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Rottlerin activates AMPK possibly through LKB1 in vascular cells and tissues

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ABSTRACT

AMP-activated protein kinase (AMPK) is a cellular energy sensor involved in multiple cell signaling pathways that has become an attractive therapeutic target for vascular diseases. It is not clear whether rottlerin, an inhibitor of protein kinase Cδ, activates AMPK in vascular cells and tissues. In the present study, we have examined the effect of rottlerin on AMPK in vascular smooth muscle cells (VSMCs) and isolated rabbit aorta. Rottlerin reduced cellular ATP and activated AMPK in VSMCs and rabbit aorta; however, inhibition of PKCδ by three different methods did not activate AMPK. Both VSMCs and rabbit aorta expressed the upstream AMPK kinase LKB1 protein, and rottlerin-induced AMPK activation was decreased in VSMCs by overexpression of dominant-negative LKB1, suggesting that LKB1 is involved in the upstream regulation of AMPK stimulated by rottlerin. These data suggest for the first time that LKB1 mediates rottlerin-induced activation of AMPK in vascular cells and tissues.

Endothelial dysfunction, as well as migration and proliferation of vascular smooth muscle cells (VSMCs) are critical events in the development and progression of atherosclerosis [1]. Protection of normal endothelial function and inhibition of VSMC proliferation have been effective in preventing clinical and experimental vascular diseases [1–3].

AMP-activated protein kinase (AMPK) is a sensor of cellular energy status that is activated by a decrease in cellular ATP levels and coordinates multiple catabolic and anabolic pathways to maintain cellular energy homeostasis [4,5]. Previously, extensive studies on this kinase demonstrated that AMPK is activated by increases in the cellular AMP:ATP ratio caused by metabolic stresses that either interfere with ATP production (e.g., anti-diabetic medicine metformin) or that accelerate ATP consumption (e.g., skeletal muscle contraction). Once activated, AMPK inhibits energy-consuming cellular processes (such as synthesis of cholesterol and fatty acids) and stimulates energy-producing pathways (such as glucose uptake and β-oxidation of fatty acids). Activation of AMPK by the upstream kinase LKB1 has been recently recognized to mediate such diverse cellular responses as the glucose-reducing effect of metformin and growth-suppressive signals in malignant tumors and non-neoplastic cells [4–6]. Therefore, AMPK has been proposed as a therapeutic target for diabetes, obesity and obesity-linked metabolic disorders [5].

AMPK activation also exhibits several salutary effects on vascular function and improves various experimental vascular abnormalities [7–11]. For example, AMPK activation improves endothelial function [7,9] and suppresses VSMC proliferation [10,11]. Thus, one of the possible interventions to achieve both improvement of endothelial function and inhibition of VSMC proliferation might be via activation of AMPK in vascular tissues.

Protein kinase Cδ (PKCδ) has been reported to be activated by elevated glucose condition [12,13], elevated free fatty acid (FFA) levels [12,14], reactive oxygen species (ROS) [15,16], and growth factors including platelet-derived growth factor [16,17], thrombin and Angiotensin II [18]. Most of the above agents considered to be atherogenic factors closely linked to diabetes, metabolic syndrome and atherosclerosis. Indeed, PKCδ activation was observed in diabetic animals [12] and was suggested to involve FFA-induced insulin resistance [14,19]. Inhibition of PKCδ can bring favorable outcomes in several experimental models including reperfusion injury in stroke [20], coronary artery spasm [21] and migration of VSMCs [17,18,22]. These observations indicate that PKCδ may play a critical role in various vascular diseases and that the inhibition of this molecule may be beneficial in these conditions.

Rottlerin, a natural product isolated from *Mallotus phillippinensis*, was described in 1994 as a specific PKCδ inhibitor [23] and has been widely used to suppress PKCδ activity in numerous studies.

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Recent studies demonstrated that the compound inhibits indirectly PKCδ through its mitochondria uncoupling effect [24,25]. Since this reagent may carry both PKCδ-inhibitory and AMPK-activating properties, it is worth being tested as a candidate of therapeutic medicines for vascular disorders linked with diabetes, metabolic syndrome and atherosclerosis. So far, potency of rottlerin to activate AMPK in vascular cells and tissues has not been tested. Therefore, in the present study, we investigated whether rottlerin activates AMPK in VSMCs and isolated rabbit aortas, and report here that rottlerin activates AMPK in vascular cells and tissues possibly through LKB1.

Materials and methods

Materials. ATP assay kit and PKC inhibitors were from Calbiochem (La Jolla, CA). DMEM and Lipofectamine RNAiMAX were from Invitrogen (Kyoto, Japan). Antibodies for β-actin and PKCδ were from Santa Cruz Biotechnology (Santa Cruz, CA). Isoform-specific antibodies for each PKC molecule were from BD biosciences. Antibodies for the phosphorylated forms of AMPK α-subunit (Thr-172) and acetyl-CoA carboxylase (ACC; Ser-79) were from Cell Signaling Technology (Beverly, MA). Aortas from male Japanese white rabbits were purchased from ARK resource Inc (Kumamoto, Japan).

Cell culture and treatment. Human and rabbit aortic SMCs (HASMCs and RASMCs, respectively) were obtained, cultured and used as described previously [10]. LKB1-deficient A549 and HeLa cells were obtained from Japanese Collection of Research Bioresources (Osaka, Japan) and ATCC, respectively. An equal number of cells were seeded. Cells were treated with the indicated concentrations of each reagent (rottlerin or other PKC inhibitors) or vehicle (DMSO) in serum-free DMEM for indicated periods.

