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The di-peptide Trp-His activates AMP-activated protein kinase and enhances glucose uptake independently of insulin in L6 myotubes

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ABSTRACT

The di-peptide Trp-His (WH) has vasorelaxant and anti-atherosclerotic functions. We hypothesized that WH has multiple biological functions and may aid AMP-activated protein kinase (AMPK) activation and affect the glucose transport system in skeletal muscle.
First, we examined whether WH or His-Trp (HW) can activate AMPKα. Treatment of L6 myotubes with WH or HW significantly increased phosphorylation of AMPKα. WH activated AMPK independently of insulin and significantly increased glucose uptake into L6 myotubes following translocation of glucose transporter 4 (Glut4) to the plasma membrane. This activation was induced by the LKB1 pathway but was independent of changes in intracellular Ca^{2+} levels and the Ca^{2+}/calmodulin-dependent kinase pathway. L6 myotubes express only one type of oligopeptide transporter, peptide/histidine transporter 1 (PHT1, also known as SLC15a4), and WH is incorporated into cells and activates AMPKα following PHT1-mediated cell uptake.
These findings indicate that (1) WH activates AMPK and insulin independently enhances glucose uptake following translocation of Glut4 to the plasma membrane, (2) activation of AMPKα by WH is mediated by the LKB1 pathway, without altering the Ca^{2+}-dependent pathway, and (3) L6 myotubes express only one type of peptide transporter (PHT1; SLC15a4), which incorporates WH into cells to activate AMPKα.

1. Introduction

Type 2 diabetes is caused by the development of insulin resistance in peripheral tissues with a failure of insulin secretion. The quantity of cell-surface glucose transporter (Glut) is a major determinant of glucose uptake by cells. Glut4 is an insulin-sensitive glucose transporter that is translocated to the cell surface from an intracellular pool in skeletal muscle and adipose tissue. Type 2 diabetes is characterized by defects in the muscle glucose transport system resulting from insulin resistance [1].

Glucose transport can also be regulated by AMP-activated protein kinase (AMPK), a heterotrimeric protein activated by phosphorylation at Thr172 of the catalytic α subunit [2]. AMPK has been shown to upregulate catabolic pathways that generate ATP and downregulate anabolic pathways that consume ATP [3]. AMPK has been identified as an important target in the prevention and treatment of obesity and type 2 diabetes [4]. Activation of AMPK via an insulin-independent mechanism can lead to increases in Glut4 expression or translocation to the plasma membrane (PM) [4].

AMPK is activated by some type 2 diabetes drugs including metformin, the thiazolidinediones, and 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) [5–7]. We previously demonstrated that AMPK is activated in type 2 diabetic mice administered anthocyanin-rich bilberry extract, which led to a decrease in blood glucose levels and increased insulin sensitivity [8]. In addition, AMPK is activated by 10-hydroxy-2-decenoic acid, a medium-chain fatty acid found in royal jelly, resulting in Glut4 translocation and increased glucose uptake into cells [9]. Furthermore, AMPK has been shown to be activated by other drugs and food-derived

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; CaMK, Ca^{2+}/calmodulin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; 2DG, 2-deoxy-D-glucose; Glut, glucose transporter; IRS-1, insulin receptor substrate-1; KRH, Krebs–Ringer–HEPES buffer; PHT1, peptide/histidine transporter 1; PM, plasma membrene; TEA, triethanolamine; VDCC, voltage-dependent calcium channel

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compounds with potentially anti-diabetic effects [10]. Taken together, these studies demonstrate the tremendous potential of AMPK activators as therapeutics for diabetes and insulin resistance.

We previously demonstrated that Trp-His (WH) produces a dose-dependent and endothelium-independent relaxation in pre-contracted rat aortic rings [11]. A number of papers have demonstrated that other di-peptides have multiple functions, including anti-atherosclerotic and anti-diabetic effects or increased diabetic wound healing [12,13]. These findings along with our previous studies on WH led us to ask whether WH has multiple biological functions, including activating AMPK, thereby affecting glucose transport in skeletal muscle cells.

In the present study, we show that WH activates AMPK via phosphorylation of Thr172 of its catalytic α-subunit, leading to an increase in glucose uptake via AMPK-dependent Glut4 translocation to the PM. Furthermore, the LKB1 pathway is involved in this AMPK. This study also demonstrates that L6 myotubes have only one type of peptide transporter peptide/histidine transporter 1 (PHT1, also known as SLC15a4) and that WH is transported into cells via PHT1 to activate AMPK.

