Laminarin From *Salicornia herbacea* Stimulates Glucose Uptake Through AMPK-p38 MAPK Pathways in L6 Muscle Cells

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**Abstract**

Laminarin is a component of brown seaweed, especially isolated from *Salicornia herbacea*. Laminarin was known to have various physiological functions, however, the molecular mechanism is still unclear. In this study, we report that laminarin stimulates an activation of AMP-activated protein kinase (AMPK) and increases glucose uptake in rat L6 myotubes. Laminarin also increases an intracellular calcium release. Inhibition of Ca²⁺ release, using with CaMKK inhibitor, STO-609, blocked laminarin-induced AMPK activity, indicating that laminarin stimulated AMPK activity via calcium. In addition, laminarin activates p38 mitogen-activated protein kinase (MAPK) signaling pathways depending on AMPK activity. Moreover, the inhibition of either AMPK or p38 MAPK blocked laminarin-induced glucose uptake in rat L6 myotubes. Taken together, these results demonstrate that the hypoglycemic effect of laminarin is caused by its ability to activate AMPK-p38 MAPK pathways in skeletal muscles.

**Keywords**

AMPK, bioactivity, laminarin, glucose, diabetes, p38 MAPK

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Seaweed has been found to comprise certain bioactive compounds with antibiotic¹² and antitumor³ effects and is used in a variety of functional agents. Particularly, seaweed polysaccharides, including bioactive compounds, contain cellulose and viscous polysaccharides. Seaweed is also composed of about 30% to 60% soluble polysaccharides, including alginates, laminarin, and fucoidan.⁴ Laminarin from *Salicornia herbacea* is a type of β-glucan and is composed of β-glucose linked with (1,3)-β-glucan. β-Glucans are a type of natural polysaccharides contained in the cell walls of cereals, yeast, bacteria, and fungi, with significantly differing physicochemical properties depending on the source. However, the molecular mechanism of laminarin in energy homeostasis remains unclear.

The AMP-activated protein kinase (AMPK) plays a key role to maintain ATP homeostasis.⁸-¹⁰ The activity of AMPK is correlated with an increase in the AMP:ATP ratio in the cells.¹¹ AMPK switches on/off of ATP-generating and ATP-consuming pathways to maintain ATP homeostasis, respectively. AMPK is a key regulator in metabolism, especially in diabetes.¹³-¹⁶ The activation of AMPK requires phosphorylation of the catalytic α subunit on Thr-172.¹⁰,¹⁷,¹⁸ As an AMPK upstream, LKB1 and calcium/calmodulin-dependent protein kinase (CaMKK) are well known as upstream regulators of AMPK.¹⁹-²³ In addition, AMPK regulates glucose uptake in skeletal muscles by physiological and pharmacological stimuli.²⁴-²⁵

The p38 mitogen-activated protein kinase (MAPK) is known to be associated with AMPK.²⁶,²⁷ p38 Mitogen-activated protein kinase involves in glucose uptake-related signals in response to insulin²⁸,²⁹ and skeletal muscle contraction.³⁰,³¹ Also, it has been demonstrated that p38 MAPK has a role as one of the downstream of AMPK.²⁸,³² In this study, we made to verify the metabolic function of laminarin by investigating the effect of laminarin on AMPK activity in muscle.

To determine whether laminarin stimulates metabolic functions in muscles, we examined that laminarin effects on glucose uptake.

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uptake. Glucose uptake was stimulated by laminarin treatment in L6 cells (Figure 1(a)). Insulin was utilized as a positive control. To investigate the molecular mechanisms of laminarin, we evaluated that laminarin effects on AMPK activation, a key molecule in glucose uptake. Laminarin administration induced a time- and dose-dependent increase in AMPK and ACC activity in muscles (Figure 1(b) and (c)). The concentration of laminarin (100 µg/mL) at 10 to 30 minutes increased the activity of AMPK and ACC to the maximum. ACC is known as a direct downstream of AMPK. In addition, to clearly investigate glucose uptake through laminarin-mediated AMPK signaling, we confirmed glucose uptake by laminarin treatment after the inhibition of AMPK activity using compound C, an AMPK inhibitor. The laminarin-induced glucose uptake was decreased by blocking AMPK activity with compound C (Figure 1(d)). These results indicate that laminarin may play some roles in glucose metabolism in L6 myotubes via upregulating an AMPK activity.

