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Capsaicin stimulates glucose uptake in C2C12 muscle cells via the reactive oxygen species (ROS)/AMPK/p38 MAPK pathway

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ABSTRACT

Capsaicin has been reported to regulate blood glucose levels and to ameliorate insulin resistance in obese mice. This study demonstrates that capsaicin increases glucose uptake directly by activating AMP-activated protein kinase (AMPK) in C2C12 muscle cells, which manifested as an attenuation of glucose uptake when compound C, an AMPK inhibitor, was co-administered with capsaicin. However, the insulin signaling molecules insulin receptor substrate-1 (IRS-1) and Akt were not affected by capsaicin. Additional results showed that p38 mitogen-activated protein kinase (MAPK) is also involved in capsaicin-induced glucose transport downstream of AMPK because capsaicin increased p38 MAPK phosphorylation significantly and its specific inhibitor SB203580 inhibited capsaicin-mediated glucose uptake. Treatment with an AMPK inhibitor reduced p38 MAPK phosphorylation, but the p38 MAPK inhibitor had no effect on AMPK. Capsaicin stimulated ROS generation in C2C12 muscle cells, and when ROS were captured using the nonspecific antioxidant NAC, the increase in both capsaicin-induced AMPK phosphorylation and capsaicin-induced glucose uptake was attenuated, suggesting that ROS function as an upstream activator of AMPK. Taken together, these results suggest that capsaicin, independent of insulin, increases glucose uptake via ROS generation and consequent AMPK and p38 MAPK activations.

1. Introduction

Blood glucose levels are tightly regulated to within a narrow range, and defects in glucose regulation lead to glucose intolerance and diabetes. The most important factor in maintaining blood glucose levels is glucose transport [1]. Approximately 75% of postprandial blood glucose clearance takes place in skeletal muscle, and two distinct mechanisms are responsible for glucose transport: the insulin signaling pathway and AMP-activated protein kinase (AMPK) activation. In type II diabetes, which is the most prevalent form of diabetes, glucose absorption is reduced even in the presence of insulin due to insulin resistance [2]. However, AMPK stimulates glucose uptake in an insulin-independent manner, and therefore, it may be an important alternative target for treatment of type II diabetes [3]. Metformin, a type II diabetic drug, regulates blood glucose level by activating AMPK [4]. Natural compounds including berberine, resveratrol, naringenin, α-lipoic acid, ginsenoside Rc, and tangeretin are also known to promote glucose uptake via AMPK [1,5–9].

Capsaicin (8-methyl-*N*-vanilyl-6-nonenamide) is a pungent major component of hot chili peppers. Capsaicin has been used as a medication to relieve arthritis pain, and other pharmacological properties including anti-inflammatory and anti-cancer effects have also been reported. [10–12] With regard to metabolic disorders, the anti-obesity effect of capsaicin is well known; capsaicin reduces body weight and fat mass via β-adrenergic action-mediated increase in energy metabolism and thermogenesis [13,14]. Recently, new results have been reported that capsaicin improves insulin resistance and hepatic steatosis in obese mice [13,15]. With respect to the molecular mechanisms underlying these functions, capsaicin decreased the inflammatory response by reducing tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), and interleukin-6 (IL-6), and it facilitated the expression of uncoupling proteins (UCPs) that were associated with fat oxidation via peroxisome proliferator-activated receptor alpha (PPARα) [13,16]. However, decreased inflammation and increased fatty acid oxidation indirectly affect the blood glucose regulation, and therefore more direct and detailed mechanism is needed.

In this study, we demonstrate that capsaicin directly increases glucose uptake in C2C12 muscle cells by activating AMPK, and that reactive oxygen species (ROS) and p38 mitogen-activated protein kinase (MAPK) are involved in the capsaicin-mediated glucose uptake.

