Adiponectin improves insulin sensitivity via activation of autophagic flux

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Abstract

Skeletal muscle insulin resistance is known to play an important role in the pathogenesis of diabetes, and one potential causative cellular mechanism is endoplasmic reticulum (ER) stress. Adiponectin mediates anti-diabetic effects via direct metabolic actions and by improving insulin sensitivity, and we recently demonstrated an important role in stimulation of autophagy by adiponectin. However, there is limited knowledge on crosstalk between autophagy and ER stress in skeletal muscle and in particular how they are regulated by adiponectin. Here, we utilized the model of high insulin/glucose (HIHG)-induced insulin resistance, determined by measuring Akt phosphorylation (T308 and S473) and glucose uptake in L6 skeletal muscle cells. HIHG reduced autophagic flux measured by LC3 and p62 Western blotting and tandem fluorescent RFP/GFP-LC3 immunofluorescence (IF). HIHG also induced ER stress assessed by thioflavin T/KDEL IF, pIRE1, pPERK, pelf2 α and ATF6 Western blotting and induction of a GRP78-mCherry reporter. Induction of autophagy by adiponectin or rapamycin attenuated HIHG-induced ER stress and improved insulin sensitivity. The functional significance of enhanced autophagy was validated by demonstrating a lack of improved insulin sensitivity in response to adiponectin in autophagy-deficient cells generated by overexpression of dominant negative mutant of Atg5. In summary, adiponectin-induced autophagy in skeletal muscle cells alleviated HIHG-induced ER stress and insulin resistance.

Key Words

- ▶ adiponectin
- ▶ insulin resistance
- ▶ autophagy
- ▶ endoplasmic reticulum stress
- ▶ unfolded protein response
- ▶ skeletal muscle

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Introduction

Diabetes currently affects over 422 million people worldwide and type 2, the most common form, arises from an initial impairment of peripheral tissue insulin sensitivity. Skeletal muscle is responsible for clearing the majority of circulating glucose in response to insulin, and it is therefore critical to fully understand the mechanisms leading to insulin resistance and how they can be alleviated (Marette *et al.* 2014). A strong inverse correlation of circulating adiponectin with diabetes and skeletal muscle insulin resistance exists in humans and animal models (Stern *et al.* 2016). Adiponectin is now established

as an anti-diabetic hormone (Cheng *et al.* 2014). Muscle is one important target tissue of adiponectin, and we have reviewed the metabolic actions of adiponectin in skeletal muscle and the mechanisms via which these have been shown to occur (Liu & Sweeney 2014). Importantly, we recently demonstrated that adiponectin stimulates autophagic flux in skeletal muscle and that increased autophagy contributed to insulin-sensitizing anti-diabetic effects (Liu *et al.* 2015, Xu & Sweeney 2015). Others have now also shown that boosting autophagy via a variety of approaches can enhance insulin sensitivity in

skeletal muscle and other tissues (Wang *et al.* 2015, Zhu *et al.* 2016, Ghareghani *et al.* 2017, Li *et al.* 2017, Rosa-Caldwell *et al.* 2017) and vice versa that decreased the levels of skeletal muscle autophagy were observed in type 2 diabetes and upon aging (Zhang *et al.* 2016, Moller *et al.* 2017, Zhou *et al.* 2017).

Until recently, the causative role of ER stress in promoting insulin resistance in skeletal muscle was much less studied than other tissues. However, there is now a strong body of evidence, which clearly indicates that various factors can elevate ER stress in skeletal muscle leading to insulin resistance. For example, inflammation or high-fat diet can lead to ER stress (Dai *et al.* 2016, Kwak *et al.* 2016, Liong & Lappas 2016). Various reports have demonstrated that treatments which alleviate skeletal muscle ER stress induced by stressors such as high-fat diet or palmitate are able to elicit a corresponding improvement in insulin sensitivity (Hwang *et al.* 2013, Salvado *et al.* 2014, Quan *et al.* 2015, Dai *et al.* 2016, Kwak *et al.* 2016). To further elucidate the mechanisms responsible for adiponectin's insulin-sensitizing effect on skeletal muscle, additional research is needed to examine crosstalk between autophagy and ER stress.

