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Coffee polyphenol caffeic acid but not chlorogenic acid increases 5′AMP-activated protein kinase and insulin-independent glucose transport in rat skeletal muscle☆,☆☆

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Abstract

Chlorogenic acid is an ester of caffeic and quinic acids, and is one of the most widely consumed polyphenols because it is abundant in foods, especially coffee. We explored whether chlorogenic acid and its metabolite, caffeic acid, act directly on skeletal muscle to stimulate 5′-adenosine monophosphate-activated protein kinase (AMPK). Incubation of rat epitrochlearis muscles with Krebs buffer containing caffeic acid (≥0.1 mM, ≥30 min) but not chlorogenic acid increased the phosphorylation of AMPKα Thr^{172}, an essential step for kinase activation, and acetyl CoA carboxylase Ser^{79}, a downstream target of AMPK, in a dose- and time-dependent manner. Analysis of isoform-specific AMPK activity revealed that AMPKα2 activity increased significantly, whereas AMPKα1 activity did not change. This enzyme activation was associated with a reduction in phosphocreatine content and an increased rate of 3-*O*-methyl-D-glucose transport activity in the absence of insulin. These results suggest that caffeic acid but not chlorogenic acid acutely stimulates skeletal muscle AMPK activity and insulin-independent glucose transport with a reduction of the intracellular energy status.
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1. Introduction

Skeletal muscle plays an important role in glucose metabolism and homeostasis in humans. Skeletal muscle relies on glucose for much of its energy requirements, and as such, it is the major site of glucose uptake in the body — about 75% of plasma glucose is cleared by the skeletal muscles [1]. In subjects with type 2 diabetes mellitus (T2DM), insulin-stimulated glucose uptake is reduced by about 50% [1]. This study supports the notion that the primary defect in insulin action in patients with T2DM resides in the skeletal muscle. Like insulin stimulation, exercise acutely increases the rate of glucose transport into contracting skeletal muscle by the translocation of glucose transporter 4 (GLUT4) to the plasma membrane and transverse tubules (reviewed in Refs. [2,3]). This phenomenon is considered to be responsible for the acute hypoglycemic effect of exercise, with glucose in the blood being taken up by contracting skeletal muscles. Indeed, exercise-stimulated GLUT4 translocation is not impaired in insulin-resistant conditions such as T2DM and obesity [4]. Thus, the insulin-independent mechanisms of exercise have been widely used to decrease blood glucose in patients with T2DM.

It has been demonstrated that exercise and insulin use distinct signaling pathways in skeletal muscle, and 5′-adenosine monophosphate-activated protein kinase (AMPK) has been identified to be involved in the mechanisms leading to exercise-stimulated glucose transport (reviewed in Ref. [5]). AMPK is a heterotrimeric kinase, consisting of a catalytic α-subunit and two regulatory subunits, β and γ. Two distinct α-isoforms (α1 and α2) exist in skeletal muscle [6], and both isoforms can be activated in response to muscle contraction, which stimulates glucose transport in the absence of insulin [7,8]. AMPK is a member of a metabolite-sensing protein kinase family and acts as an energy-sensing and signaling molecule in muscle cells by monitoring cellular energy levels, such as the AMP–adenosine triphosphate (ATP) ratio. AMPK in skeletal muscle is also implicated in a variety of antidiabetic properties of exercise, including GLUT4 expression [9,10], glycogen regulation [11,12], fatty acid oxidation [13,14], activation of peroxisome proliferator-activated receptor γ coactivator 1α and mitochondrial biogenesis [15], activation of SIRT1 [16] and enhanced insulin sensitivity [10,17]. In addition, skeletal muscle AMPK partially mediates glucose and lipid homeostasis by adipokines, including leptin and adiponectin, and the hypoglycemic effect of metformin (reviewed in Ref. [18]). Thus, through these effects in skeletal muscle, AMPK serves as a metabolic activator that reduces risk for T2DM.

