E3 Ubiquitin Ligase, WWP1, Interacts with AMPKα2 and Downregulates its Expression in Skeletal Muscle C2C12 Cells*

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*Running title: WWP1 interacts with AMPKα2 and downregulates its expression

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Background: The role of ubiquitin-proteasome pathway under high glucose condition is unclear. Results: AMPKα2 interacts with WWP1 and its expression is down regulated by ubiquitin proteasome pathway in high glucose culture condition in C2C12 cells. Conclusion: WWP1 downregulates AMPKα2 expression through direct interaction in high glucose culture condition of skeletal muscle C2C12 cells. Significance: The ubiquitin proteasome pathway may involve in high glucose-induced AMPKα2 down regulation.

Summary
It is known that activity of AMP-activated protein kinase (AMPKα2) was depressed in high glucose condition. However, whether protein expression of AMPKα2 is also down-regulated or not remain unclear. In this study, we showed that the expression of AMPKα2 down-regulated in cells cultured under high glucose conditions. Treatment of proteasome inhibitor, MG132, blocked high-glucose induced AMPKα2 downregulation. Endogenous AMPKα2 ubiquitination was detected by immunoprecipitation of AMPKα2 followed by immunoblotting detection of ubiquitin. Yeast-two hybrid (YTH) approach identified WWP1, E3 ubiquitin ligase, as AMPKα2 interacting protein in skeletal muscle cells. Interaction between AMPKα2 and WWP1 was validated by co-immunoprecipitation. Knock down of WWP1 blocked high glucose-induced AMPKα2 down regulation. Over-expression of WWP1 down regulated AMPKα2. In addition, the expression of WWP1 is increased in high glucose culture conditions in both mRNA and protein levels. Level of AMPKα2 was down-regulated in the quadriceps muscle of diabetic animal model db/db mice. Expression of WWP1 blocked metformin-induced glucose uptake. Taken together, our results demonstrated that WWP1 down-regulated AMPKα2 under high glucose culture condition via ubiquitin-proteasome pathway.

INTRODUCTION
The metabolic syndrome is a combination of metabolic risk factors, including obesity, insulin resistance, hyperglycemia and dyslipidemia and increase the risk of developing cardiovascular disease and diabetes. Maintaining energy balance depends on the efficiency of tightly regulated mechanisms of energy intake and expenditure. Hyperglycemia is a condition in which an excess amount of glucose circulates in the blood plasma. Glucose homeostasis is maintained by a balance between hepatic glucose production and glucose uptake by peripheral tissues. Activation of AMP-activated protein kinase (AMPK) enhances glucose uptake through the translocation of glucose transporter 4 (GLUT4) to the cell membrane and also through regulation of Glut4 gene expression (1-3). AMPK activator enhances glucose uptake into muscle by increasing cell surface GLUT4 levels (4-6). AMPK may regulate GLUT4 endocytosis (7,8) and GLUT4 translocation (9,10). Thus, AMPK is a well-accepted therapeutic target for the metabolic syndrome and type 2 diabetes (11,12). AMPK is an enzyme that plays a role in cellular energy homeostasis. AMPK, a heterotrimeric complex comprised of a catalytic subunit and two regulatory subunits, is activated when cellular energy is depleted (13). Activated AMPK switches on ATP-generating catabolic pathways, including glucose and fatty acid oxidation (14-16), while simultaneously switches off ATP-consuming anabolic pathways (cholesterol, fatty acid, and triacylglycerol synthesis) (17). Thus, AMPK plays as a counter-regulatory mechanism and restore the AMP: ATP ratio. In addition to its roles in energy homeostasis, AMPK has also been shown to be regulated by ubiquitination (18). This fact suggests that the expression level of AMPK may play an important role in in specific biological conditions; however, the molecular link by which ubiquitination acts in high glucose condition is obscure. It is reported that elevated glucose down regulated the activity of AMPK in pancreatic cells (19) and rat skeletal muscle (20).

Protein ubiquitination is a mechanism for targeting proteins for rapid proteasomal degradation (21). Ubiquitin is attached covalently to target proteins in a series reaction driven by ubiquitin ligase. E1 ligases load ubiquitin onto E2 ligases, which in turn modify target proteins. Substrate specificity is generated by E3 ligases, which serve as adaptors. Recent studies demonstrated that ubiquitin proteasome system may regulate AMPK activity. De-ubiquitination enzyme USP9X interacts with NUAK1 (AMPK-related kinase 5) (18), implicating the role of AMPK ubiquitination. The other group identified Cidea (cell death-
inducing DFFA-like effector α) as an E3 ubiquitin ligase for AMPK (22). Although the basic regulation of AMPK activity by kinases has been delineated, regulation by post-translational modifications such as ubiquitination remains to be determined.

