

Heat shock protein 60 (HSP60) modulates adiponectin signaling by stabilizing adiponectin receptor

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Short report

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Abstract

Adiponectin, an adipokine produced and secreted by adipocytes, is involved in regulating the development and progression of insulin resistance, diabetes, and diabetic complications. Heat shock protein 60 (HSP60) is a molecular chaperone, most commonly presenting in mitochondria and participating in the maintenance of protein homeostasis. Accumulating studies have demonstrated that the elevated circulating HSP60 and the decreased intracellular HSP60 are closely associated with diabetic complications such as diabetic cardiomyopathy. However, the underlying mechanism remains poorly understood. In the present study, we reported that HSP60 interacted directly with adiponectin receptors. Its abundance was positively associated with adiponectin action. Furthermore, HSP60 depletion markedly mitigated the protective impacts of adiponectin on high glucose-induced oxidative stress and cell apoptosis in rat cardiac H9c2 cells. In addition, HSP60 knockdown significantly enhanced proteasome activity leading to the degradation of adiponectin receptor 1. Taken together, we showed for the first time that HSP60 interacted with adiponectin receptors and mediated adiponectin signaling through stabilizing adiponectin receptor. This *in vitro* study also provides an alternative explanation for mechanism by which adiponectin exerts its action.

Background

Adiponectin is the most abundant adipokine produced and secreted by adipocytes. Through binding with its specific receptors adiponectin receptor 1 (AdipoR1) and AdipoR2, adiponectin initiates intracellular signaling pathways and exerts promising effects in the prevention or treatment of diabetes and metabolic syndrome, cardiovascular diseases, cancers, central nervous system disorders and so on [1–4]. Previous researches have confirmed that adiponectin signaling could be mediated by adaptor protein APPLs (adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper) including APPL1 and APPL2 [5, 6]. APPL1 associates with the intracellular domain of AdipoRs and positively regulates adiponectin's actions in some type of cells such as sensitizing insulin signaling in skeletal muscle cells [5, 6]. APPL1-deficiencies in mice impair adiponectin signaling and therefore cause systemic insulin resistance [7]. In contrast, APPL2 negatively regulates adiponectin signaling by competitively interacting with AdipoRs or heterodimerizing with APPL1 [6]. The "Yin and Yang" balance between APPL1 and APPL2 orchestrates adiponectin signaling and maintains normal adiponectin function [6, 8].

Heat shock protein 60 (HSP60) is classically described as a molecular chaperone, most commonly presenting in mitochondria and involving in the maintenance of protein homeostasis. Currently, accumulating evidences have linked HSP60 with diabetes mellitus and diabetic complications, although the molecular mechanisms are poorly understood [9–11]. For instance, serum HSP60 levels have been found to be significantly elevated in the patients with type 2 diabetes and morbid obesity, due to enhanced mitochondrial stress and responsible for inflammation [11–13]. A modified form of highly reactive HSP60 peptide p277 (DiaPep277) has been testing to treat type 1 diabetes [14]. In addition, the elevated serum HSP60 levels also increases cardiovascular risk in obesity individuals [13]. Therefore, HSP60 may represent a potential therapeutic target for diabetes and its complications.

Interestingly, type 2 diabetic subjects exhibit the decreased expression of HSP60 in the some tissues such as brain, heart, and subcutaneous adipose tissue [15–17]. Importantly, the decrease in HSP60 levels is closely associated with inflammation, mitochondrial dysfunction, formation of reactive oxygen species (ROS), and insulin resistance, which are usually observed in diabetic individuals and prevented by adiponectin administration [4, 15–18]. However, the status of HSP60 in adiponectin signaling is unclear.

Here, we experimentally demonstrated that HSP60 mediated adiponectin signaling in vitro by stabilizing adiponectin receptor. This finding will undoubtedly help us to deepen our understanding of adiponectin action and explore a novel therapy strategy for diabetes and diabetic complications.