We hypothesized that the interplay between adiponectin-stimulated autophagy and ER stress plays an important role in the regulation of skeletal muscle insulin sensitivity. To investigate this, we used an established cellular model of skeletal muscle insulin resistance, namely high insulin high glucose (HIHG) treatment in L6 cells (Huang *et al.* 2002) treated with or without adiponectin. We also generated an autophagy-deficient L6 cell line by stably overexpressing a dominant negative point mutant of Atg5 to allow examination of the functional significance of autophagy during the development of insulin resistance.

Materials and methods

Cell culture, stable cell lines and cellular model of insulin resistance

L6 myoblasts were maintained in alpha-minimum essential medium (AMEM; 5.5 mM glucose) supplemented with 10% fetal bovine serum (FBS) in 75 cm² flasks at 37°C in 5% CO₂. Differentiation from myoblasts to myotubes was induced by serum starvation in 2% FBS AMEM for 6 days. Prior to treatments, cells were starved for 3 h in 0.5% FBS AMEM with relevant chemical added as indicated in figure legends after 2 h of starvation, thus allowing a 1 h pre-treatment before experimental starting

point. AMEM used for growing stable cell lines contained puromycin to select for transgene-expressing cells. Insulin resistance was induced by incubating cells in high insulin (HI; 100 nM Humulin R, purchased from Eli Lilly and Company) and high glucose (HG; 25 mM D-glucose purchased from Sigma-Aldrich) containing AMEM with 0.5% FBS (HIHG) for 24 h. A stable L6 cell line transfected to overexpress GLUT4 (a kind gift from Dr Amira Klip, The Hospital for Sick Children, Toronto) was used to assess insulin-stimulated glucose uptake in myoblasts (Huang *et al.* 2002).

A stable L6 GRP78mCherry reporter cell line containing the GRP78 promoter controlling the expression of mCherry, allowing the fluorescent signal to be used as visual readout of GRP78 gene expression, was generated by retroviral infection. The GRP78mCherry target vector (a kind gift from Dr Allen Volchuk, Toronto General Research Institute/University Health Network, Canada) was incorporated into the retroviral cloning vector pQCXIP by restriction and ligation. Once purified, the viral vector was transfected into EcoPack 2-293 (Clontech Laboratories). This human embryonic kidney-derived packaging cell line express Moloney murine leukemia virus Gag, Pol and Env proteins. 48 h after transfection the culture media containing the virus was collected and 100 µL of the supernatant was added together with polybrene (4 µg/mL) to L6 cells 1 day after being seeded in 10-cm culture dishes. After being incubated with the virus for 24 h the growth media was replaced with fresh growth media containing puromycin (2 µg/mL; Sigma-Aldrich) as the selection antibiotic and the stable expression of mCherry was verified by FACSCalibur flow cytometry (BD bioscience) (Lai *et al.* 2008, Liu *et al.* 2015). L6 cells stably expressing tandem fluorescent LC3 (TFLC3) was generated as we described before (Liu *et al.* 2015). A stable L6 cell line overexpressing an Atg5-dominant negative mutant (Atg5K130R (Atg5K)), which prevents conjugation to ATG12 and blocks LC3II incorporation and elongation of the autophagosome membrane was generated as previously described by us (Liu *et al.* 2015). Cells expressing empty vector (EV) were used as control.

Materials

Adiponectin was produced as we previously described (Palanivel *et al.* 2007) and dissolved in phosphate-buffered saline (PBS), and then media for use at 5 µg/mL. All culture media and solutions were purchased from Wisent (Saint-Jean-Baptiste, QC, Canada). Tunicamycin (Sigma-Aldrich) was dissolved in DMSO to a stock concentration of 5 mM.

Rapamycin (Sigma-Aldrich) was dissolved in PBS to a stock concentration of 27.4 μ M. Bafilomycin A1 (Sigma-Aldrich) was dissolved in DMSO to a stock concentration of 100 μ M.

Western blot analysis

Upon completion of treatment, L6 cells were lysed in RIPA lysis buffer (50mM Tris, 150mM NaCl, 0.1% SDS, 1% Triton X and 0.5% sodium deoxycholate) containing 10% β -mercaptoethanol and phosphatase inhibitor cocktail set V (EMD Millipore), heated for 10min in 90°C and centrifuged at 13,600g for up to 15min. Samples were resolved by 8–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane, blocked in 3% bovine serum albumin (BSA) blocking buffer and then immunoblotted with primary antibodies (pPERK, pelf2 α , LC3, pAKT(T308), pAKT(S473), β -actin and GAPDH purchased from Cell Signaling, pIRE1 purchased from Novus Biologicals, ATF6 purchased from Santa Cruz, p62 antibody from BD Biosciences (Mississauga, ON, Canada) and KDEL purchased from Abcam. These were subsequently detected with horseradish peroxidase (HRP)-conjugated secondary antibodies purchased from Cell Signaling. Protein bands were visualized using enhanced chemiluminescence (ECL, BioRad) reagent and quantitated by densitometry using ImageJ. All values were corrected for an appropriate loading control, β -actin or GAPDH.