In this study, we found that AMPKα2 directly interacted with E3 ubiquitin ligase WWP1. We also showed that the expression level of AMPKα2 down regulated in C2C12 cells cultured under high glucose conditions, and further demonstrated that the ubiquitination is involved in high glucose-induced AMPKα2 down regulation. These findings provide novel insight into the manner in which high glucose contributes to insulin resistance through down regulation of AMPKα2 in skeletal muscle cells.

MATERIALS AND METHODS

Reagents - Anti-AMPKα2 antibody were purchased from Cell Signaling Technology (New England Biolabs, Beverly, MA, USA). Anti-WWP1 antibody was purchased from ProteinTech Group, Inc. (Chicago, IL, USA). Anti-ubiquitin antibody, anti-Flag antibody, anti-β-actin antibody, and AICAR were purchased from Cell Signaling Technology (New England Biolabs, Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Assay Designs and Stressgen (Ann Arbor, MI, USA). Metformin, MG132, and lactacystin were obtained from Calbiochem (San Diego, CA, USA). L-(-)Glucose, D-(+)Glucose, and mannitol were purchased from the Sigma Chemical Company (St Louis, MO, USA).

Cell culture and differentiation - Mouse myoblast C2C12 and human embryonic kidney (HEK) 293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in an incubator with 5% CO2. Cells were grown in culture medium consisting of 500 µl of DMEM (GIBCO™, Auckland, NZ, USA) containing 0.584 g/liter of L-glutamate and 4.5 g/liter of glucose, and mixed with 500 ml of F-12 medium containing 0.146 g/liter of L-glutamate, 1.8 g/liter of glucose, 100 µg/ml of gentamicin, 2.5 g/liter of sodium carbonate, and 10% heat-inactivated FBS. L6 myoblasts were maintained in cell monolayers in α-MEM supplemented with 10% FBS, 50 U of penicillin per ml, and 50 µg of streptomycin per ml in a 5% CO2 humidified atmosphere at 37°C. Two days after the myoblasts achieved confluence, differentiation to myotubes was induced by incubating the cells for 6-7 days in α-MEM supplemented with 2% FBS, which was changed every 2 days. The monolayer myotubes were finally serum-starved in DMEM for 2 hours, then used in the following experiments.

Animal tissue - Male C57BLKS/J-db/db mice and wild-type C57BL/6J mice were obtained from the Central Lab. Animal Inc. (Seoul, Korea) at age 6 weeks. All mice were maintained under standard light (12 hours light/dark), temperature (22 ± 2°C) and humidity (40 ± 10%) condition. The mice were anesthetized with a dry ice and quadriceps muscle was prepared. All procedures were approved by the Institutional Animal Care and Utilization Committee for Medical Science of Korea University.

Immunoblot analysis - Cells were grown on 6-well plates and serum-starved for 24 hours prior to treatment with the indicated agents. Following treatment of the cells, the media was aspirated and the cells were washed twice in ice-cold PBS and lysed in 100 µl of lysis buffer. The samples were then briefly sonicated, heated for 5 minutes at 95°C, and centrifuged for 5 minutes. The supernatants were electrophoresed on SDS-PAGE (8%) gels, and transferred to polyvinylidine difluoride membranes. The blots were incubated overnight at room temperature with primary antibodies and then washed six times in Tris-buffered saline/0.1% Tween 20 prior to 1 hour incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature. The blots were then visualized via ECL (Amersham Biosciences, Buckinghamshire, UK). In some cases, the blots were stripped and reprobed using other antibodies.

Immunoprecipitation - The amount of proteins from C212 cells or Flag-WWP1 transfected HEK293 cells was determined by the Bradford method. Cellular protein (100 µg) was mixed with 1 µg of anti-Flag or anti-AMPK antibodies and incubated overnight at 4°C. Then, 10 µl of protein A sepharose (Amersham, Uppsala, Sweden) was added to these samples and incubated for another 3 hours at 4°C. After the incubation, samples were washed three times with wash buffer (25 mM HEPES, 5 mM EDTA, 1% Triton X-100, 50 mM NaF, 150 mM NaCl,
10 mM phenylmethylsulfonyl fluoride, 1 µM leupeptin, 1 µM pepstatin, and 1 µM aprotinin A (pH 7.2)). The washed samples were resuspended in SDS sample buffer (125 mM Tris-HCl [pH 6.8], 20% [v/v] glycerol, 4% [w/v] SDS, 100 mM dithiothreitol, and 0.1% [w/v] bromophenol blue) and heated at 100°C for 5 minutes prior to electrophoresis.