The previous studies demonstrated that S. herbacea may stimulate glucose uptake in the skeletal muscle via multiple mechanisms. Especially, laminarin from S. herbacea is known to affect a diet-induced obesity and enhance glucagon-like peptide-1 (GLP-1) level.33 Glucagon-like peptide-1 is one of the secretion hormones from intestinal epithelial L cells. It regulates biological functions, such as food intake,34 glucose level,35 and insulin secretion.36,37 Glucagon-like peptide-1 is secreted by increasing intracellular calcium level ([Ca²⁺]), which is regulated with the activity of PLC signaling or calcium-related receptors.38-40 Subsequently, [Ca²⁺] stimulates several transcription factors, especially cAMP response element binding (CREB) protein,
and consequently releases GLP-1. To further characterize the mechanism underlying laminarin-mediated AMPK activation, we employed the intracellular calcium release. We observed that laminarin began to increase the intracellular calcium release at 15 seconds and decreased slowly in L6 myotubes by laminarin using the Fluo-3 calcium dye (Figure 2(a)). To understand the signal pathway of laminarin-induced intracellular calcium release, we examined intracellular calcium release by laminarin with STO-609, a selective inhibitor of CaMKK. CaMKK is well known as an upstream regulator of AMPK signaling pathway. We demonstrated that laminarin stimulated AMPK activity via calcium (Figure 2(b)). To further study calcium involvement in glucose uptake, L6 muscle cells were differentiated for 7 days and treated with laminarin and STO-609. Laminarin-induced glucose uptake was decreased significantly in the presence of STO-609 (Figure 2(c)). This result indicates that laminarin-mediated glucose uptake and AMPK activation may be associated with intracellular calcium signaling.

To explain the signal pathways involved in laminarin-mediated glucose uptake, we examined the effects of laminarin on p38 MAPK, a key agent for glucose uptake. We figured out that laminarin (100 µg/mL) activated p38 MAPK in a time- and dose-dependent manner in the L6 myotubes (Figure 3(a) and (b)). The phosphorylation level of p38 MAPK reached a highest level at 30 minutes. These results have been demonstrated as crucial activation steps in correlation in AMPK-mediated glucose uptake. To determine whether p38 MAPK is downstream of AMPK, we investigated p38 MAPK activation following the treatment of an AMPK inhibitor, compound C. Laminarin-mediated activation of p38 MAPK was decreased in the compound C pretreated L6 cells (Figure 3(c)). To validate the roles of p38 MAPK in the laminarin-mediated signaling pathway, we evaluated the pretreatment effects of inhibitors of the p38 MAPK on glucose uptake. The effect of laminarin on glucose uptake was attenuated in L6 cells in the presence of SB203580, p38 MAPK inhibitor (Figure 3(d)). Together, these results imply that p38 MAPK plays as a downstream of AMPK in the laminarin-mediated signaling pathway.

Finally, to establish physiological relevance, we corroborated Glut4 translocation to the plasma membrane using myc-tagged L6 cells. Laminarin stimulated Glut4 translocation to the plasma membrane from the cytosol in L6 cells (Figure 4(a)). Insulin was employed as a positive control for Glut4 translocation. Furthermore, we measured a Glut4 translocation from cytosol to the plasma membrane using myc-tagged-Glut4 expressed L6 cells. Using the p38 MAPK inhibitors, SB203580, we confirmed that laminarin-mediated translocation of Glut4 was inhibited when the p38 MAPK signal was blocked (Figure 4(b)). Taken together, our findings suggest that laminarin stimulates glucose uptake via Glut4 translocation to the plasma membrane in skeletal muscle L6 myotube cells.
Laminarin, glucose, insulin, and STO-609 (CaMKK inhibitor) were purchased from the Sigma Chemical Company (St. Louis, MO, United States). 2-[^H]-Deoxy-d-glucose (2-DG) was obtained from NEN Life Science Products (Boston, MA, United States). Polyclonal anti-phospho-AMPKα (Thr172)/AMPKα, anti-phospho-p38 MAPK, and p38 MAPK antibodies were purchased from Cell Signaling (Cell Signaling Technology, New England Biolabs, Beverly, MA, United States). Monoclonal β-actin antibody was purchased from Upstate Biotechnology (Lake Placid, NY, United States). C-myc and phospho-ACC antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Compound C, an AMPK inhibitor, and SB203580, a p38 MAPK inhibitor, were provided by Calbiochem (RY 70-100 Rahway, NJ, United States). The fluorescent Ca$^{2+}$ indicator Fluo-3, AM was purchased from Invitrogen (Leiden, Netherlands).

**Experimental**

**Reagents**

Laminarin, glucose, insulin, and STO-609 (CaMKK inhibitor) were purchased from the Sigma Chemical Company (St. Louis, MO, United States). 2-[^H]-Deoxy-d-glucose (2-DG) was obtained from NEN Life Science Products (Boston, MA, United States). Polyclonal anti-phospho-AMPKα (Thr172)/AMPKα, anti-phospho-p38 MAPK, and p38 MAPK antibodies were purchased from Cell Signaling (Cell Signaling Technology, New England Biolabs, Beverly, MA, United States). Monoclonal β-actin antibody was purchased from Upstate Biotechnology (Lake Placid, NY, United States). C-myc and phospho-ACC antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Compound C, an AMPK inhibitor, and SB203580, a p38 MAPK inhibitor, were provided by Calbiochem (RY 70-100 Rahway, NJ, United States). The fluorescent Ca$^{2+}$ indicator Fluo-3, AM was purchased from Invitrogen (Leiden, Netherlands).