2. Materials and methods

2.1. Chemicals

WH and His-Trp (HW) were synthesized using an Fmoc solid-phase synthesis method according to the manufacturer's instructions (Kokusan Chemicals, Tokyo, Japan). AICAR and insulin were obtained from Wako Pure Chemical Industries (Osaka, Japan). LKB1 (siRNA ID: s163339) and PHT1 (siRNA ID: s140941) siRNA oligonucleotides were purchased from Life Technologies (Tokyo, Japan). Fluo-4AM was obtained from Dojindo (Kumamoto, Japan). Compound C, 2-deoxy-D-glucose (2DG), 2DG-6-phosphate sodium salt, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, and resazurin sodium salt were obtained from Sigma–Aldrich (St. Louis, MO, USA). β-$NADP^+$ and diaphorase from *Clostridium kluyveri* were obtained from Oriental Yeast (Tokyo, Japan). The various antibodies used in this study were obtained from Cell Signaling Technology (Tokyo, Japan; phospho-AMPKα (Thr172, No. 2531), AMPKα (No. 2532), β-actin (No. 4967), phospho-Akt (Ser473, No. 4060), Akt (No. 4691), insulin receptor substrate-1 (IRS-1, No. 2382), LKB1 (No. 3047), phospho-CaMKII (Thr286, No. 3361), and CaMKII (No.4436)), Santa Cruz Biotechnology (Dallas, TX, USA; Glut1, No. SC-7938), Life Technologies (Tokyo, Japan; phospho-IRS-1 (Tyr612, 44816G)), or Abcam (Tokyo, Japan; Glut4, ab65267).

2.2. Cell culture

Rat myoblast L6 cells (JCRB9081) from the Health Science Research Resources Bank (Osaka, Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO_2. For myoblast differentiation into myotubes, 80% confluent myoblast cells were placed in DMEM containing 2% horse serum. The medium was changed every 2 days and the cells were cultured for an additional 7 days to obtain mature myotubes.

2.3. siRNA transfections

Transfection of L6 cells with negative control siRNA, LKB1, or PHT1 siRNA was performed according to the manufacturer's protocol. In brief, cells were seeded at 1.25×10^5 cells per well and transfected with siRNA. After 24 h, the medium was changed to fresh antibiotic-free differentiation medium. The cells were cultured for an additional 4 days and were then re-transfected with siRNA for 24 h. Cells were treated with WH 24 h post-transfection. The knockdown efficiencies were evaluated by measuring mRNA levels as previously described [14]. The knockdown efficiencies of LKB1 and PHT1 were 73.2 ± 1.7 and 80.1 ± 2.9%, respectively.

2.4. Myotube treatment and immunoblot analysis of AMPK and related proteins

Myotubes (differentiated for 7 days) were placed in serum-free DMEM containing 1% BSA for 3 h. After incubation, the myotubes were treated with AICAR (1 mM), WH, or vehicle (0.1% DMSO) for the indicated time or condition. After treatment, the cells were lysed [15]. Aliquots of supernatant were boiled in Laemmli sample buffer for 5 min [16]. The samples (25 μg protein) were then resolved in SDS–PAGE, transferred onto nitrocellulose membranes, and the blots were probed with various antibodies for 16 h at 4 °C. The membranes were then reacted with horseradish peroxidase-conjugated anti-rabbit or mouse IgG antibody, the immunoreactivity was visualized using ECL reagent (GE Healthcare Bioscience, Tokyo, Japan), and the relative density was evaluated with Multi Gauge Ver. 3.0 Densitograph Software (Fuji Film, Tokyo, Japan). The experiments were performed in triplicate and representative results are shown.

2.5. AMPK activity assay

AMPK activity in the samples was measured using the AMPK Kinase Assay Kit (Cyclex Co., Ltd., Nagano, Japan) according to the manufacturer's instructions.

2.6. ATP, ADP, and AMP analysis

Intracellular ATP, ADP, and AMP concentrations were measured using HPLC as previously described [17].

2.7. Preparation of the PM fraction

The PM fraction was prepared according to the method of Nishiumi and Ashida [18]. The obtained fraction was resolved in SDS–PAGE and the blots were probed for Glut4 and Glut1 as described in Section 2.4.