Materials And Methods

Antibodies and reagents

Antibodies against to AMPK α (#5831), phospho-AMPK α (Thr172) (#2535), p38 MAPK (#8690), phospho-p38 MAPK (Thr180/Tyr182)(#9216), cleaved caspase-3 (#9661), Myc-tag (#2276), ubiquitin (#3936), β -tubulin (#2146) were from Cell Signaling Technology (Billerica, MA, USA). Antibodies against to AdipoR1 (ab70362), AdipoR2 (ab77612), and HSP60 (ab46798) were obtained from Abcam (Cambridge, MA, USA). Normal IgG (sc-2025) and secondary antibodies conjugated to horseradish peroxidase or alkaline phosphatase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Abbiotec (San Diego, CA, USA), respectively. Recombinant mouse adiponectin (ALX-522-059) and recombinant rat adiponectin globular form (Catalog#: SRP4593) were acquired from Enzo Life Sciences (Farmingdale, NY, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. MG132 (HY-13259) was obtained from MedChemExpress (Monmouth Junction, NJ, USA).

Cell culture and treatment

Rat cardiac H9c2 cell (ATCC, CRL-1446) were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Mouse liver HepIR cells (kind gifts from Drs, Feng Liu and Lily Q. Dong, UTHSCSA, USA) were cultured in MEM-alpha containing 10% FBS and 0.8 μ M dexamethasone [6, 19]. All cells were maintained in a humidified incubator with 5% CO₂ and 95% air at 37°C.

High glucose treatment was performed as our described previously [20, 21]. The control group received the treatment of 5.5 mM glucose and the identical concentration of mannitol which act as osmotic control to remove a hyperosmolar effect.

Plasmid construction

The cDNAs of full-length of mouse HSP60, mouse AdipoR1, and mouse AdipoR2 were generated by PCR and subcloned into the mammalian expression vectors pcDNA3.1 (Myc-tagged), or pGEX, respectively, as described previously [6].

Small interfering RNAs and transfection

The small interfering RNAs (siRNAs) targeting rat HSP60 (NM_022229.2) and mouse HSP60 (NM_010477.4) were synthesized by Genechem Co., LTD (Shanghai, China). Transfection was performed with 120 pM of siRNA using Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies Corporation, Gaithersburg, MD, USA) according to the manufacturer's protocol. The most effective sequences of siRNAs and its paired control used in the experiments were as follows: rat HSP60, 5'-GAGAGGTGTGATGTTGGCTGTTGAT-3' and 5'-GAGTGTGGTAGGGTTTGTCTGAGAT-3'; mouse HSP60, 5'-CAAATGGAGACAAAGACATTGGGAA-3' and 5'-CAAAGGCAGAAACAGTTAGGATGAA-3'. Knockdown efficiency was assessed by western blot.

Cell immunofluorescence

Immunofluorescence staining was performed as our described previously [6, 21]. Images were acquired on an Olympus IX83 laser scanning confocal microscope and analyzed by Olympus FV1200 software.

DHE staining

The real-time formation of ROS in cells was detected by dihydroergotamine (DHE) staining as described previously [21]. Briefly, the cells were plated on the coverslips within a 24-well plate at a density of 2×10^4 cells/well, starved serum for 6 hours, and then treated with or without high glucose and/or other compound for the desired time. DHE (at a final concentration of 10 μ M) was used to stain the cells at 37°C for 30 minutes in the dark. Cells were then rinsed once with pre-warmed PBS. DHE fluorescence was captured with fluorescence microscopy and quantified by automated image analysis.

Apoptosis determination

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) was performed to detect cells undergoing apoptosis as described by the manufacturer's protocol (Roche Applied Science, Indianapolis, IN, USA).

GST pull-down, immunoprecipitation and western blot

The pull-down assay, immunoprecipitation experiments, and western blot were performed as described previously [6].

Statistical analyses

The data are presented as the means \pm SD. Differences between the groups were examined using one-way analysis of variance (ANOVA), followed by a Newman-Keuls post hoc test. The values of $p < 0.05$ were considered statistically significant.