Glucose uptake

Glucose uptake was assessed exactly as previously described (Liu & Sweeney 2014).

Thioflavin T immunofluorescence and KDEL colocalization

Thioflavin T (ThT) dye exhibits increased fluorescence when bound by protein aggregates such that the level of fluorescence has been shown to correlate with ER stress inducers (Beriault & Werstuck 2013). Here, ThT dye was added to live cells for 40min (1 μ M/mL), media was aspirated and cells were washed before fixing in 4% paraformaldehyde for 20min. Coverslips were then mounted on slides in Vectashield mounting media containing DAPI. When ThT assay was combined with KDEL immunofluorescence, after fixing, the cells were

quenched in 0.1% glycine in PBS for 10min, washed with PBS and permeabilized for 3min in 0.1% Triton X 100, followed by blocking for 30min in 3% BSA, and then incubated overnight at 4°C with 1:500 primary KDEL antibody. Cells were then washed and subsequently incubated with 1:1000 secondary antibody Alexa Fluor 594 for 1h in the dark at room temperature and washed prior to mounting on slides in DAKO mounting media. Slides were imaged with Zeiss LSM 700 Confocal Microscope. Average fluorescence per cell was quantified using Zen 2012 Blue Edition, and Manders Coefficient analysis of colocalization of ThT (green) to KDEL (red) fluorescence was assessed using ImageJ software (JaCoP plugin).

GRP78-mCherry reporter assay

After treatment, cells were fixed in 4% paraformaldehyde for 20min, washed 3 times in cold PBS and mounted on slides in DAKO mounting media. Stacked images (12 slices) were taken with Zeiss LSM 700 Confocal Microscope and images quantified using ImageJ software as average fluorescence intensity per cell for 40 cells/treatment/replicate experiment; all other images were taken as single images.

Tandem fluorescent RFP/GFP-LC3 assay

After treatment cells were fixed in 4% paraformaldehyde for 20min, washed 3 times in cold PBS and mounted on slides in DAKO mounting media. To assess autophagosome formation, green and red merged images were converted to 8-bit black and white images and number of puncta counted using ImageJ Particle Analysis. To measure autophagic flux, images were quantified as total red/green fluorescence intensity per image for 8–10 images using Zen 2012 Blue Edition.

Statistical analysis

Comparison between two groups were done using unpaired one-tailed *t*-test and comparisons between multiple groups were done by one-way ANOVA, where data was found to not be normally distributed the non-parametric equivalents were used (Mann–Whitney *U* and Kruskal–Wallis, respectively). Significant ANOVAs were followed by Tukey's HSD *post hoc* test when differences between all groups were of interest and Dunnett's *post hoc* test when all groups were compared to control. $P < 0.05$ was accepted as significant.

Results

HIHG treatment induced insulin resistance in L6 skeletal muscle cells

Insulin-stimulated glucose uptake was attenuated after 24-h HIHG treatment, although HIHG itself increased basal transport in L6 myotubes (Fig. 1A). HIHG-induced insulin resistance in L6 myotubes was also confirmed by examining the level of AKT phosphorylation in response to 100 nM of insulin at 0, 1, 3, 6, 8 and 10 min by Western blot. Insulin increased AKT phosphorylation at both the threonine 308 and serine 473 phosphorylation sites, whereas 24-h HIHG treatment clearly attenuated this response (Fig. 1B). We confirmed that there was no significant change in the total Akt protein expression after 24-h HIHG treatment (Fig. 1C). The insulin-sensitizing ability of adiponectin was then assessed with or without 24-h HIHG treatment. Results in Fig. 1D examining Akt phosphorylation on T308 demonstrated that adiponectin significantly attenuated HIHG-induced insulin resistance. Together, these data establish the cellular model of HIHG-induced insulin resistance and the beneficial effect of adiponectin in maintaining insulin sensitivity.