Silencing AMPKα2 and WWP1- C2C12 cells were seeded in 6-well plates and allowed to grow to 70% confluence for 24 hours. Transient transfections were performed with transfection reagent (Lipofectamine 2000; Invitrogen) according to the manufacturer’s protocol. Briefly, both AMPKα2 siRNA (NM_001013367; Dharmacon, CO, USA), WWP1 siRNA (Genolution Pharmaceuticals, Inc.) and non-targeted control siRNAs (Non-targeting pool; Dharmacon CO, USA) were designed. Five µl of siRNA and 5 µl of transfection reagent (Lipofectamine 2000) were each diluted with 95 µl of reduced serum media (Opti-MEM; Invitrogen), then mixed. The mixtures were allowed to incubate for 30 minutes at room temperature and then were added dropwise to each culture well containing 800 µl of reduced serum media (Opti-MEM; Invitrogen), then mixed. The cells were incubated for 24 hours and lysed, and the expression of AMPKα2 protein was assayed with Western blotting.

Immunohistochemistry- Seven micrometer-thick cryosections from db/db and control mouse quadriceps femoris muscle were fixed with 4% freshly depolymerized paraformaldehyde for 15 minutes at 4°C. After blocking with normal goat serum, sections were immunostained with antibodies against AMPKα for overnight at room temperature. After extensive washing with phosphate-buffered saline (PBS), sections were incubated with FITC-labeled secondary antibody.

Reverse transcription–polymerase chain reaction (RT-PCR) - First strand cDNA synthesis was performed using 1 µg of total RNA isolated from C2C12 at 55°C for 20 minutes using the Thermoscript II one-step RT-PCR Kit (Invitrogen, Paisly, UK). Amplification of cDNA was carried out in the same tube using the Gene Amp System 9700 thermocycler (Applied Biosystems, Warrington, UK). Heating to 94°C for 5 minutes inactivated the reverse transcriptase. The following PCR conditions were used: 27 cycles of 30 seconds at 94°C, 30 seconds at 56°C, and 30 seconds at 72°C, followed by 7 minutes at 72°C. The number of PCR cycles used was optimized to ensure amplification at the exponential phase. Ten µl samples from each RT-PCR product were removed and analyzed by agarose gel electrophoresis. Bands were stained with ethidium bromide and visualized under ultraviolet light. Band intensity quantification was determined by a gel documentation system (Gene Genius, Syngene, UK). The following primers were used: WWP1-sense (5’- TCC TGG TGA CGG AAG AAA AC-3’) and WWP1-antisense (5’- AAC CAG GTG TCT TTG CCA AC -3’); and AMPKα2-sense (5’- CGC CTC TAG TCC TTC ATC AG -3’) and AMPKα2-antisense (5’- CAG CTG TGC TGG AAT CAA AA -3’); and β-actin-sense (5’-ACT ACC TCA TGA AGA TCC TCA AGA TCC TCA -3’).

GST-pull down assay- GST fusion proteins containing different domains of AMPKα2 were engineered by PCR. The fusion proteins were expressed in Escherichia coli bacteria by incubation with 0.2 mM isopropyl β-D-galactopyranoside for 6 h at 37 °C. The bacteria were lysed by sonication, and the fusion proteins were purified with glutathione-agarose beads with buffer A followed by immunoblotting with anti-WWP1 antibody.

Glucose uptake - Glucose uptake was measured using the 2-deoxy-D-[3H]-glucose. Briefly, L6 cells were serum-starved overnight before each experiment and glucose uptake was measured on day 7. Medium was replaced in the morning (serum-free medium), and cells were exposed to drugs for 1 hour. Cells were washed twice in warm PBS before media and drugs were placed in DMEM devoid of glucose for 20 minutes. 2-Deoxy-D-[3H]-glucose (50 nM) was added for 15 minutes at 37°C, and the reactions were terminated by washing twice in ice-cold PBS. Cells were lysed in 10% SDS or 50 mM NaOH, and samples were transferred to scintillation vials with scintillant and allowed to sit at room temperature for 1 hour before being counted.