2.8. Glucose uptake assay

Glucose uptake was determined using a fluorescence assay according to the method of Yamamoto et al. [19]. Briefly, L6 myotubes were incubated with serum-free MEM containing 0.2% BSA for 18 h. The cells were incubated with WH and insulin for 1 h. The cells were rinsed twice with Krebs–Ringer–HEPES (KRH) buffer (50 mM HEPES, pH 7.4, 137 mM NaCl, 4.8 mM KCl, 1.85 mM $CaCl_2$, and 1.3 mM $MgSO_4$). 2DG uptake was performed by incubation with KRH buffer containing 0.1% BSA and 1 mM 2DG for 20 min. The cells were then washed twice with KRH buffer with 0.1% BSA and 0.1 M NaOH was added to each well. The culture plate was heated at 85 °C for 60 min. The dried cell lysate was neutralized with 0.1 M HCl and 200 mM triethanolamine (TEA) buffer (pH 8.1) was added. The cell lysate was mixed with an assay cocktail [50 mM TEA buffer (pH 8.1) containing 50 mM KCl, 0.02% (w/v) BSA, 0.1 mM β-$NADP^+$, 2 units/mL of diaphorase, 150 units/mL of glucose-6-phosphate dehydrogenase, and 2 μM resazurin] in another 96-well plate and incubated at 37 °C for 60 min. The fluorescence of resorufin was measured with excitation at 540 nm and emission at 590 nm using a Mithras LB 940 multimode microplate reader (Belthold, Bad Wildbad, Germany). The 2DG concentration in each well was calculated based on a standard curve generated with a 2DG-6-phosphate solution.

2.9. Measurement of intracellular Ca^{2+} levels

L6 myotube cells were detached from plates with a trypsin/EDTA mixture and washed with fresh cell culture medium by centrifugation at 800×*g* for 5 min. Cells were then resuspended in KRH buffer containing 2 μM Fluo-4AM and incubated for 60 min at 37 °C in the dark. Cells were washed and resuspended in KRH buffer at a concentration of 2×10^6 cells/mL and transferred to a cuvette for whole-cell population calcium measurement. The Fluo-4 fluorescence was measured using a spectrofluorophotometer (RF-5300PC; Shimadzu, Kyoto, Japan) with excitation at 495 nm and emission at 518 nm [20].

2.10. Detection of peptide transporters in rat tissues and L6 cells using RT-PCR

One male Sprague Dawley rat, 7 weeks of age, was obtained from Japan SLC (Shizuoka, Japan). The rat was housed in an animal room under controlled temperature (23 ± 3 °C) and lighting (lights on from 8:00 to 20:00 h) and allowed free access to water and a laboratory chow diet (CE-2; CLEA Japan, Tokyo, Japan). After 1 week of breeding, the rat was deprived of food for 16 h and euthanized by decapitation. The small intestine, kidney, skeletal muscle, and spleen were then removed. Tissue samples were immediately frozen using liquid nitrogen and stored at −80 °C until use. The experimental design was approved by the Animal Experiment Committee of Chubu University, and the rat was maintained in accordance with their guidelines.

Total RNA from various rat tissues and L6 myotubes was isolated using QIAzol reagent (QIAGEN, Tokyo, Japan) according to the manufacturer's directions. Total RNA (1.0 μg) was reverse-transcribed to cDNA in a final reaction volume of 20 μL using a ReverTra Dash RT-PCR Kit (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's directions. The resulting cDNA was used to perform PCR with ReverTra Dash RT-PCR Kit (Toyobo) with primers specific for PEPT1 (SLC15a1), PEPT2 (SLC15a2), PHT1 (SLC15a4), and PHT2 (SLC15a3) that were designed against rat sequences (Table 1) [21–23]. β-Actin was used as an internal control. The PCR reaction was performed using optimized PCR conditions (PEPT1: denaturation, 94 °C for 30 s; annealing, 55 °C for 2 s; extension, 72 °C for 30 s; PEPT2: denaturation, 94 °C for 30 s; annealing, 55 °C for 2 s; extension, 72 °C for 30 s; PHT1: denaturation, 94 °C for 30 s; annealing, 58 °C for 2 s; extension, 72 °C for 30 s; PHT2: denaturation, 94 °C for 30 s; annealing, 60 °C for 2 s; extension, 72 °C for 30 s; β-actin: denaturation, 94 °C for 30 s; annealing, 61 °C for 2 s; extension, 72 °C for 30 s). RT-PCR products were separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide. Gel images were visualized using a Printgraph gel imaging system (ATTO, Tokyo, Japan).