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2. Materials and methods

2.1. Reagents

[^{3}H] 2-Deoxyglucose (2-DG) was obtained from Amersham Bioscience (Piscataway, NJ). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Welgene (Daegu, Korea). Anti-phospho-acetyl CoA carboxylase (ACC) (Ser79), ACC, phospho-AMPK (Thr172), phospho-p38 mitogen-activated protein kinase (MAPK; Thr180/Tyr182), p38 MAPK, and phospho-Akt (Ser473) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-insulin receptor substrate-1 (IRS-1; Tyr989) and actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 5-Aminoimidazole-4-carboximide ribonucleoside (AICAR) and compound C were obtained from Calbiochem (San Diego, CA). Capsaicin, insulin, N-acetylcysteine (NAC), SB203580, and dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma (St. Louis, MO). All reagents were dissolved in DMSO except insulin. DMSO < 0.1% was used as a vehicle control.

2.2. Cell culture

C2C12 myocytes, obtained from American Type Culture Collection (ATCC, Manassas, VA), were maintained in DMEM containing 10% FBS in an atmosphere of 5% CO_2 at 37 °C. For differentiation into myotubes, confluent cells were cultured in DMEM supplemented with 2% horse serum for 4–5 days.

2.3. Glucose uptake assay

Glucose uptake assays were performed as described previously, with modifications [5]. Briefly, differentiated C2C12 myotubes were washed and serum-starved with low glucose DMEM (1000 mg/L) for 3 h. Cells were preincubated with each inhibitor for 20 min, followed by treatment with each stimulant (capsaicin or insulin). After 1 h, 1.0 μCi/ml of [^{3}H] 2-DG was administered for a further 30 min. Cells were washed twice using ice-cold PBS and solubilized in 0.1% SDS. Cell-associated radioactivity was measured using a scintillation counter (Bio-Rad, Melville, NY). Glucose uptake was normalized to total protein.

2.4. Western blotting

Following 12% SDS–polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membranes (GE Biosciences, Piscataway, NJ). The membranes were blocked using Tris-buffered saline containing 5% skim milk and 0.1% Tween-20 for 1 h at room temperature (RT). After blocking, the membranes were incubated with appropriate primary antibodies overnight at 4 °C. After three washes, the membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at RT. The immune complexes were visualized using an ECL detection kit (GE Biosciences).

2.5. ROS measurement

Intracellular ROS measurements were performed using the modified method described by Ka et al. [17]. After pretreatment for 30 min with the antioxidant NAC (5 mM), capsaicin or H_2O_2 was added to the cells for 2 h. The cells were then stained with 20 μM DCF-DA for 30 min at 37 °C. The cells were washed twice with ice-cold PBS, and their fluorescence intensity was then examined using a fluorescence microscope (Olympus Optical, Japan) and measured using a microplate fluorometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA; excitation at 485 nm and emission at 530 nm).

2.6. Statistical analyses

All experiments were independently repeated three times. All numeric values are represented as the mean ± S.D. Statistical analyses were performed using IBM SPSS Statistics 20 (SPSS Inc. Chicago, IL). The statistical significance of differences between data was determined using Student's *t*-tests or one-way analyses of variance (ANOVA) followed by Tukey's tests. The accepted level of significance was $P < 0.05$.

3. Results

3.1. Capsaicin increases glucose uptake by activating AMPK

Using a muscle cell line, we first evaluated the direct effect of capsaicin on glucose absorption. Capsaicin increased glucose uptake significantly in C2C12 muscle cells. In particular, the level of glucose uptake at 100 μM capsaicin was 82.0 ± 8.5% of that at 1 μM insulin (Fig. 1). Following capsaicin treatment, phosphorylation of AMPK and its downstream mediator ACC was increased in a dose-dependent manner, and these phosphorylations levels were maintained up to 6 h (Fig. 2A–B). To verify that the capsaicin-induced increase in glucose uptake was due to the activation of AMPK, C2C12 muscle cells were co-treated with 10 μM compound C, which is a specific inhibitor of AMPK. Capsaicin-induced glucose uptake was reduced in the presence of compound C (Fig. 2C). AMPK and ACC phosphorylation was also decreased following treatment with compound C (Fig. 2D). In addition to AMPK, the insulin signaling pathway facilitates the glucose uptake. Therefore, we examined whether capsaicin affects insulin signaling molecules. At doses of up to 200 μM, capsaicin did not cause any change in IRS-1 or Akt phosphorylation either when administered alone or in combination with 1 μM insulin (Fig. 2E). Taken together, these results suggest that capsaicin facilitates glucose uptake by activating AMPK, and does not involve the insulin signaling pathway.