Plasmid construction of Flag-wwp1- The open reading frames of human WWP1 was cloned into the Pemv-Tag2C vector (Stratagene) between EcoRI and SalI sites. cDNA from Hela
immunoprecipitated AMPKα2 proteasome pathway. Next, we tested whether suggesting the involvement of ubiquitin-proteasome inhibitor, lactacystin (Fig. 2B), this effect was also observed by another proteasome inhibitor, blocked high glucose-inhibition on AMPKα2 degradation - (Fig. 2A). MG132, a proteasome inhibitor, blocked high glucose-induced AMPKα2 downregulation (Fig. 2A). This effect was also observed by another proteasome inhibitor, lactacystin (Fig. 2B), suggesting the involvement of ubiquitin-proteasome pathway. Next, we tested whether AMPKα2 was ubiquitinated in cells. We immunoprecipitated AMPKα2 from cell lysates, and then analyzed with antibody against ubiquitin. As can be seen, the multi-ubiquitination of AMPKα2 was detected in immunoprecipitated sample (Fig. 2C). These results demonstrate that proteasome pathway is involved in high glucose-mediated AMPKα2 downregulation. 

AMPKα2 interacts with WWP1 - To explain the mechanism of high glucose-mediated AMPKα2 downregulation, we tried to identify AMPKα2-interacting protein. To identify protein that interacts with catalytic domain of AMPKα2, we carried out a yeast-two-hybrid (YTH) screen of a skeletal muscle C2C12 cDNA library. In result, WWP1, an E3 ubiquitin ligase, was identified as AMPKα2-interacting protein. To determine whether WWP1 interacts with AMPKα2 in mammalian cells, Flag-AMPKα2 was transfected into HEK293 cells. Lysates from transfected cells were immunoprecipitated with antibody against Flag. A band corresponding to WWP1 was detected in the immunoprecipitates, demonstrating interaction (Fig. 3A). Moreover, to assess in vivo interaction, we performed immunoprecipitation of skeletal muscle C2C12 cells protein using an AMPKα2 antibody, followed by Western blotting with an antibody to WWP1. AMPKα2 co-immunoprecipitated with WWP1 from endogenous C2C12 cells (Fig. 3B). Immunoprecipitation of Flag-WWP1 transfected HEK293 cells using a Flag antibody showed that AMPKα2 was co-immunoprecipitated with WWP1 (Fig. 3C). These results demonstrated that the interaction detected in the YTH also occurs in vivo and in vitro also.

Expression level of AMPKα2 is regulated by expression status of WWP1 - To investigate the relationship between WWP1 and AMPKα2, we first examined the effect of WWP1 knock down on high glucose-induced AMPKα2 downregulation. High glucose-induced downregulation of AMPKα2 was blocked in WWP1 knock-down condition. However, AMPKα2 downregulation was appeared in scramble siRNA, implying that WWP1 is important to down-regulate AMPKα2 (Fig. 4A). Interestingly, the anti-WWP1 antibody recognized two protein bands in the C2C12 cell lysates. The intensity of the two bands changed in similar patterns, indicating that WWP1 may be recognized by both protein bands. Next, to further study the function of WWP1, we

RESULTS

AMPKα2 downregulated in C2C12 cells cultured under high glucose conditions - High glucose concentrations are known to have detrimental effects on many cells types. To gain insight into the effect of high glucose conditions on skeletal muscle system, we evaluated the effect of high glucose concentration on the expression of AMPKα2 in skeletal muscle C2C12 cells. The expression of AMPKα2 significantly down regulated in high glucose concentration in cell culture medium. Mannitol is used as the isotonic control. L-glucose is used as the D-glucose specificity control. The level of AMPKα2 was not affected by these two agents, suggesting that AMPKα2 down-regulation is D-glucose-specific (Fig. 1A). We also assessed the effects of high concentration of glucose in time dependently. Exposure to 30 mM concentration of glucose for 3 days dramatically down regulated AMPKα2 expression (Fig. 1B). Together, these results demonstrate that high glucose condition down-regulates AMPKα2 expression in skeletal muscle cells.