2.11. Statistical analysis

Data are expressed as mean ± SEM. The differences between control and WH groups in Table 2 (ATP, ADP, AMP and AMP/ATP ratio), and mRNA levels of L6 myotubes treated with PHT1 siRNA in Fig. 5A vs. negative control were compared by the Student's *t* test. In other cases, differences among means were analyzed by the Turkey–Kramer test. Differences with *P* values <0.05 were considered significant.

Table 2
ATP, ADP, and AMP concentrations and the AMP:ATP ratio in L6 myotubes treated with WH for 60 min.

	ATP	ADP	AMP	AMP:ATP ratio
	(nmol/mg protein)			
Control	3.84 ± 0.11	0.20 ± 0.01	0.05 ± 0.01	0.013 ± 0.001
WH	2.73 ± 0.09*	0.15 ± 0.01*	0.05 ± 0.01	0.017 ± 0.001*

* Mean values are significantly different from those of the control ($P < 0.05$) ($n = 6$).

3. Results

3.1. WH activates AMPKα in L6 myotubes

First, we examined whether WH activates AMPKα. WH significantly increased phosphorylated AMPKα levels in a time- and dose-dependent manner in L6 myotubes (Fig. 1A and B). Similarly, the administration of HW also significantly induced phosphorylation of AMPKα (data not shown). To further elucidate the effects of WH on AMPK, AMPK activity was examined in myotubes treated with WH. AMPK activity was also significantly increased by WH in a time- and dose-dependent manner (Fig. 1C and D).

3.2. WH promotes insulin-independent Glut4 translocation and glucose uptake in L6 myotubes

Glut4 translocation to the PM and the subsequent increase in glucose uptake into skeletal muscle are stimulated by activated AMPK in an insulin-independent manner. To evaluate the effect of WH on this pathway, we analyzed L6 myotube lysates and the PM fraction for an increase in the relative proportion of Glut4 in the PM by immunoblotting. Our results show that, similarly to insulin, WH alone significantly stimulated Glut4 translocation to the PM; pretreatment with the AMPK inhibitor compound C markedly inhibited this WH-dependent translocation (Fig. 2A), and nullified the WH-induced phosphorylation of AMPK (Fig. 2B). As expected, insulin stimulated an increase in the phosphorylation of Akt (Ser473) and IRS-1 (Tyr612), while WH had no discernible effect (Fig. 2C).

To further confirm the role of AMPK activation in the translocation of Glut4 to the PM, we tested whether WH stimulates the uptake of 2DG in the presence or absence of compound C. Insulin alone significantly increased the uptake of 2DG uptake, which was not affected by compound C. WH also significantly increased 2DG uptake into cells (Fig. 2D); however, the effect of WH, unlike that of insulin, was dependent on AMPK, as confirmed by the pretreatment with compound C, which markedly reversed the effect of WH (Fig. 2D).

3.3. AMPKα activation induced by WH depends on LKB1 as well as the ratio of AMP to ATP

A key factor in the regulation of AMPK is the phosphorylation of Thr172 of AMPKα by upstream kinases, resulting in the activation of

Table 1
Primer sequences of SLC15 transporters and β-actin.

Gene name	Sense primer 5′–3′	Antisense primer 5′–3′	PCR product size (bp)	Ref. No.
PEPT1 (Slc15a1)	ATCTACCATACGTTTGTTGC	CTGGGGCTGAAACTTCTT	523	[21]
PEPT2 (Slc15a2)	GCTGCCTACTGAAGCCAAATGCTTG	AGAGGCTGCTGAAGGCATGGT	341	[22]
PHT2 (Slc15a3)	GAGTCTGGGTCACGGAGAC	GAGGCCCACGATGCTG	739	[23]
PHT1 (Slc15a4)	GAGGGCCGTTCACAGAGGA	TGAGGCCTTATAGTCTGCAG	859	[23]
β-Actin	CGTGACATCAAGGAGAAGCT	ATCCACATCTGCTGGAAGGT	444	[23]