HIHG attenuated autophagic flux

In order to examine alterations in autophagy and their functional significance, we first treated cells with

HIHG for 2, 6 and 24 h and confirmed reduced insulin-stimulated AKT phosphorylation on T308 at all times, with 24 h having the biggest effect (Fig. 2A). HIHG also decreased content of the autophagosome marker protein LC3II at each time point (Fig. 2B). Bafilomycin A (BafA), an inhibitor of autophagosome lysosome fusion, was used here as a positive control to attenuate autophagic flux and thus elicit an accumulation of LC3II. We also observed by Western blotting that p62 levels accumulated after HIHG treatment (Fig. 2C). We observed that 24-h HIHG treatment reduced LC3-II levels (Fig. 2D). Tandem fluorescence LC3 (TFLC3) expressing cells were then used here to monitor the process of autophagic flux in more detail. This assay is based on the fact that the acidic environment in the lysosome quenches green but not red fluorescence, thus, a higher red/green fluorescence ratio indicates more autophagic flux. We observed a reduced red/green fluorescence ratio observed following BafA treatment, indicating less autophagosome fusion with lysosomes and also observed that HIHG had a similar effect in reducing autophagic flux (Fig. 2E). To further examine the inhibitory effect of HIHG on autophagic flux, we examined the number of fluorescent 'puncta' using ImageJ particle analysis (Fig. 2F) demonstrating an expected elevation in LC3-positive puncta in BafA-treated cells but with a reduced number of LC3-positive puncta after HIHG, suggesting that HIHG attenuates the early stage of autophagy initiation.

Figure 1

Skeletal muscle insulin resistance was induced by exposing L6 cells to HIHG condition and reversed by adiponectin treatment. (A) Insulin (100 nM) stimulated (3H)2-deoxy-d-glucose uptake with or without 24-h HIHG treatment in L6 skeletal muscle cells; (B) Representative images of AKT phosphorylation in control or HIHG condition in response to 0, 1, 3, 6, 8 and 10 min of 100 nM insulin stimulation; (C) Representative image of total AKT in control or HIHG condition. (D) Representative images and densitometry analysis of 5 min 100 nM insulin-stimulated AKT phosphorylation upon HIHG treatment with or without adiponectin pre-treatment. $n=3$ for each experiment. Values are mean $\pm$ S.E.M., * $P<0.05$.

Figure 2

Insulin resistance in skeletal muscle cells was associated with impairment in autophagy. (A) Representative images and densitometry analysis of 5 min 100nM insulin-stimulated AKT phosphorylation upon 2-, 6- and 24-h HIHG treatment, $n=4$; (B) Representative images and densitometry analysis of LC3 in HIHG condition for 0, 2, 6, and 24 h, $n=4$; (C) Representative images and densitometry analysis of P62 in 24 h of HIHG treatment compared to control, $n=6$. (D) Representative images and densitometry analysis of LC3 in 24 h HIHG treatment compared to BafA-treated L6 muscle cells, $n=4$; (E) Representative confocal images of tandem fluorescence-GFP-RFP-LC3 L6 cells and quantitative statistical analysis of red-to-green fluorescence ratio in HIHG or BafA-treated cells, $n=7$; (F) Representative images and quantification of puncta using ImageJ Particle Analysis with or without HIHG or BafA treatment, $n=7$. Values are mean $\pm$ s.e.m., $*P<0.05$. A full color version of this figure is available at <http://dx.doi.org/10.1530/JME-17-0096>.

HIHG elevated ER stress and UPR activation

We next performed a series of experiments to examine the effect of HIHG on ER stress and related unfolded protein response (UPR) signaling pathway in skeletal muscle cells. Thioflavin T (ThT) was used to visualize misfolded protein aggregates and thus quantify levels of ER stress and indicated a small but significant increase in ER stress after 24, but not 4, hours of HIHG treatment (Fig. 3A). Increased ER stress was accompanied by an increase in UPR activation, determined by transfecting

L6 cells with a GRP78mCherry reporter. The increased level of mCherry fluorescence in cells treated with HIHG for 24 h compared to control cells indicated an increase in UPR activation (Fig. 3B). Tunicamycin, a specific inhibitor of N-linked glycosylation, was used here as a positive control for the activation of ER stress in both assays (Fig. 3A and B). Further analysis of UPR pathways was examined by Western blot. Figure 3C demonstrated that HIHG significantly increased phosphorylation of