Proteasome degradation pathway is involved in high glucose induced AMPKα2 degradation - To check the involvement of proteasome degradation in AMPKα2 down-regulation, we analyzed the effect of proteasome inhibition on AMPKα2 expression. MG132, a proteasome inhibitor, blocked high glucose-induced AMPKα2 downregulation (Fig. 2A). This effect was also observed by another proteasome inhibitor, lactacystin (Fig. 2B), suggesting the involvement of ubiquitin-proteasome pathway. Next, we tested whether AMPKα2 was ubiquitinated in cells. We immunoprecipitated AMPKα2 from cell lysates,
transiently transfected with Flag-WWP1. The level of AMPKα2 down-regulated in cells which WWP1-overexpressed. This down-regulation was blocked by treatment with proteasome inhibitor (Fig.4B), suggesting that ubiquitin-proteasome pathway is critical for AMPKα2 downregulation. To determine the interaction domain, we constructed GST fused whole AMPKα2, C-terminal AMPKα2, and N-terminal AMPKα2. We incubated GST fusion proteins with cell lysates from C2C12 cells. Targeted AMPKα2 proteins (asterisk) were detected by Coomassie blue staining. Immunoblotting with anti-WWP1 antibody showed that C-terminal AMPKα2 interacted with WWP1 (Fig.4C). Interaction between AMPKα2 and WWP1 was enhanced by high glucose incubation (Fig.4D). Together, these results indicate that WWP1 play an important role in AMPKα2 downregulation in high glucose culture condition.

**WWP1 increased in high glucose conditions**

To gain insight into the role of WWP1 in high glucose culture condition, we examined the effect of high glucose concentrations on expression of WWP1 both in protein and mRNA levels. When cells were cultured in the high concentrations of glucose, Western blot analysis showed that the level of WWP1 protein expression increased in dose-dependently (Fig. 5A). In experiment with RT-PCR, mRNA levels of WWP1 were also increased by incubation of C2C12 cells with 30 mM concentration of glucose conditions (Fig. 5B). Together, these results suggest that WWP1 may be regulated by glucose concentration status.

**AMPKα2 decreased in db/db mouse**

To examine the effect of high glucose on AMPKα2 in vivo, we used type 2 diabetes animal model, db/db mouse. Proteins of the quadriceps from wild type and db/db mice were prepared. Each group has 5 mice. The non-fasting blood glucose levels in db/db mice were significantly higher than those of wild mice (Data not shown). Insulin levels of db/db mice were also increased (Data not shown). Hyperinsulinemic-hyperglycemia of db/db mice indicates that these animals show insulin resistance, a primary characteristic of type 2 diabetes. The expression of AMPKα2 was down-regulated in quadriceps muscle of these mice (Fig. 6A). Furthermore, level of AMPKα2 mRNA was also down-regulated (Fig. 6B). To confirm whether AMPKα2 is regulated at the transcriptional level, we performed RT-PCR experiment in C2C12 cells. The level of AMPKα2 mRNA was slightly down-regulated at 12 hours after incubation with high glucose concentration, suggesting that AMPKα2 may be regulated by transcriptional level (Fig.6C). Immunostaining of db/db mouse and wild type mouse identified AMPKα2 in quadriceps muscle. In hyperglycemic db/db mouse, the level of AMPKα2 was downregulated compared with euglycemic wild type mouse. (Fig. 6D). The results of diabetic animal model was correlated with the result of biochemical data, thus these results indicate that the WWP1 may have a key function in AMPKα2 downregulation in high glucose conditions.

**Expression of WWP1 suppressed the metformin-induced glucose uptake** - Finally, to provide physiological relevance of WWP1, we investigated the effect of WWP1 expression on glucose uptake. Metformin-induced glucose uptake was blocked in WWP1 overexpression (Fig.7A). To gain insight into the role of high glucose-mediated AMPKα2 downregulation, we examined the effect of metformin on glucose uptake under high glucose culture condition. Attenuation of glucose uptake under high glucose culture condition was recovered by treatment with metformin, a first choice for the treatment of type 2 diabetes (Fig.7B). These results indicated that expression of WWP1 is important for metformin-mediated glucose uptake through regulating AMPKα2 expression.

**DISCUSSION**

The principal finding of this study was that WWP1 is involved in high glucose-induced AMPKα2 downregulation. Specifically, we demonstrated that direct interaction between AMPKα2 and WWP1 is instrumental in AMPKα2 downregulation.

The anti-diabetic role of AMPK has previously been evaluated in conjunction with as its activity. Dysfunction of AMPK activity may lead to metabolic syndrome. There is correlation between low activation state of AMPK with metabolic disorders associated with insulin resistance and obesity (23,24). AMPK activity is also suppressed in muscle and liver by sustained hyperglycemia (25). Moreover, many agents those are useful in treating diabetes, including TZDs (26,27), metformin (28), have been shown to stimulate AMPK activity. The contribution of AMPK activity to the anti-diabetic role has
raised questions as to which of the protein expression of AMPK may be relevant to its metabolic role. In the present study, we have established that high glucose culture condition down-regulated AMPK expression. Additionally, we have demonstrated that WWPL down regulated AMPKα2 through direct interaction. The relationship of AMPK with ubiquitination has been suggested. Collectively, our results indicate that AMPK may down regulate in high glucose culture condition via the ubiquitination pathway.