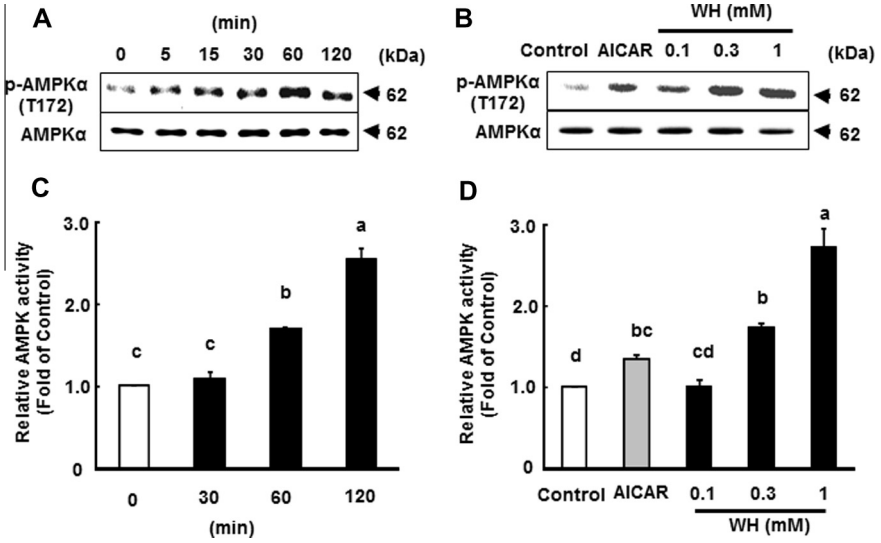

Fig. 1. Immunoblot analysis of the effect of WH duration (A) and dose (B) on total and phosphorylated AMPKα and the effect of WH time (C) and dose (D) on AMPK activity. The cells were treated with 0.3 mM WH for various durations (A) and (C), or with vehicle (0.1% DMSO), AICAR (1 mM), or WH for 60 min (B) and (D). AMPK activity (C and D) was expressed relative to the control (=1.0). Values are means ± SEM; $n = 4–8$. Mean values without a common letter are significantly different at $P < 0.05$.

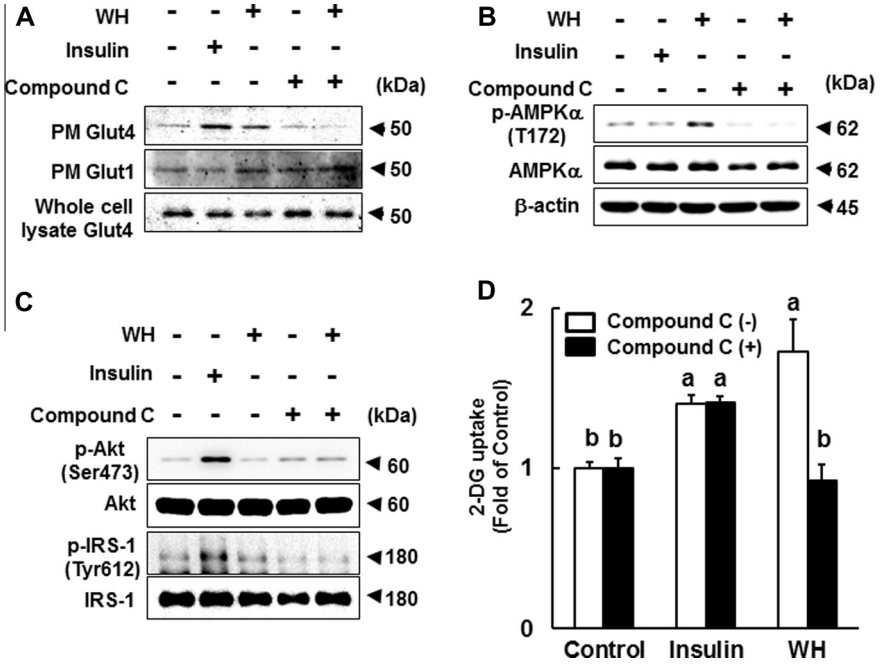

Fig. 2. Immunoblot analysis of Glut4 and Glut1 (A), AMPKα and β-actin (B), and insulin signaling proteins (C) in L6 myotubes. Cells were treated with insulin (100 nM) or WH (0.3 mM) for 60 min in the presence or absence of compound C (20 μM), prior to isolation of the PM fraction or the whole-cell lysate of treated cells. Panel D shows the uptake of 2DG in L6 myotubes. Cells were treated with vehicle (0.1% DMSO), insulin (100 nM), or WH (0.3 mM) for 60 min in the presence or absence of compound C (20 μM). Values are means ± SEM; $n = 3–9$. Mean values without a common letter are significantly different at $P < 0.05$.