3.2. p38 MAPK is involved in capsaicin-induced glucose uptake

p38 MAPK is known to be activated by insulin and AMPK and for its facilitation of glucose uptake by regulating the activity or

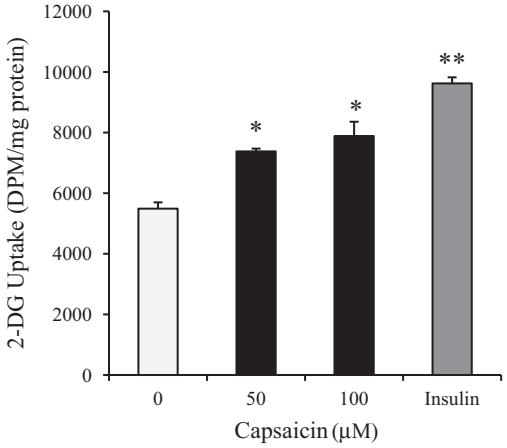

Fig. 1. Capsaicin increases glucose uptake in C2C12 muscle cells. After 2 h of serum starvation, differentiated C2C12 muscle cells were treated with capsaicin or 1 mM insulin for 1 h and then administered with 1.0 μCi/ml [^{3}H]-2-DG for 30 min. The radioactivity was measured using a scintillation counter, and glucose uptake was normalized to total protein. *, $p < 0.05$; **, $p < 0.01$ compared with control. 2-DG, 2-deoxyglucose.