Results And Discussion

HSP60 associated with adiponectin receptors

Adiponectin receptors are of two specific receptor subtypes, AdipoR1 and AdipoR2 [22]. AdipoR1 is expressed ubiquitously and constitutively in most tissues and cells including adult cardiomyocytes and rat cardiac H9c2 cells [23, 24], while AdipoR2 is mainly expressed in the liver [25]. To identify the association between AdipoRs with HSP60, we firstly detected the localization of AdipoRs and HSP60 in H9c2 cells and mouse liver Hep1R cells [19]. Immunofluorescence staining revealed that endogenous HSP60 co-localized with endogenous AdipoR1 in H9c2 cells (Fig. 1a) and overexpressed HSP60 also co-localized with overexpressed AdipoR2 in Hep1R cells (Fig. 1b), respectively. To investigate whether HSP60 and AdipoRs associate directly, GST pull-down and co-immunoprecipitation assays were performed. As shown in Fig. 1c and 1d, endogenous AdipoR1 in H9c2 cells and AdipoR2 in Hep1R cells interacted with GST-HSP60 but not with GST control proteins. Co-immunoprecipitation experiments revealed that overexpressed HSP60 interacted specifically with endogenous AdipoR1 in H9c2 cells (Fig. 1e) and endogenous AdipoR2 in Hep1R cells (Fig. 1f). These findings indicate that HSP60 interacts directly with adiponectin receptors.

HSP60 mediated adiponectin action

To study the functional role of HSP60 in adiponectin action, intracellular HSP60 protein levels were increased by overexpression (OE) or decreased by siRNA knockdown (KD), respectively. The cells were then starved serum for 6 h, followed by stimulation with 1 μ g/ml adiponectin for 30 min. It is well known that AdipoR1-mediated adiponectin signaling could be activated by globular adiponectin (gADPN) whereas AdipoR2 only bind with full-length adiponectin (fADPN) [22, 25]. Therefore, H9c2 cells and Hep1R cells were treated with gADPN and fADPN, respectively. We found that phosphorylation of AMPK and p38 MAPK in response to adiponectin stimulation were greatly suppressed in HSP60-KD H9c2 cells (Fig. 2a and 2b) and Hep1R cells (Fig. 2c and 2d) but obviously enhanced in HSP60-OE H9c2 cells (Additional file 1: Figure S1a and S1b) and Hep1R cells (Additional file 1: Figure S1c and S1d), respectively. Given that

adiponectin can effectively activate AMPK in cardiomyocytes and p38 MAPK in hepatocytes [26, 27], our results demonstrate that HSP60 positively modulates adiponectin signaling.

HSP60 knockdown mitigated the protective effects of adiponectin on high glucose-induced oxidative stress and cell apoptosis in H9c2 cells

Hyperglycemia is a hallmark feature of both type 1 and type 2 diabetes. Previous study has evidenced that high levels of glucose induce oxidative stress and cell apoptosis in cardiomyocytes [21, 28], which can be protected by adiponectin administration [29]. Using this model, we wanted to further confirm the role of HSP60 in mediating adiponectin signaling.

H9c2 cells were starved serum for 6 h, and then incubated with 5.5 mM (normal glucose control) or 33 mM glucose (high glucose, HG) in the presence or absence of 1 μ g/ml of gADPN for another 48 h. TUNEL and DHE staining assays were carried out to detect cell apoptosis and real-time formation of ROS, respectively. The cleaved caspase-3 was detected by western blot to confirm the progression of apoptosis.

We found that HSP60 depletion significantly increased cell apoptosis, even on normal glucose (Fig. 3a, Additional file 2: Figure S2a, and Fig. 3b). This finding is consistent with previous study showing that the deletion of HSP60 in adult cardiomyocytes results in the impairment of structure and function of cardiac muscle cell [30]. Furthermore, adiponectin administration markedly inhibited HG-induced apoptosis in siRNA control cells (Fig. 3a, Additional file 2: Figure S2a, and Fig. 3b). However, these protective effects were almost completely diminished in HSP60-KD cells (Fig. 3a, Additional file 2: Figure S2a, and Fig. 3b). The similar effects on ROS formation were found in siRNA control or HSP60-KD cells treated with or without adiponectin (Fig. 3c and Additional file 2: Figure S2b). These findings further confirm the HSP60 regulation on adiponectin signaling.

HSP60 stabilized adiponectin receptor through a proteasome-dependent mechanism

HSP60 has been found to positively regulate insulin-like growth factor-1 (IGF-1) signaling, through maintaining the abundance of IGF-1 receptor in cardiac muscle cells [31]. To figure out whether the similar mechanism exists in adiponectin receptors, we observed the effects of HSP60 KD on the protein levels of AdipoR1 in cardiac H9c2 cells. The cells were starved serum for 6 h, followed by stimulation with 1 μ g/ml of gADPN for 18 h. We found that AdipoR1 expression was significantly reduced by HSP60 depletion but not affected by adiponectin treatment (Fig. 4a and Fig. 4b), suggesting that HSP60 depletion induced AdipoR1 degradation.