Coffee is one of the most commonly consumed beverages in the world. Many epidemiological studies have indicated that long-term

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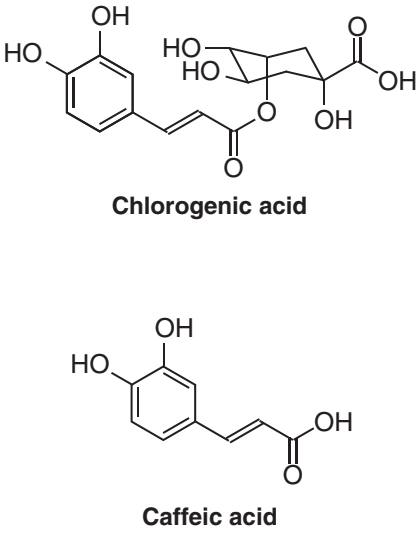

Fig. 1. Structures of chlorogenic acid and caffeic acid.

coffee consumption is associated with a reduced risk of developing T2DM [19,20]. Our recent studies [21,22] reported that caffeine, the major constituent of coffee, acutely increases AMPKα Thr^{172} phosphorylation, an essential step for kinase activation [23] and insulin-independent glucose transport in rat skeletal muscles. Although our results suggest that caffeine is an active compound responsible for the antidiabetic effect of coffee, coffee is a complex mixture of hundreds of chemicals that occur naturally or are formed during the roasting process. In fact, a reduced risk of developing T2DM has been associated with the consumption of decaffeinated coffee [24]. Thus, it is reasonable to speculate that coffee components other than caffeine have beneficial effects on glucose homeostasis in humans.

Phenolic compounds of plant origin, particularly chlorogenic acid (5-*O*-caffeoylquinic acid) and caffeic acid (3,4-dihydroxycinnamic acid) (Fig. 1), have been investigated for their antihyperglycemic properties [25–29]. Coffee is a major dietary source of chlorogenic acid, which reduces blood glucose concentrations in animal models [25–27]. Chlorogenic acid is an ester of caffeic and quinic acids, and caffeic acid has also been reported to decrease blood glucose in animals [28,29].

We have hypothesized that, like caffeine stimulation [21,22], chlorogenic acid and its major metabolite caffeic acid have antidiabetic properties by acting directly on skeletal muscle to stimulate AMPK. To test this hypothesis, we evaluated the effects of chlorogenic acid and caffeic acid on AMPKα Thr^{172} phosphorylation and α1- and α2-isoform-specific AMPK activities in isolated rat skeletal muscles incubated *in vitro*. We found that caffeic acid but not chlorogenic acid increased AMPK phosphorylation and its activity in a dose- and time-dependent manner with a corresponding decrease in muscle energy status and increase in glucose transport activity.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 100 to 120 g were obtained from Shimizu Breeding Laboratories (Kyoto, Japan). Animals were housed in an animal room maintained at 22°C–24°C with a 12:12-h light–dark cycle and fed a standard laboratory diet (Certified Diet MF; Oriental Koubo, Tokyo, Japan) and water *ad libitum*. Rats were fasted overnight before the experiments and were randomly assigned to the experimental groups.

All protocols for animal use and euthanasia followed the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences (Physiological Society of Japan) in accordance with international guidelines, and were reviewed and approved by the Kyoto University Graduate School of Human and Environmental Studies and Kyoto University Radioisotope Research Center.

2.2. Muscle incubation

Muscles were treated as we described previously [7,30]. Rats were killed by cervical dislocation without anesthesia, and the epitrochlearis muscles were rapidly and gently removed. Both ends of each muscle were tied with sutures (silk 3-0; Nitcho Kogyo, Tokyo, Japan), and the muscles were mounted on an incubation apparatus with a tension set to 0.5 g. The buffers were continuously gassed with 95% O_2–5% CO_2 and maintained at 37°C. Muscles were preincubated in 7 ml of Krebs-Ringer bicarbonate buffer (KRB) (117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24.6 mM $NaHCO_3$) containing 2 mM pyruvate (KRBP) for 40 min.

For the time- and dose-dependent effects, muscles were then randomly assigned to incubation in 7 ml of fresh buffer in the presence of 1 mM chlorogenic acid or 1 mM caffeic acid for up to 60 min, or in 7 ml of fresh buffer in the absence or presence of 0.01 to 1 mM chlorogenic acid or caffeic acid for 30 min, respectively. Immediately after incubation, muscles were either used for the measurement of glucose transport or immediately frozen in liquid nitrogen and subsequently subjected to Western blot analysis. Some frozen muscles were also assayed for ATP and phosphocreatine (PCr) and isoform-specific AMPK activity.