The main goal of the present study was to ascertain whether or not AMPKα2 is directly regulated by high glucose, and if so, to determine which mechanisms are involved in this process. Our data has revealed a novel role for WWPL of AMPKα2 downregulation in high glucose conditions. Our identification of an AMPKα2-WWPL interaction in high glucose-treated skeletal muscle cells led us to hypothesize that WWPL might play a role in the high glucose-mediated down regulation of AMPKα2. Indeed, this is the first report of a link between AMPKα2 and WWPL in skeletal muscle cells. It is thus tempting to speculate that WWPL-mediated regulation of AMPKα2 plays a role in diabetes since both AMPKα2 expression has been implicated in the insulin resistance. Overall, although the mechanism by which high glucose influences AMPKα2 remains unknown, the findings in this report suggest that high glucose may down regulates AMPKα2 expression through the WWPL as part of the process of high glucose-mediated insulin resistance. In the present study, we further demonstrated that high glucose induces expression of WWPL and overexpression of the WWPL decreased AMPKα2 expression. In addition, wild type WWPL block metformin mediated GLUT4 translocation. These results demonstrate that WWPL has an important role in glucose homeostasis through regulating AMPKα2 expression. Regulation of AMPK by ubiquitination has been suggested (29,30). The AMPK kinases NUAK1 and MARK4 are polyubiquitinated, and expression of NUAK and MARK4 mutants did not interact with the de-ubiquitinating enzyme USP9X de-ubiquitinating enzyme (DUB) and thus resulting in AMPK activity (18). This result implies that an important role for de-ubiquitination in AMPK activity regulation and further raises the question of what E3 ubiquitin ligases are involved. Because E3 ubiquitin ligases function to recognize specific substrates and to transfer ubiquitin to target protein. Thus, the specificity of the ubiquitin process is found in the E3 ubiquitin ligases. In the present study, we found an interaction of AMPKα2 with E3 ubiquitin ligase WWPL. This interaction is presumably required for the high glucose-mediated AMPKα2 downregulation. Combined with the recent report of interaction between proteasome itself and AMPK (31), protein-protein interaction is become a module for ubiquitin-mediated AMPK regulation.

On the basis of our present results, we propose a novel mechanism to explain how the expression of AMPK is down regulated in high glucose culture conditions. In this study revealed that AMPKα2 is down regulated in hyperglycemic animal db/db mice, while its interaction partner WWPL is up regulated. In conclusion, WWPL appears to be involved in insulin resistance through suppressing AMPKα2 expression. In conclusion, the present study supports the hypothesis that WWPL may have important role in hyperglycemic situations, such as diabetes and insulin resistance, through direct interaction with AMPKα2 and down regulates its expression.
References


FOOTNOTES

*This study was supported by the National Research Foundation of Korea funded by the Korea government (2010-0011053).
1Conflict of interest: We have no conflicts of interest to declare.
2The abbreviations used as follows: AICAR, 5-aminoimidazole-4-carboxy-amide-1-D-ribofuranoside; AMPK, AMP-activated protein kinase; ACC, acetyl CoA carboxylase; ACO, acyl-CoA oxidase; GLUT4, glucose transporter 4, YTH, yeast two hybrid
Figure Legends

Figure 1. AMPKα2 down-regulated in C2C12 cells cultured under high glucose conditions. A. C2C12 cells were stimulated for the indicated agents, such as D-glucose (30 mM), L-glucose (30 mM), and mannitol (30 mM) for 6 days. The cell lysates (20 μg) were analyzed via Western blotting for anti-AMPKα2 antibody. Blotting with anti-β-actin antibody was conducted as a protein loading control. B. C2C12 cells were stimulated for the indicated times with 30 mM high glucose. The cell lysates (20 μg) were analyzed via Western blotting for anti-AMPKα2 antibody. Blotting with anti-β-actin antibody was conducted as a protein loading control.

