AMP-Activated Protein Kinase Inhibits Angiotensin II–Stimulated Vascular Smooth Muscle Cell Proliferation

Daisuke Nagata, MD, PhD; Ryo Takeda, MD; Masataka SATA, MD, PhD; Hiroshi SATONAKA, MD; Etsu Suzuki, MD, PhD; Tetsuo Nagano, PhD; Yasunobu Hirata, MD, PhD

Background—AMP-activated protein kinase (AMPK) is a stress-activated protein kinase that works as a metabolic sensor of cellular ATP levels. Here, we investigated whether AMPK signaling has a role in the regulation of the angiotensin II (Ang II)–induced proliferation signal in rat vascular smooth muscle cells (VSMCs).

Methods and Results—Aminoimidazole-4-carboxamide-1-/H9252-ribofuranoside (AICAR) activated AMPK in rat VSMCs and inhibited Ang II–induced extracellular signal–regulated kinase 1/2 phosphorylation but not that of p38 MAPK or Akt/PKB. Although Ang II activated AMPK, this activation was significantly inhibited by catalase, N-acetylcysteine, and diphenyleneiodonium chloride, an NADPH oxidase inhibitor. Moreover, the observation that AMPK was activated by H2O2 suggests that AMPK is redox sensitive. The Ang II type 1 receptor antagonist valsartan but not the Ang II type 2 receptor antagonist PD123319 significantly inhibited Ang II–induced AMPK activation, suggesting that Ang II–induced AMPK activation was Ang II type 1 receptor dependent. Whereas 3H-thymidine incorporation by VSMCs treated with Ang II was significantly inhibited when the cells were pretreated with 1 mmol/L AICAR, the inhibition of AMPK by dominant-negative AMPK overexpression augmented Ang II–induced cell proliferation. Subcutaneous injection of AICAR (1 mg/g body weight per day) for 2 weeks suppressed neointimal formation after transluminal mechanical injury of the rat femoral artery.

Conclusions—Our findings indicate that Ang II–induced AMPK activation is synchronized with extracellular signal-regulated kinase signaling and that AMPK works as an inhibitor of the Ang II proliferative pathway. AMPK signaling might serve as a new therapeutic target of vascular remodeling in cardiovascular diseases. (Circulation. 2004;110:444-451.)

Key Words: atherosclerosis ■ angiotensin ■ muscle, smooth ■ free radicals ■ signal transduction

Vascular smooth muscle cell (VSMC) proliferation is involved in the pathogenesis of various conditions such as atherosclerosis, post-PTCA restenosis, and vasculitis. A large body of evidence has shown that the renin-angiotensin system is a central player modulating not only the tonus of vasculature but also the proliferation of vascular cells.1 To date, several clinical trials have shown that the angiotensin II (Ang II) type 1 receptor (AT1R) antagonists prevent cardiovascular events, suggesting that AT1R is one of the important therapeutic targets to prevent cardiovascular diseases.2,3 Although reactive oxygen species (ROS) may derive from mitochondria, xanthine oxidase, cyclooxygenase, nitric oxide synthase, heme oxygenases, or peroxidases, it has often been shown that NAD(P)H oxidases are the primary producers of ROS in vascular tissues.4,5 Recent publications have suggested that ROS mediate a large part of Ang II effects in the cardiovascular system.6,7 Several investigators have shown that AMP-activated protein kinase (AMPK) is also a redox-sensitive enzyme.8–10 AMPK was identified as a homologue of yeast SNF1 and is known as a metabolite-sensing protein kinase.11 In mammalian cells, AMPK is activated by increases in the AMP-to-ATP ratio, which occur under hypoxia/anoxia.12 When the AMP-to-ATP ratio increases, AMPK is activated by AMPK kinase and by a conformational change after combining with AMP.13 Activated AMPK phosphorylates and downregulates several anabolic enzymes, including HMG-CoA reductase or acetyl-CoA carboxylase, and shuts off the ATP-consuming synthetic pathway.13 Because AMPK is an enzyme that inhibits the anabolic enzymes described above and because some investigators have shown that AMPK inhibits the extracellular signal-regulated kinase (ERK) 1/2 pathway,14,15 we speculate that AMPK may work as a negative regulator of the Ang II–induced proliferative signaling.