Figure 3

ER stress and UPR activation was increased in insulin-resistant skeletal muscle cells. (A) ThT assay measuring ER stress with representative confocal images and quantitative and statistical analysis of total green fluorescence per cell for 4 and 24 h HIHG-treated cells compared to control, $n=3$. Tunicamycin was used here as positive control to induce ER stress; (B) Representative confocal images of L6 GRP78mCherry UPR-reporter and statistical analysis of mean red fluorescence per cell for HIHG condition compared to control, $n=7$. Tunicamycin was used here as positive control to induce ER stress; (C) Representative images and densitometry analysis of the UPR markers pIRE1, pPERK, pelf2 α and ATF6 in HIHG condition compared to control, $n=4$. Values are mean $\pm$ s.e.m., * $P<0.05$. A full color version of this figure is available at <http://dx.doi.org/10.1530/JME-17-0096>.

IRE1, PERK and the cleaved form of ATF6. However, an unexpected decrease in phosphorylation of pelf2 α was observed (Fig. 3C).

Rapamycin reduced HIHG-induced ER stress in an autophagy-dependent manner

In order to study the inter-relationship between ER stress and autophagy in response to HIHG treatment in skeletal muscle cells, we first used a gain of function approach with rapamycin as a well-established inducer of autophagy. Again using TFLC3 assay, rapamycin treatment significantly induced autophagic flux and restored the impairment in autophagy caused by HIHG (Fig. 4A). This ability of rapamycin to induce autophagy and recover HIHG-impaired autophagy was also confirmed by Western blotting analysis of LC3II levels (Fig. 4B). We then used a loss-of-function approach by generating an autophagy-deficient cell line (Atg5K130R cells). As expected, the ability of rapamycin to induce autophagy $\pm$ HIHG was absent in Atg5K130R cells (Fig. 4B). Having characterized the approaches for gain or loss of function of autophagy, we then used them to examine HIHG-induced ER stress. ThT assay demonstrated that rapamycin significantly reduced HIHG-induced ER stress (Fig. 4C). However, this effect of rapamycin was lost in autophagy-deficient cells (Fig. 4C). These data indicate that targeting autophagy is an effective means to reduce HIHG-induced ER stress in skeletal muscle cells.

Rapamycin increased autophagy and attenuated HIHG-induced insulin resistance in control but not autophagy-deficient cells

The importance of autophagy in determining insulin sensitivity was investigated and AKT phosphorylation (S473) data showing that both HIHG and BafA induced insulin resistance, which could suggest that reduced autophagy correlated with reduced insulin sensitivity (Fig. 5A). Both HIHG and BafA increased ER stress, indicated by ThT fluorescence (Fig. 5B). Next, the effect of bafilomycin or rapamycin on insulin sensitivity was examined in both EV and Atg5K cells. Rapamycin directly enhanced insulin sensitivity, and most importantly, we observed that rapamycin also significantly improved HIHG-induced insulin resistance in normal but not autophagy-deficient cells (Fig. 5C). Bafilomycin directly reduced insulin sensitivity and also exacerbated HIHG-induced insulin resistance (Fig. 5C).

Adiponectin alleviated HIHG-induced ER stress and insulin resistance in an autophagy-dependent manner

Adiponectin has been shown previously to stimulate autophagy and here we observed that adiponectin prevented the HIHG-induced decrease in LC3II (Fig. 6A). TFLC3 assay red/green fluorescence (Fig. 6B) and TFLC3 puncta analysis (Fig. 6C) also indicated that adiponectin prevented the HIHG-induced decrease in autophagic flux. Furthermore, adiponectin alleviated HIHG-induced

Figure 4

Rapamycin treatment increased autophagy and decreased HIHG-induced ER stress in EV but not Atg5K cells. (A) Quantitative and statistical analysis of red-to-green fluorescence ratio in tandem fluorescence-GFP-RFP-LC3 L6 cells in HIHG condition compared to control, with or without rapamycin pre-treatment, $n=4$; (B) Representative images and densitometry analysis of LC3 in response to rapamycin pre-treatment in HIHG-treated EV and Atg5K cells, $n=3$; (C) Representative confocal images and quantitative and statistical analysis of ThT green fluorescence comparing the effect of rapamycin pre-treatment in HIHG condition in EV and Atg5K cells respectively, $n=4$. Values are mean $\pm$ s.e.m., $*P<0.05$. A full color version of this figure is available at <http://dx.doi.org/10.1530/JME-17-0096>.

