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# Aspirin-induced AMP-activated protein kinase activation regulates the proliferation of vascular smooth muscle cells from spontaneously hypertensive rats

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ABSTRACT

Acetylsalicylic acid (aspirin), used to reduce risk of cardiovascular disease, plays an important role in the regulation of cellular proliferation. However, mechanisms responsible for aspirin-induced growth inhibition are not fully understood. Here, we investigated whether aspirin may exert therapeutic effects via AMP-activated protein kinase (AMPK) activation in vascular smooth muscle cells (VSMC) from wistar kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Aspirin increased AMPK and acetyl-CoA carboxylase phosphorylation in a time- and dose-dependent manner in VSMCs from WKY and SHR, but with greater efficacy in SHR. In SHR, a low basal phosphorylation status of AMPK resulted in increased VSMC proliferation and aspirin-induced AMPK phosphorylation inhibited proliferation of VSMCs. Compound C, an AMPK inhibitor, and AMPK siRNA reduced the aspirin-mediated inhibition of VSMC proliferation, this effect was more pronounced in SHR than in WKY. In VSMCs from SHR, aspirin increased p53 and p21 expression and inhibited the expression of cell cycle associated proteins, such as p-Rb, cyclin D, and cyclin E. These results indicate that in SHR VSMCs aspirin exerts anti-proliferative effects through the induction of AMPK phosphorylation.



## 1. Introduction

Proliferation of vascular smooth muscle cells (VSMCs) is an important pathogenic factor in vascular diseases such as atherosclerosis, restenosis, and hypertension [1,2]. Proliferation of VSMCs is activated by various growth factors and cytokines. VSMCs play a major role in the pathogenesis of hypertension-induced vascular remodeling [3–5]. Thus, inhibition of VSMC proliferation may reduce the development of vascular proliferative disease.

AMP-activated protein kinase (AMPK) is a heterotrimeric complex, consisting of a catalytic α subunit and regulatory β and γ subunits, that functions as a serine/threonine protein kinase. The composition of the AMPK structure is highly conserved in eukaryotes and functions as a sensor of cellular energy [6,7]. AMPK activates and phosphorylates a number of metabolic enzymes involved in ATP-consuming cellular events including fatty acid, cholesterol and protein synthesis; AMPK is also involved in the activation of ATP-generating processes, including the uptake and oxidation of glucose [8]. A number of molecules and AMPK-associated signaling pathways have been reported to be regulated by direct phosphorylation by AMPK and indirectly by AMPK-activated gene regulation [9]. An important focus of these studies has been the suppression of VSMC proliferation by inhibition of cell cycle progression and regulation of mitosis by AMPK [10,11]. In addition, AMPK inhibits protein synthesis and cell growth through its ability to regulate the mammalian target of rapamycin (mTOR) [12]. Therefore, understanding the effect of AMPK activation on cellular proliferation is important for the prevention and treatment of vascular proliferative diseases as well as cancer [13].

Aspirin inhibits VSMC proliferation via cell cycle regulation, and salicylate also exerts direct anti-proliferative effects at high doses (>1 mmol/L) [14,15]. However, mechanisms by which non-steroidal anti-inflammatory drugs (NSAIDs) inhibit neointimal hyperplasia and thrombosis are not fully understood [16,17]. We compared the anti-proliferative effects of aspirin in VSMCs from wistar kyoto rats (WKY) and spontaneously hypertensive rats (SHR), because these two rat models have different pathophysiologic responses with regard to blood pressure, VSMC proliferation, and inflammatory processes. VSMCs from SHR proliferate faster than those from the normotensive model, WKY [18,19]. Furthermore, it is not clear how aspirin regulates VSMC proliferation in these two animal models of blood pressure.

We therefore tested whether aspirin induces AMPK activation as a regulator of VSMC proliferation. We compared the potency of AMPK activation and the anti-proliferative effects of aspirin in VSMCs from WKY and SHR, as they show differences in blood pressure and proliferation of VSMCs. The purpose of this study is to determine whether aspirin-activated AMPK inhibits VSMC proliferation and to determine whether these responses are altered in

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VSMCs from hypertensive (SHR) animals. In this study, we show that AMPK may be a therapeutic target for disorders like atherosclerosis. Moreover, we show that AMPK may be involved in mechanisms responsible for different VSMC proliferation rates in WKY and SHR.

## 2. Materials and methods

### 2.1. Materials

Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (South Logan, UT, USA). ECL plus western blot detection reagent was purchased from GE Healthcare (Piscataway, NJ, USA). Anti-AMPK, anti-phospho-AMPK, anti-acetyl-CoA carboxylase (ACC), anti-phospho-ACC, anti-p53, anti-Rb, and anti-phospho-Rb antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-p21, anti-cyclin D, and anti-cyclin E antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) and an anti-β-actin antibody were purchased from Sigma–Aldrich (St. Louis, MO, USA). Compound C was purchased from Calbiochem (San Diego, CA, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA).