Western blots. Western blotting was performed essentially as previously reported [10]. Same amounts of protein was resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes and immunoblotting was performed with the indicated antibodies. Following incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were detected with ECL plus kit (Amersham, Piscataway, NJ) according to the manufacturer's instructions. Immunoreactive bands were quantified by NIH image analysis software.

Measurement of intracellular ATP concentrations. After indicated treatment, cells were washed with phosphate-buffered saline (PBS) three times and lysed in a lysis buffer equipped in ATP assay kit [24]. The assay was performed according to manufacturer's instructions. ATP standard curve was prepared using a series of dilutions of ATP stock solution in triplicate and ATP concentration in each sample was estimated using the standard curve.

Experiments using RNA interference. A mixture of small interfering RNAs (siRNAs) against human PKCδ, On-TARGETplus PKCδ siRNA SMARTpool (L-003524; siPKCδ) and the negative control On-TARGETplus siCONTROL Non-targeting pool (D-001810; siNC) were purchased from Dharmacon (Denver, CO). HASMCs seeded at 60–70% confluence on 6-well dishes were transfected without siRNA (for transfection control experiments) or with siPKCδ or siNC using Lipofectamine RNAiMAX according to the instruction supplied by Invitrogen. After 48 h, cells were then subjected to the indicated experiments.

Experiments using adenoviruses. Adenoviral vectors expressing dominant negative (DN)- and wild type (WT)-LKB1 [26] were used as described previously [10]. An adenoviral vector expressing green fluorescence protein (GFP) was used for the infection control experiments. 3 days after adenoviral infection, HASMCs were treated with rottlerin as indicated.

Statistical analysis. Data were expressed as the means ± SD. Differences between groups were examined for statistical significance by one-factor ANOVA. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Rottlerin activates AMPK in vascular smooth muscle cells

The impact of rottlerin on AMPK was investigated in human VSMCs. In a dose-dependent manner, treatment with rottlerin for 2 h increased Thr-172 phosphorylation of AMPK, indicative of AMPK activation (Fig. 1A). The enzymatic activation of AMPK was also confirmed by examining Ser-79 phosphorylation of ACC, a major downstream target protein in the AMPK signaling cascade. Consistent with AMPK phosphorylation and activation, phosphorylation of ACC was elevated in rottlerin-treated HASMCs (Fig. 1A). We further investigated the time course of the effect of rottlerin on phosphorylation of AMPK and ACC. AMPK phosphorylation by rottlerin was sustained over 24 h in HASMCs (Fig. 1B). In rabbit VSMCs, similar effects of rottlerin on these molecules were observed (Fig. 1C). Next, we investigated the effect in isolated rabbit aortas. Rottlerin activated AMPK in aortic strips (Fig. 4A). These results indicate that rottlerin activates AMPK and regulates its downstream pathways in primary-cultured VSMCs and vascular tissues.

Rottlerin reduced cellular ATP concentrations in HASMCs

A decrease in cellular ATP causes AMPK activation in many mammalian cells [4–6]. To investigate the mechanism of rottlerin-induced AMPK activation, we measured intracellular ATP concentrations in vehicle- and rottlerin-treated HASMCs. Rottlerin significantly reduced intracellular ATP concentrations within 2 h (Fig. 2A). The effect of rottlerin on cellular ATP was dose-dependent and sustained for at least 24 h (Fig. 2A and B). ATP concentration in rabbit aortic strips treated with 5 μM rottlerin was also decreased (72 ± 8.4% of vehicle-treated, $n = 3$ each). These results suggest that rottlerin may decrease cellular ATP through the inhibition of mitochondrial ATP production as previously reported [24,25], resulting in AMPK activation in HASMCs and isolated aortas.

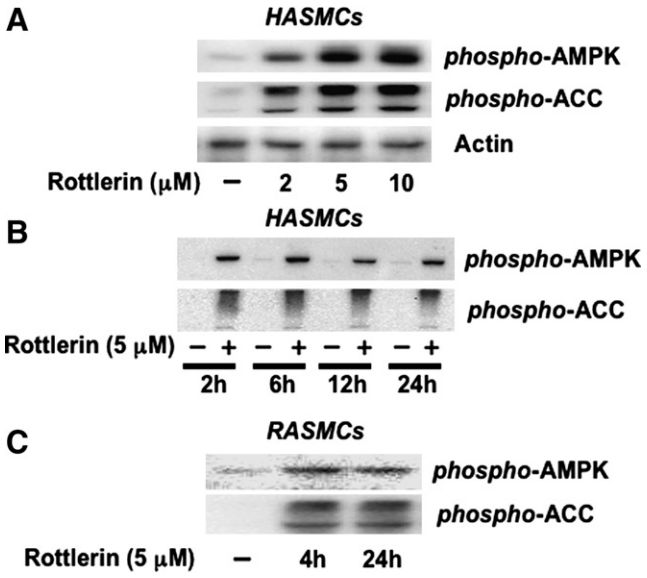

Fig. 1. Rottlerin activates AMPK in HASMCs (A and B) and RASMCs (C). VSMCs were treated with the indicated concentrations of rottlerin or vehicle (–) for 2 h (A) or for indicated periods (B and C). Representative immunoblots of more than three independent experiments are shown.