Figure 2. Proteasome degradation pathway is involved in high glucose induced AMPKα2 degradation. A. C2C12 cells were pre-treated 5 μM MG132 for 1 hour and stimulated with 30 mM glucose incubation for 48 hours. The cell lysates (20 μg) were analyzed via Western blotting for anti-AMPKα2 antibody. Blotting with anti-β-actin antibody was conducted as a protein loading control. B. C2C12 cells were pre-treated 5 μM lactacystin for 1 hour and incubated with 30 mM glucose for 48 hours. The cell lysates (20 μg) were analyzed via Western blotting for anti-AMPKα2 antibody. Blotting with anti-β-actin antibody was conducted as a protein loading control. C. C2C12 cells were immunoprecipitated with anti-AMPKα2 antibody. The immunoprecipitates were analyzed via Western blotting for anti-ubiquitin and AMPKα2 antibodies. TCL, total cell lysates.

Figure 3. AMPKα2 interacts with WWP1. A. HEK293 cells were transiently transfected with Flag-AMPKα2 for 48 hours and immunoprecipitated with anti-Flag antibody. The immunoprecipitates were analyzed via Western blotting for anti-WWP1 and Flag antibodies. B. The cell lysates (1 mg) of C2C12 cells were immunoprecipitated with anti-AMPKα2 antibody. The immunoprecipitates were analyzed via Western blotting for anti-WWP1 and AMPKα2 antibodies. C. HEK293 cells were transiently transfected with Flag-WWP1 for 48 hours and immunoprecipitated with anti-Flag antibody. The immunoprecipitates were analyzed via Western blotting for anti-AMPKα2 and Flag antibodies. TCL, total cell lysates.

Figure 4. Expression level of AMPKα2 is regulated by expression status of WWP1. A. C2C12 cells were transiently transfected with 50 nM siRNA WWP1 and scramble siRNA for 48 hours and then exposed to 30 mM glucose incubation for 48 hours. The cell lysates (20 μg) were analyzed via Western blotting for anti-AMPKα2, anti-WWP1 antibodies. Blotting with anti-β-actin antibody was conducted as protein loading control. B. C2C12 cells were pre-treated 5 μM MG132 for 1 hour and then cells were transiently transfected with Flag-WWP1 for 48 hours. The cell lysates (20 μg) were analyzed via Western blotting for anti-AMPKα2, anti-Flag antibodies. Blotting with anti-β-actin antibody was conducted as protein loading control. C. C2C12 cells were prepared and incubated with GST fusion proteins of various AMPKα2. Formed protein complexes were isolated by glutathione beads and washed three times with washing buffer, and analyzed by SDS/PAGE and subsequent Coomassie staining. The pull-down sample was also immunoblotted with anti-WWP1 antibody. D. C2C12 cells were exposed to 30 mM glucose incubation for 48 hours. The cell lysates (1 mg) of normal and high glucose conditions C2C12 cells were immunoprecipitated with anti-AMPKα2 antibody. The immunoprecipitates were analyzed via Western blotting for anti-WWP1 and AMPKα2 antibodies.

Figure 5. WWP1 increased in high glucose conditions. A. C2C12 cells were stimulated for the indicated doses of glucose for 48 hours. The cell lysates (20 μg) were analyzed via Western blotting for anti-WWP1 antibody. Blotting with anti-β-actin antibody was conducted as a protein loading control. B. Total mRNA was prepared for these cells after 30 mM glucose incubation and RT-PCR was conducted using specific WWP1 primers. The PCR product was then gel-run in 1% agarose, and visualized in UV. Beta-actin was employed as a positive control.
Figure 6. AMPKα2 down-regulated in db/db mouse. A. Western blot analysis of quadriceps muscles of wild type and db/db mice. The tissue lysates (40 μg) were analyzed via Western blotting for anti-AMPKα2 antibody. Blotting with anti-β-actin antibody was conducted as a protein loading control. Densitometry analysis (n = 5) of values are means ± SEM values of the ratios of densities (AMPKα2/β-actin). *p< 0.05 vs wild mouse. B. RT-PCR analysis of quadriceps muscles of wild type and db/db mice. Total mRNA was prepared for these mice and RT-PCR was conducted using specific AMPKα2 primers. The PCR product was then gel-run in 2% agarose, and visualized in UV. β-actin RNA was employed as a positive control. Densitometry analysis (n = 5) of values are means ± SEM values of the ratios of densities (AMPKα2 mRNA/β-actin mRNA). *p< 0.05 vs wild mouse. C. Total mRNA was prepared for these cells after 30 mM glucose incubation and RT-PCR was conducted using specific AMPKα2 primers. The PCR product was run in 1% agarose gel, and visualized in UV. Beta-actin was employed as a positive control. D. Histochemistry of the quadriceps muscle of wild type and db/db mice. Each cryosection was stained with anti-AMPKα2 antibody.