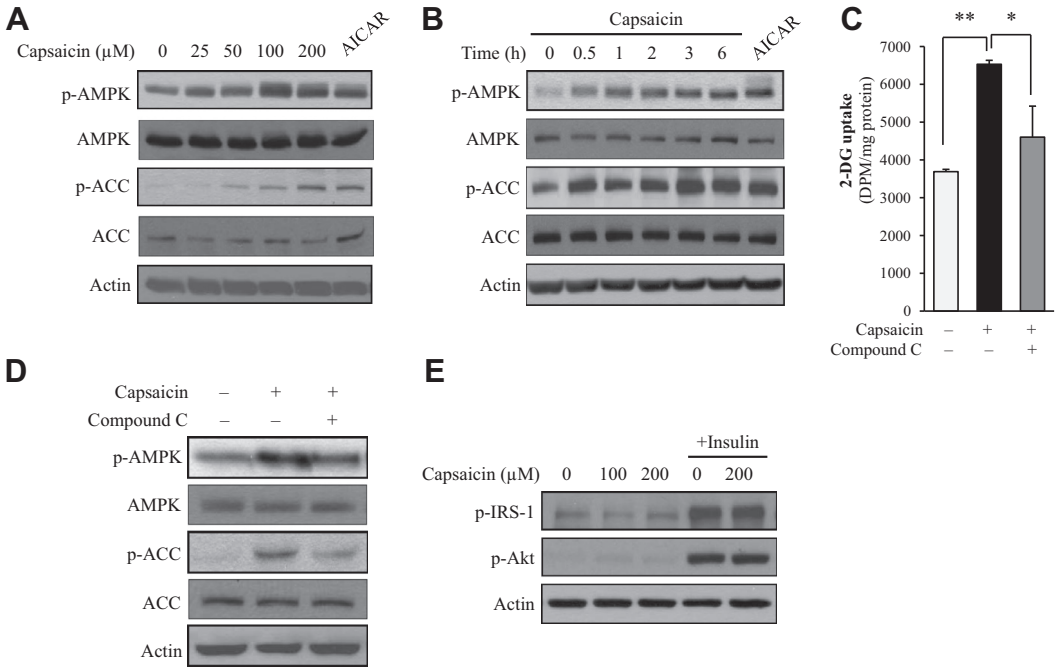

Fig. 2. Capsaicin increases AMPK phosphorylation significantly but has no effect on insulin signaling molecules. (A) Differentiated C2C12 cells were treated with varying doses of capsaicin for 30 min. (B) Capsaicin (100 μM) was administered to cells for the times indicated. AICAR, an AMPK activator, (0.5 mM) was used as a positive control. The cell lysates were analyzed using Western blotting with anti-phospho-AMPK (Thr172) and anti-phospho-ACC (Ser79) antibodies. Protein detected with anti-actin was used as the loading control. (C) After 20 min pretreatment with compound C, which is an AMPK inhibitor, the C2C12 muscle cells were exposed to capsaicin for 1 h and then administered 1.0 μCi/ml [^{3}H]-2-DG for 30 min. The radioactivity was measured using a scintillation counter, and glucose uptake was normalized to total protein. *, $p < 0.05$; **, $p < 0.01$. (D) After 20 min pretreatment with 20 μM compound C, 100 μM capsaicin was added to the cells for 30 min. (E) Capsaicin was added to the cells in the absence or presence of 1 μM insulin for 30 min. The cell lysates were analyzed using Western blotting with anti-phospho-IRS-1 (Tyr989) and anti-phospho-Akt (Ser476) antibodies.

translocation of glucose transporter type 4 (GLUT4) [18]. Thus, we investigated whether p38 MAPK is also involved in capsaicin-induced glucose uptake. Capsaicin increased p38 MAPK phosphorylation in a dose-dependent manner (Fig. 3A). In addition, the capsaicin-stimulated glucose uptake was inhibited by the presence of 10 μM SB203580, which is a specific inhibitor of p38 MAPK (Fig. 3B). Moreover, whereas the AMPK inhibitor compound C reduced capsaicin-induced p38 MAPK phosphorylation, SB203580 did not affect AMPK or ACC phosphorylation, as shown in Fig. 3C. These results suggest that p38 MAPK mediates capsaicin-induced glucose uptake downstream of AMPK.

3.3. ROS generated by capsaicin function as an upstream signaling mediator of AMPK

Within cells ROS facilitates glucose uptake. Exercise, some medications, and phytochemicals are known to regulate glucose metabolism, such as gluconeogenesis and glucose uptake, via the generation of ROS [19–21]. Thus, we hypothesized that capsaicin activated AMPK by generating ROS. As expected, capsaicin significantly increased the generation of ROS in C2C12 muscle cells. Following treatment with 200 μM capsaicin, ROS levels were 1.44-fold ±0.11 higher when compared with vehicle control (Fig. 4A–B). Moreover, co-treatment of capsaicin with the nonspecific antioxidant NAC (5 mM) abolished both the increase in glucose uptake and AMPK phosphorylation induced by capsaicin (Fig. 4C–D). In particular, the reduction in AMPK phosphorylation following treatment with NAC suggests that ROS play a critical role as upstream regulators of AMPK phosphorylation. Taken together, these results strongly suggest that capsaicin stimulates glucose uptake by generating ROS and consequently activating AMPK.

4. Discussion

Activation of AMPK has been proposed as the mechanism by which several natural compounds increase glucose uptake [1,8,9,22]. As an example, berberine, a plant-derived isoquinoline alkaloid, enhances glucose uptake in insulin-resistant muscle cells by activating AMPK and PKCζ thereby overcoming insulin resistance [22]. In this study, we demonstrate that capsaicin also increases glucose uptake in C2C12 muscle cells by activating AMPK. In contrast, capsaicin had no effect on insulin signaling molecules, such as IRS-1 and Akt. AMPK and insulin signaling pathways are distinct pathways; however, these two pathways meet at p38 MAPK. p38 MAPK regulates the activity and translocation of GLUT4 and, as a consequence, facilitates glucose absorption [23,24]. In this study, we observed that p38 MAPK is involved in capsaicin-induced glucose transport signaling downstream of AMPK, which was demonstrated by the phosphorylation of AMPK and p38 MAPK and glucose uptake in the absence or presence of SB203580 and compound C.