ER stress as indicated by significantly decreased ThT fluorescence in HIHG and adiponectin treatment compared to HIHG alone (Fig. 6D). To test whether adiponectin restored HIHG-impaired insulin signaling and induced ER stress was autophagy dependent or not, we used the autophagic deficient Atg5K130R cell line. Adiponectin maintained insulin sensitivity, measured by phosphorylation of AKT T308, in HIHG treated normal but not autophagy-deficient cells (Fig. 6E).

Discussion

Metabolic dysfunction, occurring at least in part via insulin resistance, in skeletal muscle plays a major role in the development of the metabolic syndrome and type 2 diabetes (Marette *et al.* 2014). New knowledge contributing

to our understanding of the mechanisms responsible for development of skeletal muscle insulin resistance is thus of great importance in order to facilitate development of improved therapeutic approaches. Various mechanisms have been suggested to contribute to the pathophysiology of metabolic dysfunction, and our previous studies have helped to establish skeletal muscle as an important target tissue for the actions of adiponectin, an anti-diabetic hormone, which normally circulates at high levels, but which is reduced in obesity and diabetes (Liu & Sweeney 2014, Marette *et al.* 2014, Stern *et al.* 2016). In skeletal muscle, adiponectin mediates direct metabolic effects as well as improved insulin sensitivity. The cellular signaling pathways via which adiponectin confers beneficial metabolic effects have been well characterized and many effects are mediated via an AdipoR/APPL1/AMPK

Figure 5

Inhibition of autophagy with bafilomycin treatment increased ER stress and decreased AKT phosphorylation. (A) Representative images and densitometry analysis of pAKT in BafA-treated cells compared to HIHG condition and control, $n=3$. (B) Representative confocal images and quantitative and statistical analysis of ThT green fluorescence in BafA-treated cells, $n=4$. (C) Representative images and densitometry analysis of insulin-stimulated pAKT in EV and Atg5K cells in response to BafA and rapamycin treatment, $n=3$. Values are mean $\pm$ s.e.m., $*P<0.05$. A full color version of this figure is available at <http://dx.doi.org/10.1530/JME-17-0096>.

axis (Cheng *et al.* 2014). Indeed, it is expected that development of strategies to mimic adiponectin action can translate into effective therapeutic applications. For example, a small molecule adiponectin receptor agonist

has been shown to activate AMPK via AdipoR's and ameliorate insulin resistance and glucose intolerance in db/db mice or mice fed a high-fat diet (Okada-Iwabu *et al.* 2013). To study novel mechanisms via which adiponectin

Figure 6

Adiponectin alleviates ER stress and improves insulin sensitivity in an autophagy-dependent manner. (A) Representative images and densitometry analysis of LC3 in HIHG condition with or without adiponectin pre-treatment, $n=3$; (B) Quantitative and statistical analysis of red-to-green fluorescence ratio in tandem fluorescence-GFP-RFP-LC3 L6 cells in HIHG condition compared to control, with or without adiponectin pre-treatment, $n=4$; (C) Quantitative analysis of TFLC3 puncta signal in HIHG condition compared to control, with or without adiponectin pre-treatment, $n=4$; (D) Representative confocal images and quantitative and statistical analysis of ThT green fluorescence in HIHG condition with or without adiponectin pre-treatment, $n=4$; (E) Representative images and densitometry analysis of pAKT in HIHG condition comparing adiponectin pre-treatment in EV and Atg5K cells, $n=3$; (F) Quantitative and statistical analysis of ThT green fluorescence comparing the effect of adiponectin pre-treatment in HIHG condition in EV and Atg5K cells respectively, $n=4$. Values are mean $\pm$ s.e.m., $*P<0.05$. A full color version of this figure is available at <http://dx.doi.org/10.1530/JME-17-0096>.

promoted insulin sensitivity, we used L6 skeletal muscle cells treated with HIHG to induce insulin resistance (Huang *et al.* 2002). Importantly, we first demonstrated that adiponectin treatment improved insulin signaling in this model, which is in keeping with previous work showing that adiponectin increased insulin sensitivity in other models (Liu *et al.* 2015).

