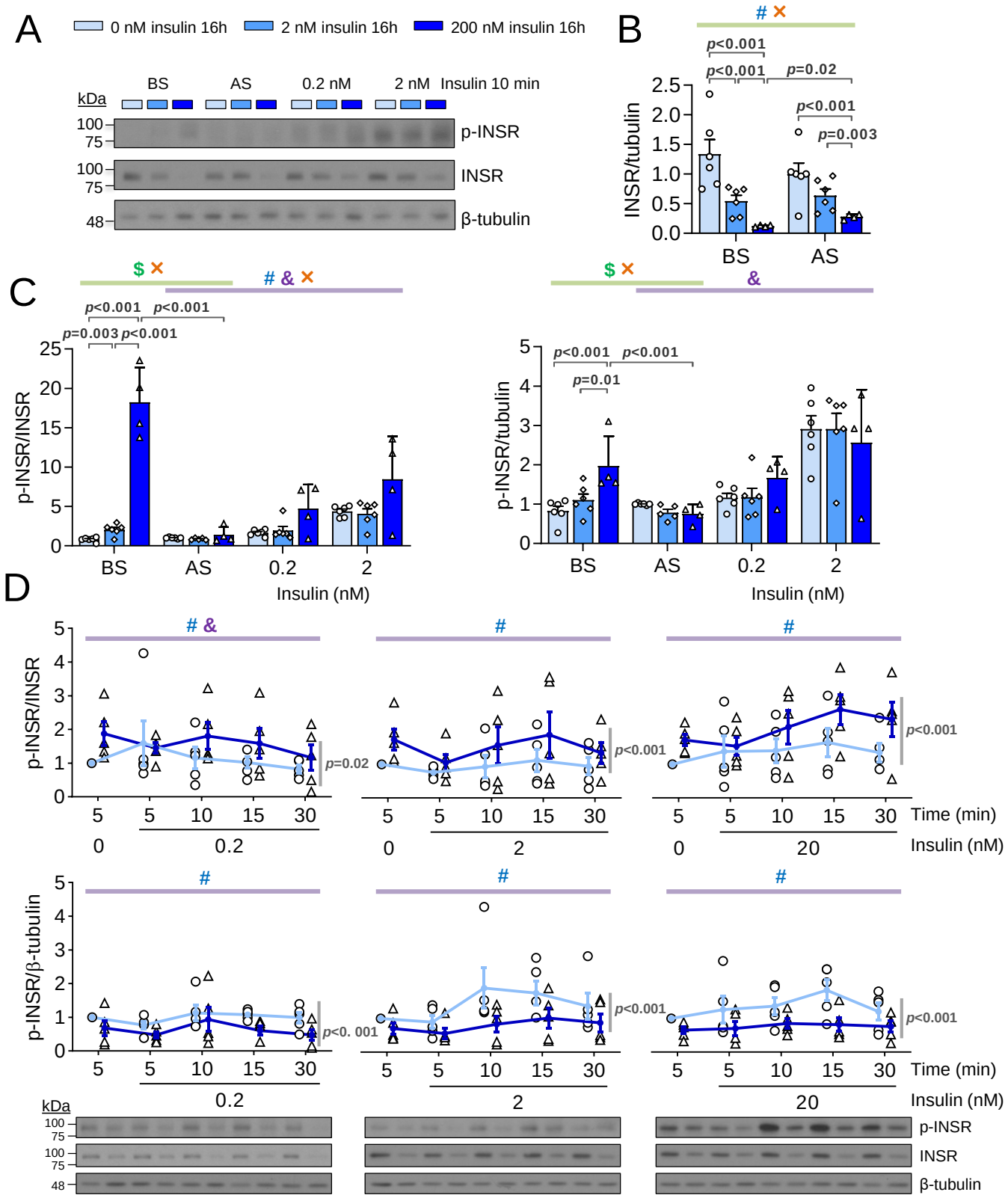


**Figure 3. Effects of hyperinsulinemia and serum starvation on INSR abundance and phosphorylation .**

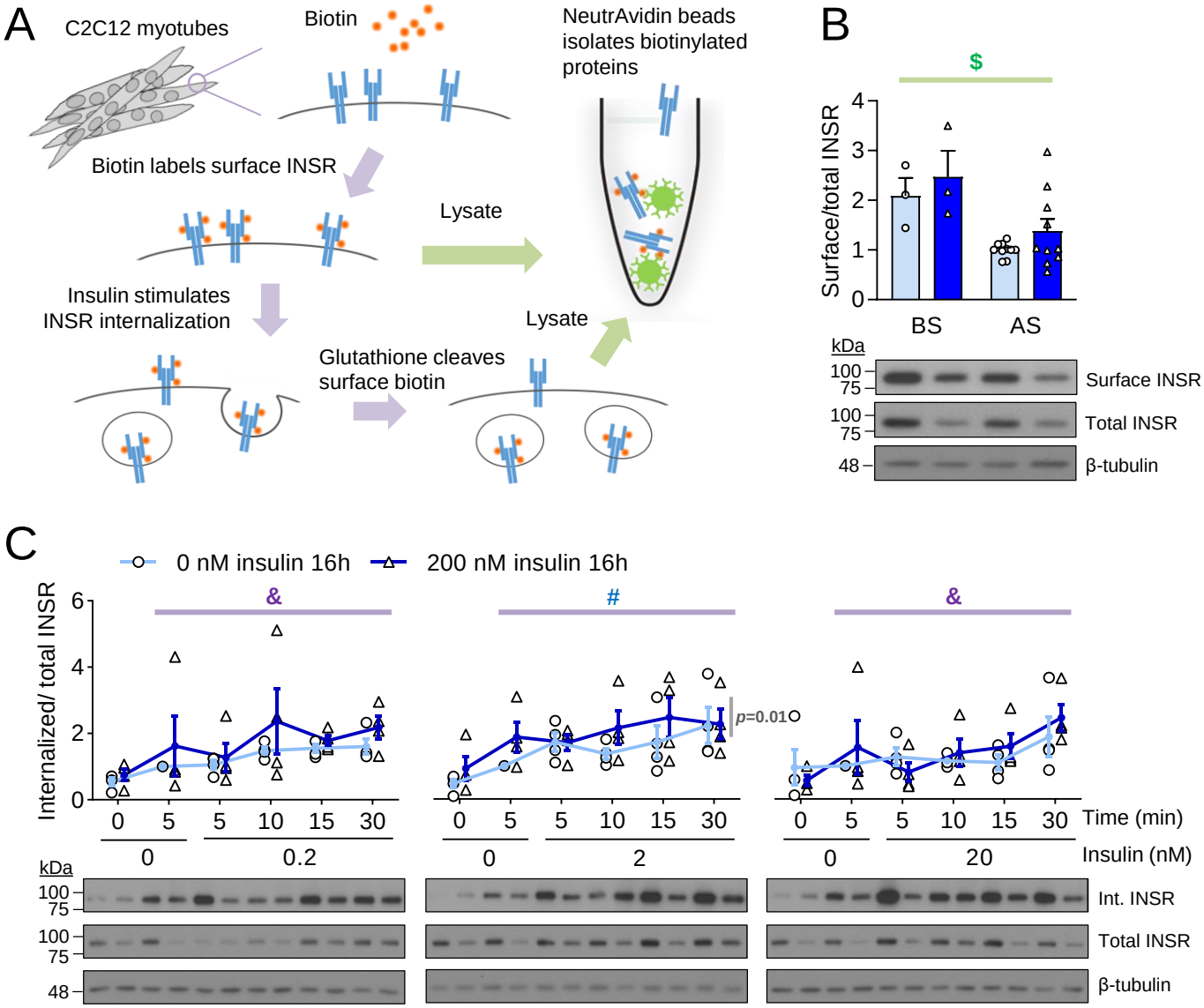