### 2.2. Cell culture

WKY and SHR were anesthetized with pentobarbital (50 mg/kg). VSMCs were isolated from the thoracic aorta, and the connective tissue was removed. Tissue was processed using a 1 mm chop setting in a 10 cm culture dish and was cultured in DMEM, 10% FBS, and 1% antibiotic–antimycotic solution (penicillin 10,000 U/ml, amphotericin B 25 μg/ml, streptomycin 10,000 μg/ml). Primary cultures of VSMCs were obtained from 10- to 14-week old WKY and SHR (200–250 g). Both cell types were maintained at 37 °C and 5% $CO_2$. We used VSMCs from passages 5–8 at 70–90% confluence. Cell growth was arrested by incubating the cells in serum-free DMEM for 16–24 h prior to use.

### 2.3. Western blot analysis

Protein extracts were obtained from PRO-PREP protein extract solution (iNtRON Biotechnology, Sungnam, Korea). The protein concentration was quantified using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were mixed with sodium dodecyl sulfate (SDS) sample buffer and incubated for 5 min at 100 °C. Total protein samples (30 μg) were then subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on an 8% gel at 100 V for 1.5 h. The separated proteins were electrophoretically transferred onto a PVDF membrane at 100 V for 1.5 h. The membranes were blocked with 5% non-fat milk in PBS containing 0.05% Tween 20 (PBS-T) for 2 h at room temperature. The membranes were then incubated overnight at 4 °C with the primary antibodies at a 1:1000 dilution in PBS. The membranes were then washed four times with wash buffer and incubated for 1 h at room temperature in PBS containing anti-rabbit IgG (Stressgen, Ann Arbor, MI, USA). Finally, after four more rinses with wash buffer, the membranes were exposed to ECL or ECL plus western blot analysis detection reagents.

### 2.4. Cell proliferation assay

Cell proliferation was analyzed using the MTT assay. VSMCs were seeded on 24-well plates at $1 \times 10^4$ cells per well in DMEM supplemented with 10% FBS. After treatment with aspirin and/or compound C, 50 μl of 1 mg/ml MTT solution was added to each well (0.1 mg/well) and incubated for 4 h. The supernatants were aspirated and the formazan crystals in each well were solubilized with 200 μl dimethyl sulfoxide. An aliquot of this solution (100 μl) was placed in the 96-well plates. Cell proliferation was assessed by measuring the absorbance at 570 nm using a microplate reader.

Cell number was determined using a hemocytometer. Cells were extensively rinsed in tris-buffered saline to remove any loosely attached or floating cells. The cells were then harvested by trypsinization and counted.

### 2.5. Transfection of AMPK siRNA

Transfection of VSMCs with siRNA was performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Aliquots of $1 \times 10^4$ cells were plated on 6-well plates the day before transfection and grown to about 70% confluence. The cells were then transfected with 10 μM AMPK siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) plus 100 pmol of Lipofectamine for 6 h in Opti-MEM®I reduced serum medium (Invitrogen, Carlsbad, CA, USA). Following an incubation period of 48 h, the AMPK protein level was measured using western blot analysis and cell proliferation was analyzed using the MTT assay.

### 2.6. Statistical analysis

All data are represented as mean ± S.E.M. Differences between data sets were assessed using analysis of variance (ANOVA) followed by Bonferoni's *t*-test. A difference was considered statistically significant at $P < 0.05$.

## 3. Results

### 3.1. Aspirin-induced phosphorylation of AMPK and ACC in a time-dependent manner

Aspirin (10 μM) produced a time-dependent increase in phosphorylation of AMPK at Thr-172 and of ACC at Ser-79 (at 10, 30, and 60 min) in VSMCs from WKY (Fig. 1A) and SHR (Fig. 1B). However, when values were expressed as a fold increase of their respective controls, the increase in AMPK phosphorylation was greater in SHR than in WKY. Phosphorylations of AMPK and ACC were expressed as the phosphorylated-to-total protein ratio.

### 3.2. Dose-dependent phosphorylation of AMPK and ACC by aspirin

Phosphorylation of AMPK is required for enzymatic activation and phosphorylation of ACC. The levels of phosphorylation of AMPK at Thr-172 were increased significantly over control, with increasing concentrations of aspirin (1 and 10 μM) in VSMCs from WKY (Fig. 2A) and SHR (Fig. 2B). These effects were more enhanced in VSMCs from SHR than from WKY. Aspirin also induced phosphorylation of ACC at Ser-79 in a dose-dependent manner, a major downstream target protein in the AMPK signaling cascade.

### 3.3. Aspirin-induced AMPK activation inhibits the proliferation of VSMCs from SHR

To investigate whether aspirin-induced AMPK activation directly regulates the proliferation of VSMCs, we performed the MTT assay and cell counting. VSMCs from SHR proliferated faster than those from WKY. Aspirin treatment for 72 h significantly decreased VSMC proliferation from SHR in the MTT assay