2.3. Western blot analysis

Sample preparation and Western blot analysis for detection of phosphorylated AMPKα, total AMPKα and phosphorylated acetyl CoA carboxylase (ACC), total ACC, phosphorylated Akt and total Akt were performed as described previously [30,31]. Muscles were homogenized in ice-cold lysis buffer (1:40 wt/vol) containing 20 mM Tris-HCl (pH 7.4), 1% Triton X, 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 2 mM dithiothreitol, 4 mg/L leupeptin, 50 mg/L trypsin inhibitor, 0.1 mM benzamidine and 0.5 mM phenylmethylsulfonyl fluoride (buffer A) and centrifuged at 16,000*g* for 40 min at 4°C. Denatured lysates (10 μg of protein) were separated on either 10% polyacrylamide gel for AMPK and Akt or 7.5% gel for ACC. Proteins were then transferred to polyvinylidene difluoride membranes (PolyScreen; PerkinElmer, Wellesley, MA, USA) at 100 V for 1 h. Membranes were blocked for 1 h at room temperature in TBS-T (TBS with 0.1% Tween 20) containing 5% nonfat dry milk and were then incubated overnight at 4°C with appropriate antibody [phosphospecific AMPKα Thr^{172} (#2531; Cell Signaling Technology, Beverly, MA, USA) diluted 1:1000, AMPKα (#2532; Cell Signaling Technology) diluted 1:1000, phosphospecific ACC Ser^{79} (#07-303; Upstate Biotechnology, Lake Placid, NY, USA) diluted 1:1000, ACC (#3662; Cell Signaling Technology) diluted 1:1000, phosphospecific Akt Ser^{473} (#9271; Cell Signaling Technology) diluted 1:1000 and Akt (#9272; Cell Signaling Technology) diluted 1:1000]. The membranes were then washed, reacted with anti-rabbit IgG coupled to peroxidase and developed with enhanced chemiluminescence reagents according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). The protein signals were detected with ImageCapture G3 (Liponics, Tokyo, Japan) and quantified using ImageJ [32]. The mean intensity of control samples in each membrane was used as a reference for controlling gel-to-gel variation. Equal protein loading and transfer were confirmed by Coomassie brilliant blue staining of the membranes.

2.4. Isoform-specific AMPK activity assay

The AMPK activity assay was performed as described previously [30,33]. Muscles were homogenized as described in Western Blot Analysis, and resultant supernatants (100 μg of protein) were immunoprecipitated with isoform-specific antibodies directed against the α1 or α2 catalytic subunits of AMPK [30] and protein A-Sepharose beads (GE Healthcare). Immunoprecipitates were washed twice both in buffer A and in wash buffer (240 mM HEPES and 480 mM NaCl). Kinase reactions were performed in 40 mM HEPES (pH 7.0), 0.1 mM SAMS peptide [7,30], 0.2 mM AMP, 80 mM NaCl, 0.8 mM dithiothreitol, 5 mM $MgCl_2$ and 0.2 mM ATP (2 μCi of [γ-^{32}P] ATP/sample) (PerkinElmer, Wellesley, MA, USA) in a final volume of 40 μl for 20 min at 30°C. At the end of the reaction, a 15-μl aliquot was removed and spotted onto Whatman P81 paper (Whatman International, Maidstone, UK). The papers were washed six times in 1% phosphoric acid and once in acetone. ^{32}P incorporation was quantitated with a scintillation counter, and kinase activity was expressed as fold increases relative to the basal samples.

2.5. ATP and PCr assay

Frozen muscles were homogenized in 0.2 M $HClO_4$ (3:25 w/v) in an ethanol–dry ice bath (−20°C to −30°C) and centrifuged at 16,000*g* for 2 min at −9°C. The supernatant of the homogenate was neutralized with a solution of 2 M KOH, 0.4 M KCl and 0.4 M imidazole, centrifuged at 16,000*g* for 2 min at −9°C and then subjected to enzymatic analysis [34]. ATP and PCr content was expressed as nanomoles per milligram wet weight of muscle.

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