To investigate the functions of AMPK in rat VSMCs, we used 5-aminoimidazole-4-carboxamide-1-β-ribofuranoside
(AICAR), which is an AMPK activator, and a newly constructed replication-defective adenoviral vector expressing dominant-negative (dn) AMPK. We investigated the effect of AMPK on the ERK pathway activated by Ang II in cultured rat VSMCs as well as on neointimal formation in the rat femoral artery wire-injury model.

**Methods**

**Materials**

AMPK pan α, phospho-AMPK α (Thr172), phospho-ERK 1/2, ERK 1/2, phospho-p38, p38 antibody, and U0126 (MEK 1/2 inhibitor) were purchased from Cell Signaling Technology. Phospho-acetyl CoA carboxylase (ACC) (Ser 79), ACC, and phospho-serine/threonine antibody were purchased from Upstate Biotechnology. AICAR, which is an AMPK activator, and a newly constructed replication-defective adenoviral vector expressing dominant-negative (dn) AMPK. We investigated the effect of AMPK on the ERK pathway activated by Ang II in cultured rat VSMCs as well as on neointimal formation in the rat femoral artery wire-injury model.

**Cell Culture and Adenoviral Vectors Transduction**

Rat VSMCs were cultured from rat thoracic aortas following the explant method as previously described.8 See Part 1 of the online Data Supplement, available at http://circulationaha.org, for more details.

The construction of a replication-defective adenoviral vector expressing dnAMPK was described previously.9,17 An adenoviral vector expressing green fluorescence protein was obtained from Qbiogene and used for the infection-control experiment. An adenovirus vector expressing catalase was a gift from Dr Colucci (Boston University). If necessary, VSMCs were transduced with the indicated replication-defective adenoviral vectors at a multiplicity of infection of 50 plaque forming units (50 MOI) for 1 day. The medium was then changed to DMEM containing 0.1% FBS to reduce the effects of serum mitogens. After incubation in low-serum media for 2 days, transduced cells were stimulated by treatment with Ang II at 10−7 mol/L, with or without 2-hour pretreatment using inhibitors specific pathways or receptors such as N-acetylcysteine (NAC; 10−2 mol/L), diphenyleneiodonium chloride (DPI; 10−5 mol/L), valsartan (10−5 mol/L), or PD123319 (10−5 mol/L). When the effects of AICAR were examined, cells were pretreated with this AMPK activator for 4 hours before the stimulation with Ang II.

**In Vitro AMPK assay**

This assay is described in detail in Part 2 of the online Data Supplement, available at http://circulationaha.org.org.

**Western Blot Analysis**

Western blot analysis and immunoprecipitation were carried out as previously described.16,17 Details can be found in Part 3 of the online Data Supplement, available at http://circulationaha.org.org.

**Measurement of 3H-Thymidine Incorporation**

Rat VSMCs were serum starved in DMEM/0.1% FBS for 48 hours and restimulated with or without 10−7 mol/L Ang II for 20 hours. 3H-thymidine (2 μCi/mL, Amersham) was added to each well 2 hours before the end of the incubation period (see Part 4 of the online Data Supplement, available at http://circulationaha.org.org).

**Rat Femoral Artery Injury**

Transluminal mechanical injury of the rat femoral artery was induced as previously described with slight modifications.18,19 Male Wistar rats (8 weeks old; 6 rats in each group) were anesthetized by pentobarbital injected intraperitoneally, and a groin incision was made under a surgical microscope. A guidewire (0.46-mm diameter) was introduced through a small muscular branch of the femoral artery proximally to the aortic bifurcation and withdrawn. AICAR dissolved in 0.9% NaCl or 0.9% NaCl alone was injected subcutaneously once daily for 14 days starting just after the injury. Rats were anesthetized by intraperitoneal injection of pentobarbital ~24 hours after the last AICAR injection. The aorta, femoral artery, heart, and liver were collected and subjected to Western blot or histochemical analysis. The dosing schedule of AICAR (1 mg/g body weight per day) was set on the basis of results of previous reports.20

**Histochemical Analysis**

The femoral arteries were fixed by perfusing 4% paraformaldehyde and processed for paraffin embedding. Cross sections (2 μm thick) were sliced, deparaffinized, rehydrated, and stained with hematoxylin and eosin. Images were captured with a digital camera. The degree of the intima-to-media (I/M) ratio was quantified by measuring the areas of the intima and media with the NIH Image program.