Previous studies have suggested that capsaicin may regulate blood glucose levels by activating AMPK. Kang et al. reported that insulin resistance improved when high-fat diet-induced obese mice were fed 0.015% capsaicin for 10 weeks [13]. Plasma glucose and insulin levels decreased when genetically obese diabetic KKAy mice were fed the same concentration of capsaicin for 3 weeks [15]. Several molecular mechanisms have been proposed to underlie this hypoglycemic effect of capsaicin; capsaicin improves insulin sensitivity by reducing the inflammatory cytokines levels such as TNF-α, IL-6, and MCP-1, and facilitates fatty acid oxidation by stimulating adiponectin expression [13,15]. Moreover, the AMPK pathway has been suggested as additional mechanism because AMPK phosphorylation was increased in the liver of a capsaicin-fed group [15]. Furthermore, Joo et al. proposed AMPK as a key

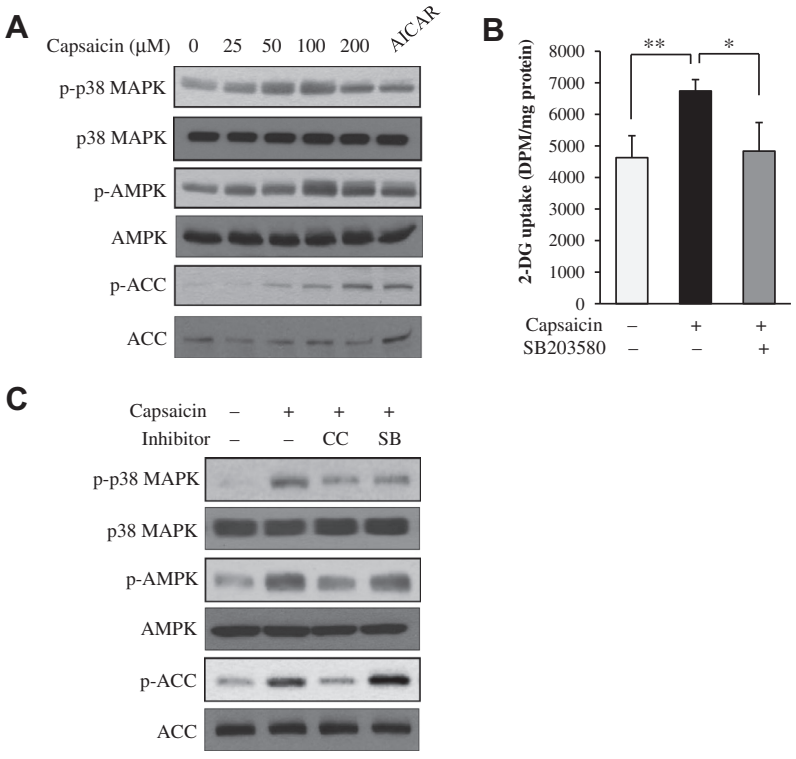

Fig. 3. p38 MAPK is involved in capsaicin-induced glucose uptake. (A) Differentiated C2C12 muscle cells were treated with capsaicin or 0.5 mM AICAR for 30 min, and cell lysates were analyzed using Western blotting. (B) The cells were pretreated with 10 μM SB203580, which is a specific inhibitor of p38 MAPK, for 20 min and then treated with 100 μM capsaicin for 1 h. *, $p < 0.05$; **, $p < 0.01$. (C) After 20 min pretreatment with each inhibitor, 100 μM capsaicin was added to the cells for 30 min. CC, compound C, 20 μM; SB, SB203580, 10 μM.