It is well-known that intracellular protein degradation is mainly induced by two cellular routes: the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system [32]. HSP60 has been reported to modulate proteasome activity and protein ubiquitination [31, 33]. We thus investigated the potential effects of HSP60 on the UPS. Indeed, HSP60 depletion markedly decreased the ubiquitination of total proteins (Fig. 4c). In addition, 20S proteasome activity was also greatly enhanced in HSP60-KD H9c2

cells (Additional file 3: Figure S3). Consistent with study performed in yeast [33], these findings demonstrate that HSP60 can inhibit proteasome activity in mammalian cells.

When H9c2 cells were starved serum for 6 h, followed by incubation with 0.1 μ M of MG132, a specific proteasome inhibitor for 18 h, we found that proteasome inhibition significantly restored HSP60 depletion-reduced protein levels of AdipoR1 (Fig. 4d and 4e). Proteasome inhibition also significantly increased the ubiquitination of total proteins when compared with HSP60-KD cells (Fig. 4f). These findings further suggested that HSP60 depletion-induced AdipoR1 degradation is mediated by a proteasome-dependent mechanism.

Conclusion

We showed for the first time that HSP60 interacted with adiponectin receptors and mediated adiponectin signaling. It is highly notable that HSP60 could stabilize AdipoR1 expression through suppressing proteasome activity. This in vitro study also provided an alternative explanation for the mechanism underlying adiponectin action.

Abbreviations

HSP60:Heat shock protein 60; AdipoR1:adiponectin receptor 1; ROS:reactive oxygen species; TUNEL:Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; UPS:ubiquitin-proteasome system; HG:high glucose.

Declarations

Ethics approval and consent to participate

No applicable.

Consent for publication

Not applicable.

Availability of supporting data

All supporting data are included in this published article.

Competing interests

The authors declare no competing or financial interests.

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Author's contributions

DZ, ZW, and CW conceived and designed the study; DZ, HL, YZ, JW, YF, YZ, and JL performed the experiments; DZ, YZ, HL, and ZW analyzed and interpreted the Data; ZW and CW supervised the project and wrote the manuscript. All authors read and approved the final manuscript.

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Figures



Figure 1

HSP60 interacted with adiponectin receptors. A Colocalization of HSP60 and AdipoR1 in H9c2 cells. B Colocalization of HSP60 and AdipoR2 in HepIR cells. C Pull-down of endogenous AdipoR1 with GST-HSP60 in H9c2 cells. D Pull-down of endogenous AdipoR2 with GST-HSP60 in HepIR cells. E Coimmunoprecipitation of AdipoR1 with HSP60 in H9c2 cells. F Coimmunoprecipitation of AdipoR2 with HSP60 in HepIR cells.



Figure 2

HSP60 knockdown attenuated adiponectin action. A Effects of HSP60 knockdown on adiponectin-stimulated phosphorylation of AMPK in H9c2 cells. B Quantification of phosphor-AMPK/AMPK in a. C Effects of HSP60 knockdown on adiponectin-stimulated phosphorylation of p38MAPK in HepIR cells. D Quantification of phosphor-p38 MAPK/p38 MAPK in c. Results are mean \pm SD. n=4. *P<0.05, **P<0.01 compared with the indicated group (one-way ANOVA).



Figure 3

HSP60 knockdown abolished the protective effects of adiponectin on high glucose-induced apoptosis and ROS formation in H9c2 cells. A Quantification of cell apoptosis. B Effects of HSP60 knockdown on cleaved caspase-3 levels. C Quantification of DHE staining. Results are mean \pm SD. n=4. ***P<0.001 compared with the indicated group (one-way ANOVA). ns: no statistical significance.



Figure 4

HSP60 knockdown reduced AdipoR1 levels in H9c2 cells. A Effects of HSP60 knockdown on AdipoR1 expression. B Quantification of AdipoR1 protein levels in a. C Effects of HSP60 knockdown on the ubiquitination of total proteins. D Effects of proteasome inhibitor MG132 on HSP60 knockdown-induced reduction of AdipoR1 expression. E Quantification of AdipoR1 protein levels in d. F Effects of MG132 on the ubiquitination of total proteins. Results are mean \pm SD. n=4. *P<0.05, **P<0.01 compared with the indicated group (one-way ANOVA). ns: no statistical significance.

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