**Statistical Analyses**

Values are expressed as mean±SEM. Statistical comparisons were performed with ANOVA with the Scheffé F procedure for post hoc analysis. P<0.05 was considered statistically significant.

**Results**

**AICAR Activates AMPK in Rat VSMCs**

We tested the effect of treatment with 1 mmol/L AICAR for 2 hours or 10−7 mol/L Ang II for 30 minutes on AMPK activity in rat VSMCs by the AMPK assay using SAMS peptide as the substrate. AICAR activated AMPK compared with the control (Figure 1A). Overexpression of dnAMPK suppressed this activation completely, suggesting that AICAR could be used as a chemical stimulator of AMPK in rat VSMCs. SAMS peptide phosphorylation induced by Ang II was also inhibited by dnAMPK overexpression (Figure 1A). These results showed that Ang II activated AMPK in rat VSMCs.

**AMPK Activated by AICAR Inhibits Ang II–Induced MEK/ERK Pathway Activation**

We studied the effects of pretreatment with 1 mmol/L AICAR for 4 hours on ERK phosphorylation induced by 10−7 mol/L Ang II. Ang II induced ERK phosphorylation in a time-dependent manner, and its phosphorylation reached a maximum at the 30-minute time point (Figure 1B). However, when AMPK was activated by AICAR, as estimated from AMPK α (Figure 1C) and ACC (Figure 1D) phosphorylation levels, ERK phosphorylation was reduced by ∼55% at 30 minutes after stimulation with Ang II (Figure 1E). However, AMPK activation did not change the phosphorylated p38 MAPK or Akt/PKB (Figure 1B), which are known to be the other proliferative or hypertrophic signals. AMPK was significantly activated after the induction of Ang II stimulation, even though its activation levels were much lower than those observed when AMPK was activated by AICAR (Figure 1B, 1C, and 1D). Next, we examined the effect of AICAR on the Ang II–induced ERK phosphorylation. After preincubation with AICAR at the indicated concentrations for 4 hours, we stimulated VSMCs with Ang II for 30 minutes. AICAR inhibited Ang II–induced ERK phosphorylation in a dose-dependent manner, and its maximal effect was observed at 1 mmol/L (Figure 1F). Next, we tested the effect of dnAMPK
Figure 1. AMPK inhibits Ang II–induced MAPK/ERK phosphorylation. When AMPK was activated by AICAR, ERK phosphorylation induced by Ang II was significantly inhibited in time- and dose-dependent manner. A, Ang II or AICAR treatment increased SAMS peptide phosphorylation that shows activity of AMPK. Overexpression of dnAMPK inhibited these activations. B, Phosphorylated AMPK and ACC closely reflect AMPK activity. ERK phosphorylation induced by Ang II was substantially inhibited when AMPK was activated by AICAR; however, phosphorylation status of p38 MAPK or Akt/PKB did not change under same conditions. C, D, Phosphorylated AMPK and ACC levels increased significantly in Ang II– and/or AICAR-stimulated VSMCs. Ang II–induced ERK phosphorylation was significantly inhibited by pretreatment with AICAR. E, Ang II–induced ERK phosphorylation was significantly inhibited by pretreatment with AICAR. F, AICAR pretreatment resulted in dose-dependent inhibition of phosphorylation of ERK induced by Ang II. G, dnAMPK overexpression augmented Ang II–induced ERK phosphorylation and suppressed inhibitory effect of AICAR. Each bar represents mean±SEM (n=4). *P<0.001; †P<0.01.
overexpression on ERK and ACC phosphorylations induced by Ang II. Transduction of dnAMPK, which has a myc epitope tag at the N-terminal, in rat VSMCs inhibited ACC phosphorylation and augmented ERK phosphorylation after Ang II stimulation (Figure 1G). The facts show the specificity of the effect of AICAR on AMPK activation.

MEK/ERK Pathway Activation Does Not Contribute to Ang II–Induced AMPK Activation

The online Data Supplement, Part 5 and the Figure (available at http://circulationaha.org), give details.