Figure 7. Expression of WWP1 blocked metformin-induced glucose uptake. A. L6 myoblast cells were differentiated for 7 days. Cells were transiently transfected with Flag-WWP1 for 48 hours and then treated with metformin for 16 hours. Glucose uptake was measured using 2-deoxy-D-[3H]-glucose. ∗p < 0.05 vs basal. B. L6 myoblast cells were differentiated for 7 days. Cells were incubated to 30 mM glucose for 48 hours in the presence of or absence of metformin for 16 hours. Glucose uptake was measured using 2-deoxy-D-[3H]-glucose. ∗p < 0.05 vs high glucose.
**Fig. 1.**

**A**

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**B**

Glucose (30 mM)  

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<td>IB: β-actin</td>
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Fig. 2.

A

Glucose  
MG132  
IB: AMPKα2
IB: β-actin

B

Glucose  
Lactacystin  
IB: AMPKα2
IB: β-actin

C

TCL  
IP: IgG  
IP: AMPKα2
IB: Ubiquitin

by guest on September 16, 2017 http://www.jbc.org/ Downloaded from
Fig. 3.

A

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AMPKα2-Flag

IB: WWP1

IB: Flag

Heavy chain

B

TCL

IP: IgG

IP: AMPKα2

IB: WWP1

IB: AMPKα2

C

<table>
<thead>
<tr>
<th>TCL</th>
<th>IP: IgG</th>
<th>IP: Flag</th>
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<tbody>
<tr>
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<td>+</td>
<td>+</td>
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</tbody>
</table>

WWP1-Flag

IB: AMPKα2

IB: Flag
**C**

IB: WWP1

Stain: Coomassie brilliant blue

**D**

High glucose

<table>
<thead>
<tr>
<th>TCL</th>
<th>IP: AMPKα2</th>
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<tbody>
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<tr>
<td>+</td>
<td>-</td>
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</tbody>
</table>

IB: WWP1

IB: AMPKα2

**Fig. 4**
Fig. 5.

A

Glucose (mM) 5 10 15 20 25 30

IB: WWP1

IB: β-actin

B

Glucose (30 mM) 0 0.5 1 3 6

WWP1 mRNA

β-actin mRNA
Fig. 6. Ratios of AMPKα2/β-actin in Wild mouse and db/db mouse samples.

IB: AMPKα2

IB: β-actin

Ratio (AMPKα2/β-actin)

Wild mouse  |  db/db mouse

0  |  0.2  |  0.4  |  0.6  |  0.8  |  1  |  1.2

wt-1  |  db-1  |  wt-2  |  db-2  |  wt-3  |  db-3  |  wt-4  |  db-4  |  wt-5  |  db-5

*
Fig. 6.

**B**

<table>
<thead>
<tr>
<th></th>
<th>Wild mouse muscle</th>
<th>db/db mouse muscle</th>
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<tbody>
<tr>
<td></td>
<td>1  2  3  4  5</td>
<td>1  2  3  4  5</td>
</tr>
<tr>
<td>AMPKα2 mRNA</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>β-actin mRNA</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

**C**

Glucose (30 mM) 0  1  3  6  12 (h)

<table>
<thead>
<tr>
<th>mRNA</th>
<th>AMPKα2</th>
<th>β-actin</th>
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</thead>
<tbody>
<tr>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

*Fig. 6.
D

Wild mouse muscle

$\text{db/db}$ mouse muscle

AMPK$\alpha_2$

Fig. 6.
**Fig. 7.**

**A**

<table>
<thead>
<tr>
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<th>Metformin</th>
<th>WWP1-Flag</th>
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<tr>
<td>+</td>
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</tbody>
</table>

2-deoxy-D-glucose uptake (dpm)

**B**

<table>
<thead>
<tr>
<th></th>
<th>High glucose</th>
<th>Metformin</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
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<td>+</td>
</tr>
<tr>
<td>+</td>
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</table>

2-deoxy-D-glucose uptake (dpm)
E3 ubiquitin ligase, WWP1, interacts with AMPKalpha2 and downregulates its expression in skeletal muscle C2C12 cells
Jung Ok Lee, Soo Kyung Lee, Nami Kim, Ji Hae Kim, Ga Young You, Ji Wook Moon, Sha Jie, Su Jin Kim, Yong Woo Lee, Ho Jin Kang, Yongchul Lim, Sun Hwa Park and Hyeon Soo Kim

J. Biol. Chem. published online January 4, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M112.406009

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