AMPK [24]. Interestingly, increasing the ratio of AMP to ATP, which reduces inactivation by dephosphorylation, results in the activation of AMPK [25]. AMP regulates AMPK activation at the initial phosphorylation step, and myristoylation of the β-subunit of AMPK is required for initiation of the signaling cascade as this lipid modification allows the upstream kinase LKB1 to phosphorylate AMPK [26].

We examined whether WH could regulate LKB1 as well as the AMP:ATP ratio to shed light on the mechanism of the activation of AMPK by WH. We first measured the intracellular concentrations of ATP, ADP, and AMP in L6 myotubes, and found that WH significantly decreased intracellular ATP concentrations and resulted in an increased AMP:ATP ratio in (Table 2). Next, we knocked down the expression of LKB1 in L6 myotubes with siRNA to examine whether LKB1 is required for the WH-induced activation of AMPK. In these cells, we found that the WH-dependent phosphorylation of AMPKα was significantly reduced (Fig. 3A).

3.4. Intracellular Ca^{2+} response of L6 myotube cells to WH

Thr172 of AMPKα is also phosphorylated by Ca^{2+}/calmodulin-dependent kinase kinase (CaMKK) or CaMKII and activates AMPK in intact cells [24,27,28]. Activation of CaMKK and CaMKII requires calcium ions, which can be provided by influx of extracellular Ca^{2+}

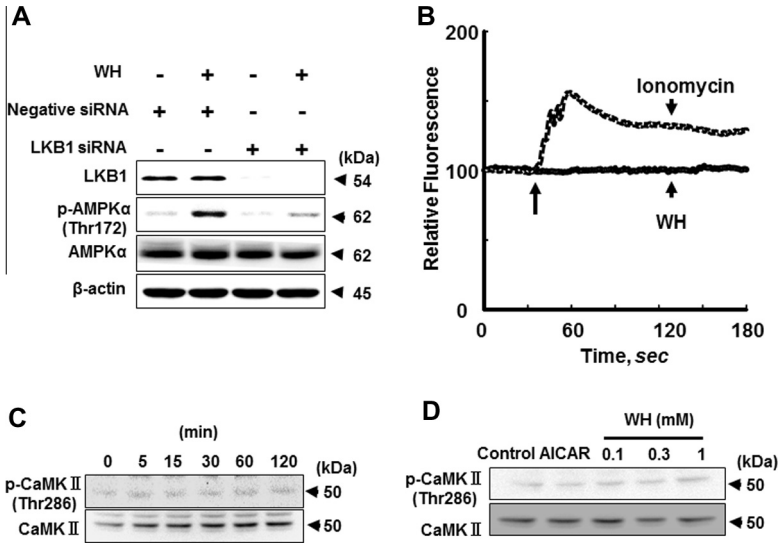

Fig. 3. Immunoblot analysis of AMPKα and CaMKII and its related proteins in L6 myotubes treated with WH under various conditions (A, C, and D) and intracellular Ca^{2+} response of L6 myotubes treated with WH (B). Cells were treated with WH (0.3 mM) for 60 min in the presence or absence of LKB1 siRNA (A). The effect of WH duration (C) and dose (D) on total and phosphorylated CaMKII. (B) Cells were loaded with Fluo4-AM and fluorescence was analyzed after treatment with WH (0.3 mM) or ionomycin (4.2 μM) as a positive control.

via calcium channels or mobilization of intracellular stores of Ca^{2+}. To investigate whether WH affected intracellular Ca^{2+} levels, cells were loaded with Fluo4-AM and the fluorescence was followed during the application of WH. The administration of WH (0.3 mM) did not alter the fluorescence from that of the basal level (Fig. 3B). CaMKII is activated by Ca^{2+} by binding Ca^{2+} and calmodulin and increases Glut4 translocation and glucose uptake [28–30]. In this study, the treatment of L6 myotube cells with WH did not affect CaMKII activation (Fig. 3C and D).