**Cell Culture**

Rat L6 myoblast cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Auckland, NZ; containing 0.584 g/L of l-glutamate and 4.5 g/L of glucose, mixed with 500 mL of F-12 medium containing 0.146 g/L of l-glutamate, 1.8 g/L of glucose, 100 µg/mL of gentamicin, and 2.5 g/L of sodium carbonate) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% antibiotics (penicillin/streptomycin) at 37°C in 100 mm cell culture dishes (Corning, NY, United States) under a humidified air atmosphere containing 5% CO$_2$. L6 cells were passaged by using a 0.25% trypsin, 0.02% EDTA solution every 2 to 3 days. L6 myoblast and over-expressing myc tagged Glu4 L6 cells were differentiated for 7
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days by incubating and changing with a fresh DMEM containing 2% FBS and 1% penicillin/streptomycin every 2 days.

**Western Blot Analysis**

L6 myoblast cells were grown in 6-well plates. The cells were subjected to 6 hours of serum starvation prior to all experiments at 37°C. Next, the cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in 100 µL of lysis buffer (0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 150 mM NaCl, and 50 mM Tris-HCl, pH 8) containing proteinase inhibitors (0.5 µM aprotinin, 1 µM phenyl-methyl-sulfonyl-fluoride, and 1 µM leupeptin) (Sigma). The supernatants were sonicated briefly, heated for 5 minutes at 95°C, centrifuged for 5 minutes, separated on SDS-PAGE (8% to 16%) gels, and finally transferred to polyvinylidene difluoride membranes. The blots were then incubated overnight at 4°C with primary antibodies and washed 3 times in Tris-buffered saline/0.1% Tween 20 prior to 1 hour of probing with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature. The blots were then visualized with ECL (Amersham Biosciences, Buckinghamshire, United Kingdom).

**Intracellular Ca^{2+} Measurement Using Fluo-3, AM**

Ca^{2+} concentration was determined using confocal microscopy (Zeiss LSM 510 Meta; Zeiss, Oberkochen, Germany). The cells were loaded with 5 mM Fluo-3, AM for 30 minutes. The culture plates were then placed on a microscope stage and observed with 200× microscope magnification. The excitation and emission wavelengths for signal detection were 488 and 515 nm, respectively.

**Immunocytochemistry**

Briefly, myc-Grut4-expressing L6 myoblasts were seeded onto collagen-coated glass cover slips. After 24 hours, the cells were serum-starved for 3 hours and stimulated with either 100 nM insulin or 100 µg/mL laminarin for 30 minutes at 37°C. The cells were then incubated with 4% paraformaldehyde in PBS for 20 minutes at 4°C. The cells were then incubated for 3 hours with primary c-myc antibody at a dilution of 1:200 in buffer (3% BSA in PBS) at 4°C, followed by incubation with Cy3-conjugated secondary antibody at a dilution of 1:1000 for 1 hour at room temperature. The cells were observed using a Zeiss LSM 700 confocal fluorescence microscope with 400× microscope magnification.

**2-Deoxyglucose (DG) Uptake Assay**

Myotube L6 cells were grown in DMEM containing 2 % v/v FBS until fully differentiated. The uptake of 2-deoxyglucose (DG) was examined. 100 nM insulin was used as a positive control. In brief, the cells were washed twice in PBS containing 2.5 mM MgCl_{2}, 1 mM CaCl_{2}, and 20 mM HEPES at pH 7.4, followed by incubation in test compounds in the same buffer at 37°C. The transport assay was then initiated via addition of 2-[^{3}H]-deoxy-d-glucose (25 mM; 10 µCi/mL) to each well for 15 minutes at 37°C. The assay was terminated via addition and subsequent washing of the cells with ice-cold PBS. The cells were lysed in 10% SDS or 50 mM NaOH. Radioactivity was evaluated via the scintillation counting of SDS-extracted lysates, whereas total protein contents were determined in NaOH-extracted lysates, via the Bradford procedure (Bio-Rad Laboratory, Richmond, CA, United States).
Immunodetection of Myc-Glu4

Myotube L6 overexpressing myc tagged Glut4 cells were grown in DMEM containing 2% v/v FBS for 6 to 7 days. The cell surface expression of myc-Glu4 was quantified using an antibody-coupled colorimetric absorbance assay. After stimulation with laminarin and the p38 MAPK inhibitor, L6 myoblasts stably expressing myc-Glu4 were incubated with anti-myc antibody (1:1000) for 1 hour, and then incubated with HRP-conjugated anti-mouse IgG (1:1000) for 1 hour. Cells were washed with PBS and incubated in 200 µL of OPD reagent (0.4 mg/mL) (Sigma-Aldrich, St. Louis, MO, United States) for 30 minutes at room temperature in the dark. The absorbance of the supernatant was measured at 450 nm.

Data Analysis

We used one-way ANOVA and Holm-Sidak comparisons followed by the post hoc Fisher's test and analyzed band intensity. The mean values were considered statistically significant when the probability of the event was determined to be below 5% (*P < 0.05).

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