We recently showed that one important cellular mechanism induced by adiponectin is autophagy (Jahng *et al.* 2015, Liu *et al.* 2015, Xu & Sweeney 2015). Adiponectin directly stimulated autophagy in skeletal muscle cells and mice lacking adiponectin had reduced high-fat diet-induced changes in skeletal muscle autophagy (Liu *et al.* 2015). Nevertheless, we believe it is important to appreciate that alterations in autophagy under conditions regulating insulin sensitivity are likely to be highly time and tissue dependent. For example, in skeletal muscle 6 weeks of caloric restriction in diet-induced obese mice conferred beneficial metabolic effects in close correlation with changes in autophagy yet 16 weeks of high-fat diet induced only minor changes in markers of autophagy (Cui *et al.* 2013, Campbell *et al.* 2015). Conflicting data exist in adipose tissue, where 8 weeks of high-fat diet attenuated autophagy and 17 weeks of high-fat diet did not significantly alter autophagy (Zhou *et al.* 2016, Rocha-Rodrigues *et al.* 2017). Furthermore, adiponectin or pharmacological strategies to boost autophagy improved insulin sensitivity but adiponectin-stimulated improvements in insulin sensitivity were lost in autophagy-deficient muscle cells (Liu *et al.* 2015). Here we show that adiponectin was able to counteract the decrease in autophagy which occurred in response to HIHG treatment of L6 cells. Our observations are in keeping with a growing body of recent studies which have shown that promoting autophagy by various approaches is beneficial in terms of improving insulin sensitivity (Wang *et al.* 2015, Zhu *et al.* 2016, Ghareghani *et al.* 2017, Li *et al.* 2017, Rosa-Caldwell *et al.* 2017). For example, using a model of palmitate-induced insulin resistance, it was shown that stress-inducible protein Sestrin2 (Sesn2) up-regulation maintained insulin sensitivity and glucose metabolism via AMPK-dependent autophagic activation (Li *et al.* 2017). It is also of interest to note other data which support the general dogma that autophagy positively correlates with insulin sensitivity. In skeletal muscle from severely insulin-resistant patients with T2D, there was decreased expression of autophagy-related gene (ATG14, RB1CC1/FIP200, GABARAP1, SQSTM1/p62, and WIPI1) and protein (LC3BII, SQSTM1/p62 and ATG5) (Moller *et al.* 2017). Furthermore, in aged

mice, there also was a decline in LC3B-I conjugation to phosphatidylethanolamine (PE), possibly due to decreased protein levels of ATG3 and ATG12-ATG5 (Zhou *et al.* 2017). We then speculated that crosstalk occurred between autophagy, induced by adiponectin, and the UPR and next investigated this.

Numerous studies have now clearly established that induction of ER stress in skeletal muscle can elicit insulin resistance (Quan *et al.* 2015, Dai *et al.* 2016, Kwak *et al.* 2016, Liong & Lappas 2016). For example, tunicamycin or palmitate stimulated ER stress and insulin resistance while suppression of ER stress, using ER stress inhibitor tauroursodeoxycholic acid (TUDCA), melatonin or siRNA knockdown of IRE1 α and GRP78, significantly downregulated insulin resistance in human skeletal muscle (Quan *et al.* 2015, Dai *et al.* 2016, Liong & Lappas 2016). The signaling protein tribbles 3 was shown to mediate endoplasmic reticulum stress-induced insulin resistance in skeletal muscle (Koh *et al.* 2013). Skeletal muscle kidney-enriched inositol polyphosphate phosphatase (SKIP), a PIP3 (phosphatidylinositol-3,4,5-trisphosphate) phosphatase, has also been proposed to link ER stress to insulin resistance in skeletal muscle (Ijuin *et al.* 2016). Here, we have shown that HIHG induced ER stress. Difficulties with directly measuring ER stress has often necessitated investigators measuring UPR activation as an indication of ER stress, which can be misleading, and so here we used thioflavin T to detect protein aggregates (Beriault & Werstuck 2013) and colocalized this signal with KDEL immunofluorescence to localize ER. Our data showed that HIHG enhanced ER-localized protein aggregates. We also found that HIHG-induced the expression of a GRP78 promoter-dependent fluorescent reporter and pIRE1, pPERK and ATF6. ER stress induced induction of activating transcription factor 6 (ATF6) and X-box-binding protein 1 (XBP1)-dependent expression of SKIP was previously shown to decrease insulin sensitivity (Ijuin *et al.* 2016), and this is in keeping with the elevated ATF6, which we observed.