3.5. WH is incorporated into cells via PHT1 and activates AMPK

Transport of WH into cells may be mediated by members of the SLC15 H^+/oligopeptide cotransporter family [31]. To date, four peptide transporters have been cloned in mammals, namely PEPT1, PEPT2, PHT1, and PHT2. However, the tissue distribution and subcellular localization of these transporters are limited and there are no reports on what oligopeptide transporters are expressed in L6 myotubes. Therefore, we first determined which oligopeptide transporters are expressed in L6 myotubes. RT-PCR analysis using specific primers demonstrated that L6 myotubes express only PHT1 (Fig. 4). These results suggest that WH can be incorporated into cells via PHT1, to activate AMPK and increase insulin-independent Glut4 translocation and glucose uptake. To confirm whether PHT1 is required for WH-induced AMPK activation, L6 myotubes with siRNA knockdown of PHT1 were treated with WH, and phosphorylated AMPKα protein levels were determined. To date, there is no credible specific antibody for PHT1. Therefore, the efficiency of siRNA-mediated PHT1 knockdown was evaluated by mRNA level. In this study, the knockdown efficiency of PHT1 siRNA was 80.1 ± 2.9% (Fig. 5A). WH-induced phosphorylation of AMPKα was significantly reduced by PHT1 knockdown (Fig. 5B).

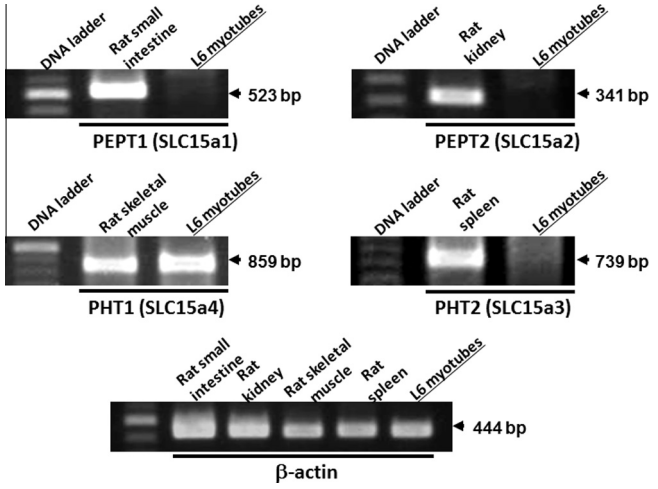

Fig. 4. Detection of PEPT1, PEPT2, PHT1, and PHT2 mRNAs by RT-PCR in various rat tissues and L6 myotube cells. RT-PCR was performed using specific primers for PEPT1 (A), PEPT2 (B), PHT1 (C), and PHT2 (D). The primer sequences are shown in Table 1. Total RNAs from rat small intestine, kidney, skeletal muscle, and spleen were used as positive controls for PEPT1, PEPT2, PHT1, and PHT2, respectively. Specific primers for rat β-actin were used to generate an internal control in each sample.

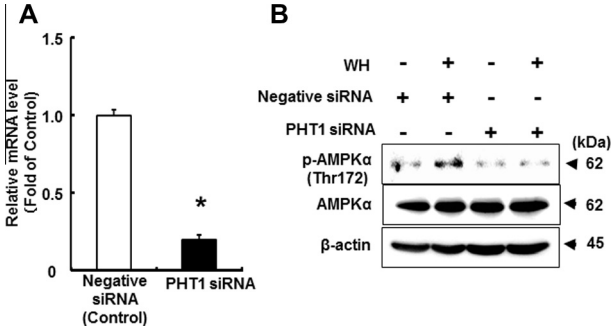

Fig. 5. Level of PHT1 gene expression in L6 myotubes treated with negative control siRNA or PHT1 siRNA (A) and immunoblot analysis of AMPKα and β-actin in L6 myotubes treated with WH (0.3 mM) for 60 min in the presence or absence of PHT1 siRNA (B). The gene expression level was expressed relative to the negative control siRNA (=1.0) after normalization using the 18s rRNA gene expression level. Values are means ± SEM; $n = 3$. *Significantly different from negative control siRNA ($P < 0.05$).