**Figure 4. Effects of *in vitro* hyperinsulinemia on INSR internalization.**

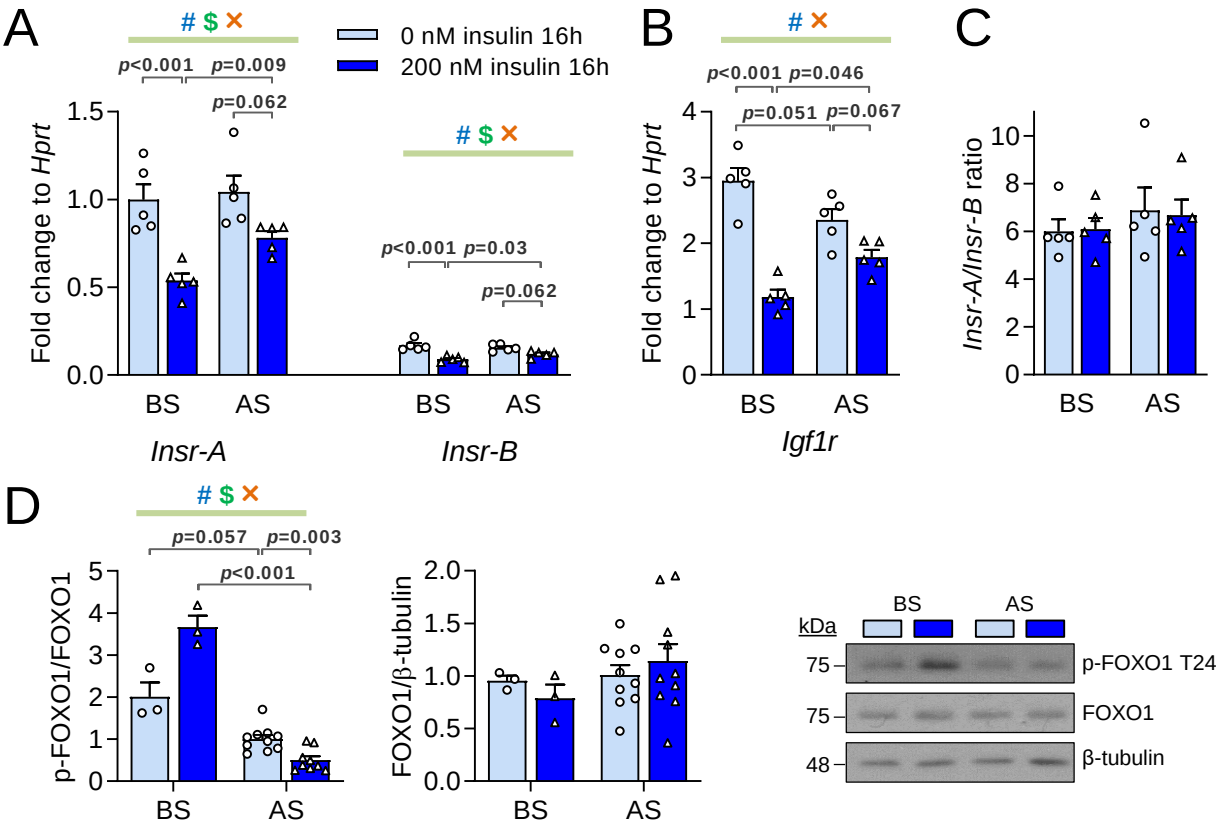


**Figure 5. Effects of prolonged hyperinsulinemia and starvation on *Insr* transcription and