Most importantly, a phenomenon which is clearly emerging from recent studies is that activating autophagy can attenuate ER stress-induced decreases in insulin sensitivity in various tissues (Hwang *et al.* 2013, Wang *et al.* 2015, Kwak *et al.* 2016). An important role for AMPK in this process is apparent. We showed that adiponectin-stimulated autophagy in muscle cells occurred via an AMPK-dependent signaling mechanism (Liu *et al.* 2015). Huang and coworkers showed that decreased insulin-stimulated signaling and glucose uptake due to ER stress was rescued with the specific ERK inhibitor U0126 via

Figure 7

Schematic representation of conclusions. (A) Summary of main findings of this study in wild type/empty vector (WT/EV) or autophagy-deficient (Atg5K) cells. (B) Depiction of potential crosstalk between ER stress, the UPR, autophagy and insulin signaling. Briefly, disruption of normal ER function leads to an accumulation of unfolded protein. Increasing ER stress causes activation of the UPR. There are three branches of the UPR controlled by three ER transmembrane proteins: PERK, IRE1 and ATF6. In normal conditions, the ER chaperone GRP78 is bound to their ER luminal domain maintaining them in an inactive state; however, the increased levels of unfolded proteins in ER stress conditions cause GRP78 to dissociate. This allows their activation (phosphorylation of PERK and IRE1 and cleavage of ATF6) and initiation of their respective downstream signaling cascades. The function of these signaling cascades is to increase the capacity for protein folding and assembly but also to decrease the protein folding load by increasing ER associated degradation of unfolded proteins. In keeping with this, it has been suggested that the UPR potentially induces autophagy via activation of eIF2α downstream of PERK, such that unfolded proteins associated with ER stress are degraded also by autophagy. In our study, we showed that disruption of autophagy caused ER stress and insulin resistance and that by restoring autophagy there was reduced ER stress and improved insulin sensitivity. Further, we demonstrated a decrease in p70S6 after HIHG treatment, potentially suggesting that chronically elevated ER stress leads to a downregulation of UPR-initiated autophagy. Future studies to further examine the link between the UPR and induction of autophagy would yield insight into the role this mechanism plays in skeletal muscle insulin resistance in this cellular model. A full color version of this figure is available at <http://dx.doi.org/10.1530/JME-17-0096>.

AMPK activation (Huang *et al.* 2002). Furthermore, in db/db mice, U0126 administration increased AMPK phosphorylation and improved insulin sensitivity in muscle tissues (Hwang *et al.* 2013). Similarly, the *in vivo* administration of bortezomib to ob/ob mice reduced ER stress marker levels, increased AMPK phosphorylation and improved insulin sensitivity in skeletal muscle (Kwak *et al.* 2016). In this study, stimulating autophagy with adiponectin or rapamycin alleviated ER stress and insulin resistance in normal but not autophagy-deficient cells, demonstrating their beneficial effect on insulin sensitivity was autophagy dependent.

Interestingly, the downregulation of autophagy, which we observed in response to HIHG might in part be due to altered UPR response. While PERK phosphorylation was elevated, we observed a notable and unexpected downregulation of p70S6, a downstream target of PERK. Based on this, future research should examine other inputs to regulation of p70S6 and if they are altered in insulin-resistant skeletal muscle cells. An additional interpretation is that since autophagy plays an important role in clearing unfolded proteins and alleviating ER stress (Pallet *et al.* 2008, Wang *et al.* 2015, Dash *et al.* 2016), impairment in autophagy might directly contribute to the observed increase in ER stress. In keeping with this, we demonstrated that both pharmacological blocking

of autophagy using bafilomycin as well as molecular impairment of autophagy in the Atg5K autophagy-deficient cell line caused an increase in ER stress. This conforms to previous findings that demonstrated autophagy-deficient models to be more susceptible to developing ER stress and insulin resistance (Munch *et al.* 2014, Wang *et al.* 2015).

In conclusion, our study demonstrated that HIHG-induced ER stress, reduced autophagy and insulin sensitivity in L6 skeletal muscle cells. Adiponectin treatment alleviated ER stress and improved insulin sensitivity in an autophagy-dependent manner (Fig. 7). These data further demonstrate an important role of autophagy in promoting skeletal muscle insulin sensitivity, particularly in response to adiponectin in skeletal muscle. This work develops our understanding of the cellular crosstalk between autophagy and ER stress in regulating insulin resistance and metabolic dysfunction in skeletal muscle. Via new knowledge such as this, we can generate new and improved therapies for metabolic complications in type 2 diabetes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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