4. Discussion

In our previous study, WH was demonstrated to exhibit potent vasorelaxant and antiproliferative effects in vascular smooth muscle cells [32]. Moreover, WH reduced elevated intracellular Ca^{2+} levels by binding to an extracellular site of the voltage-dependent calcium channel (VDCC) and inhibiting VDCC phosphorylation by blocking CaMKII activity [33]. A recent report showed that nifedipine, a dihydropyridine-type VDCC blocker, activated AMPK in vascular smooth muscle cells via LKB1; this activation did not require calcium-related upstream kinases [34]. In addition, a branched-chain amino acid-containing di-peptide enhanced glucose uptake in skeletal muscle cells [35].

We hypothesized that WH has multiple biological functions, including activation of AMPK and increasing the translocation of Glut4 to the PM with subsequent glucose uptake in skeletal muscle cells. However, to our knowledge there have been no reports of changes in glucose metabolism directly resulting from vasorelaxant di-peptide-induced activation of AMPK. The present study demonstrated that WH has a significant effect on AMPK activation, which at least partly explains its capacity for regulating insulin-independent glucose uptake.

The results of the present study show that activation of AMPKα by WH resulted in elevated glucose uptake via an increase in Glut4 translocation to the PM, both of which were via mechanisms that do not rely on insulin signaling pathways. These findings led us to speculate on the mechanisms by which WH might activate AMPK. There are at least two possible molecular mechanisms involved: (1) WH modulates intracellular energy charge, resulting in phosphorylation of Thr172 in the α-subunit via LKB1, which is one of the upstream AMPK kinases [25], or (2) WH increases the concentration of free intracellular Ca^{2+} with subsequent activation of CaMKK or CaMKII, which in turn phosphorylates AMPKα via an LKB1-independent pathway [24,27,28]. In this study, the administration of WH induced the activation of AMPK in skeletal muscle cells and led to a decrease in cellular ATP levels. The mechanism by which WH produces a drop in cellular ATP levels is currently unknown. However, it is known that metformin, a potent anti-hyperglycemic agent, inhibits mitochondrial respiratory chain complex I, resulting in AMPK activation [36]. Therefore, one plausible mechanism is that WH suppresses ATP production via inhibition of mitochondrial respiratory chain complex I, and then LKB1 phosphorylates AMPKα. Administration of WH affected neither the level of free intracellular Ca^{2+} nor CaMKII activation, indicating that the significant AMPK activation induced by WH was not due to calcium-dependent signaling pathway.

These findings led us to ask how WH can be incorporated into cells and impact AMPK activation and subsequent insulin-independent glucose uptake. Some di-peptides can be incorporated into cells via peptide transporters [37,38]. However, reports on the identities of oligopeptide transporters that are expressed in L6 myotubes have thus far been lacking. Therefore, we first identified which oligopeptide transporters are expressed in L6 myotubes, and found only PHT1. In addition, WH-stimulated phosphorylation of AMPKα was significantly reduced in PHT1-knockdown L6 myotubes. Thus, it is likely that WH is transported into cells via PHT1 to modulate AMPK signaling.

Two open questions are whether WH would be a significant activator of AMPK *in vivo*, and how the WH dose in our experiments relates to bioavailability in *in vivo* systems. Small peptides are not completely hydrolyzed by intestinal peptidases to be absorbed as free amino acids but are transported by peptide transporters in the brush border of the small intestine [39]. Carnosine (β-Ala-His) and anserine (β-Ala-π-methyl-His) are two bioactive di-peptides that are absorbed intact into human plasma [38,40]. In our previous study, long-term oral administration of WH to apolipoprotein E-deficient mice significantly reduced atherosclerotic lesion formation [41]. These results indicate that WH can be directly absorbed and distributed to various tissues via the blood and are therefore expected to modulate whole-body metabolism.

5. Conclusion

WH, a vasorelaxant and anti-atherosclerotic di-peptide, activates AMPK, and insulin independently enhances glucose uptake following translocation of Glut4 to the PM in L6 myotubes. These actions involve the LKB1 pathway, not calcium-dependent signaling pathway. This study also demonstrated that L6 myotubes express only one type of oligopeptide transporter (PHT1), which transports WH into cells, allowing WH to induce activation of AMPK. Our findings provide a new insight into the biochemical basis of the effects of this vasorelaxant di-peptide and also have important implications for the prevention and treatment of type 2 diabetes.

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