FOXO1 phosphorylation *in vitro*.**

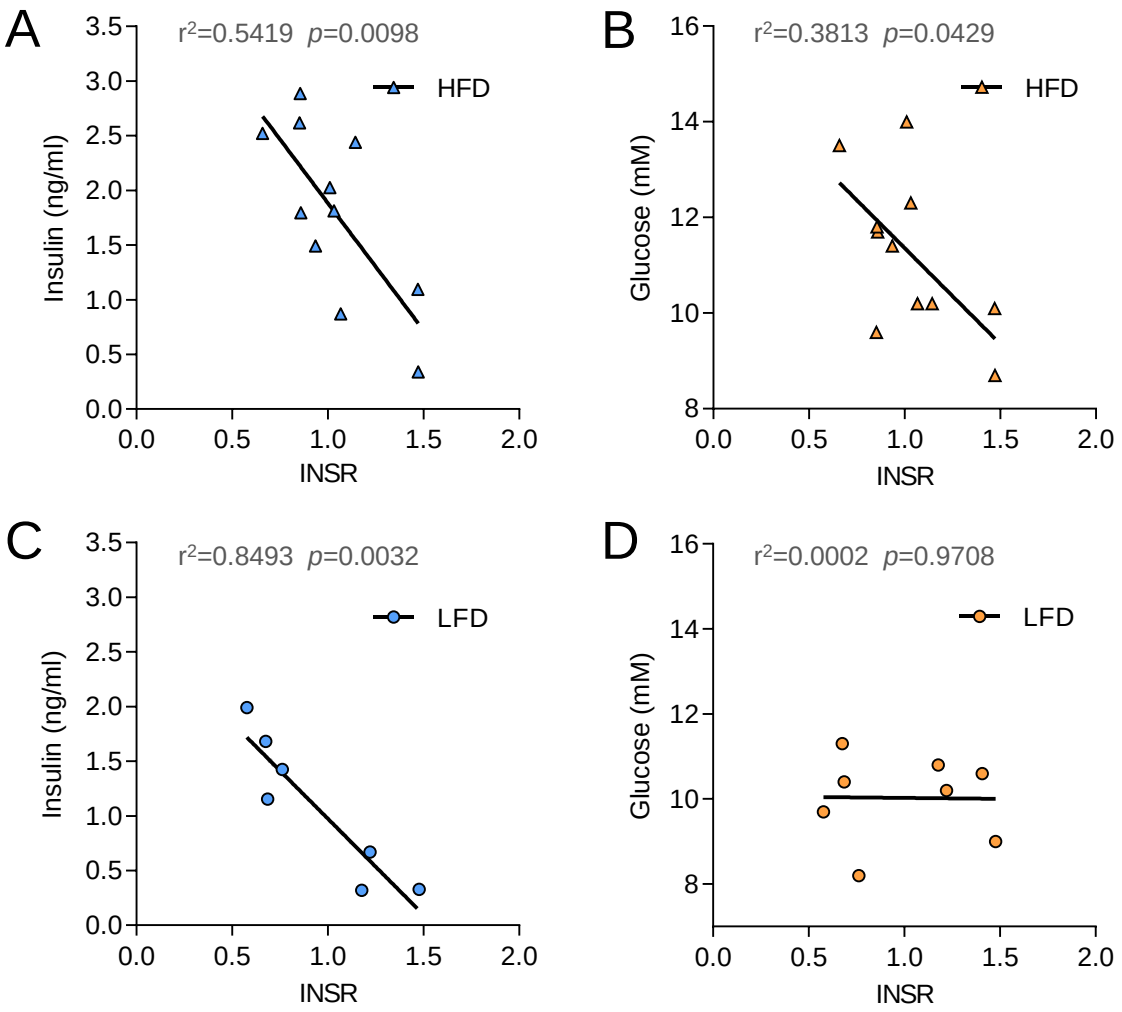


**Figure 6.** ***In vivo*** **correlation between INSR abundance and fasting insulin or glucose in mouse skeletal muscle.**