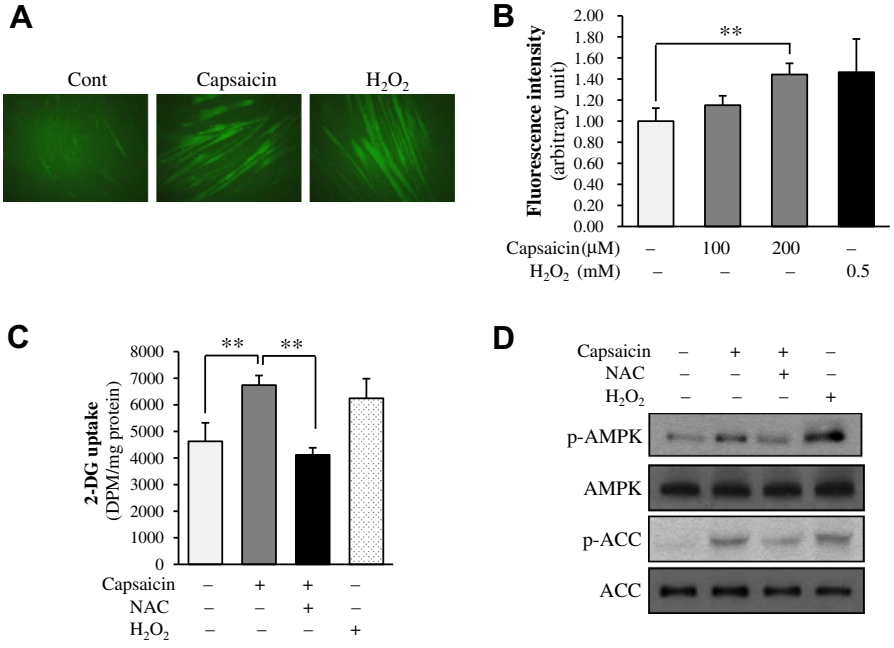

Fig. 4. Capsaicin generates ROS, which cause AMPK phosphorylation and glucose uptake. (A–B) After treatment with 100 μM capsaicin or 0.5 mM H_2O_2 (positive control) for 30 min, intracellular ROS were stained using DCF-DA. The fluorescence intensity was measured using a microplate fluorometer and normalized to total protein. (C–D) Cells were pretreated with 5 mM NAC for 20 min prior to incubation with 100 μM capsaicin or 0.5 mM H_2O_2 for 1 h (C) or for 30 min (D). **, $p < 0.01$.

molecule responsible for the reduction in body weight in rats fed a high-fat diet that were administered 10 mg/kg/day capsaicin for 9 weeks because AMPK phosphorylation and UCPs expression were increased in epididymal adipose tissue of the capsaicin-fed group compared with those in pair-fed group [16]. These previous studies presented the possibility that capsaicin may regulate blood glucose levels by activating AMPK. In this study, we demonstrate for the first time that capsaicin directly stimulates glucose uptake in muscle cells and present its mechanism, which involves AMPK activation.

AMPK is known to be activated by metabolic stress conditions such as energy deprivation, hypoxia, and oxidative stress [25,26].

These stress conditions increase AMP levels and lead to AMPK activation. Moreover, LKB1 or calcium/calmodulin-dependent protein kinase kinase (CaMKK) regulates AMPK activity as upstream kinases [25,27]. Although we verified the activation of AMPK by capsaicin, the upstream mechanism by which capsaicin activates AMPK has not yet been elucidated. We hypothesize that one possible mechanism may be that capsaicin increases ROS generation directly. As expected, capsaicin significantly stimulated ROS production in C2C12 muscle cells. We also confirmed that these ROS play a critical role in capsaicin-induced AMPK activation and glucose uptake by being as an upstream signal of AMPK because these capsaicin-induced effects were abolished when ROS were captured by the nonspecific antioxidant NAC (Fig. 4). ROS-mediated glucose uptake is known as the principal mechanism underlying blood glucose regulation in skeletal muscle and adipose tissue during exercise. One report indicated that ROS generated by muscle contraction increased Akt phosphorylation, but most studies have reported that ROS stimulate glucose uptake via AMPK activation [19,21,28]. ROS have been proposed to activate AMPK by activating CaMKK, which is an upstream kinase of AMPK, and AMPK is sequentially activated by CaMKK [25,27,29]. Because the activation of oxidant-mediated AMPK occurred in an AMP-independent manner [30], capsaicin is also expected to deliver the signal via the ROS-CaMKK-AMPK pathway, independently of AMP:ATP ratio. Glucose uptake via the ROS-AMPK pathway underlies glucose transport in low-blood-glucose states and exercise [31]. In addition, functional phytochemicals, epigallocatechin gallate (EGCG) and ginsenoside Rc, have been reported to facilitate glucose uptake by generating ROS and subsequently activating AMPK [8,20].

In conclusion, this study identifies for the first time the molecular mechanism by which capsaicin increases glucose uptake. Capsaicin treatment generates ROS and subsequently activates AMPK and p38 MAPK independent of insulin. Moreover, this study reestablishes the blood-glucose-regulating function of capsaicin, which is one of the heavily consumed spices worldwide. In addition to the direct uptake of glucose via activation of AMPK, capsaicin is known to increase insulin sensitivity by reducing inflammation and enhancing fatty acid oxidation [13,16]. Given these multiple-functions, capsaicin may be used as a valuable food supplement for regulating blood glucose.

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