Ang II Activates AMPK via the AT1R-NADPH Oxidase Axis

Although we have demonstrated that Ang II activates AMPK in VSMCs, the mechanism of this effect remains unknown. Recently, we and other groups have reported that some kinds of ROS upregulate AMPK activity,\(^8\)–\(^10\) suggesting that Ang II might activate AMPK via the AT1R-ROS axis. In NIH 3T3 cells, H\(_2\)O\(_2\) was reported to activate AMPK,\(^8\)–\(^10\) so we investigated the effect of catalase overexpression on the Ang II–induced phosphorylation of ACC and AMPK using a catalase-expression adenoviral vector (Figure 2A and 2B). Catalase overexpression significantly inhibited AMPK activation. An antioxidant NAC and an inhibitor of NADPH oxidase, DPI, also significantly inhibited AMPK activation (Figure 2A and 2B), suggesting that H\(_2\)O\(_2\) converted from O\(_2\) activated AMPK in VSMCs. To investigate which type of receptor, AT1R or AT2R, mediated these reactions, we used valsartan and PD123319 to antagonize AT1R and AT2R, respectively. Valsartan but not PD123319 significantly inhibited ACC phosphorylation, showing that AMPK activation was brought about by AT1R stimulation. These observations indicate that Ang II stimulated AMPK via the AT1R–NADPH oxidase–O\(_2^–\)–H\(_2\)O\(_2\) axis.

H\(_2\)O\(_2\) Stimulation Activates AMPK in VSMCs

Because our experiments using catalase overexpression suggested that H\(_2\)O\(_2\) might be a mediator of AMPK activation, we investigated whether H\(_2\)O\(_2\) itself could activate AMPK in VSMCs. Treatment with 250 \(\mu\)mol/L H\(_2\)O\(_2\) activated AMPK in a time-dependent manner, and the maximum activation was observed at the 10-minute time point (Figure 3A). At that time point, AMPK was activated by H\(_2\)O\(_2\) treatment in a dose-dependent manner (Figure 3B); and the increase in band density of phospho-ACC was statistically significant (250 \(\mu\)mol/L; Figure 3C).

AMPK Suppresses Ang II–Induced \(^3\)H-Thymidine Incorporation in VSMCs

We observed that AMPK inhibited the MAPK/ERK pathway, so next we investigated whether AMPK activation could suppress Ang II–induced cell proliferation. We measured \(^3\)H-thymidine incorporation in VSMCs after 20 hours of incubation with 10\(^{−7}\) mol/L Ang II. In controls, Ang II stimulation increased \(^3\)H-thymidine incorporation by \(\approx 2.5\) times. When AMPK was activated by AICAR pretreatment, \(^3\)H-thymidine incorporation was significantly suppressed to almost the basal level (Figure 4). When AMPK activity was suppressed by dnAMPK, Ang II–induced \(^3\)H-thymidine incorporation was augmented by
30% compared with controls (Figure 4). This was a mirror-image phenomenon of AICAR treatment and was compatible with the result presented in Figure 1G, which shows that dnAMPK overexpression was associated with Ang II–induced ERK phosphorylation. Valsartan but not PD123319 completely inhibited Ang II–induced 3H-thymidine incorporation. These results are compatible with the consensus that AT1R is a main receptor subtype that transduces Ang II signal to cell proliferation.

In Vivo Administration of AICAR Reduced Neointimal Formation After a Mechanically Induced Injury of the Vessels

Finally, we examined the in vivo effect of AICAR on neointimal formation in the rat femoral artery after transluminal wire-induced injury, followed by histochemical analyses. At the same time, we tested whether subcutaneously injected AICAR activated AMPK in rat aorta by Western blot analyses. AICAR significantly increased AMPK activity in the rat aorta (Figure 5A and 5B), just as in the heart and liver.

Mechanical injury caused marked neointimal formation in the femoral artery. Although AICAR did not alter blood pressure, body weight, heart weight, or serum lipid/glucose profiles of the rats (Part 6 of the online Data Supplement, available at http://www.circulationaha.org), it significantly suppressed neointimal formation in the femoral artery (Figure 6A). The I/M ratio was significantly lower in rats treated with AICAR than in those treated with the vehicle (Figure 6B).

Discussion

AMPK is a stress-activated protein kinase that works as a metabolic sensor of cellular ATP levels.21 In the present study, we have shown that AMPK is activated by Ang II synchronized with ERK signaling and, at the same time, functions as an inhibitory modulator of the ERK pathway. AICAR activated AMPK in rat VSMCs and inhibited Ang II–induced ERK phosphorylation but not p38 MAPK or Akt/PKB phosphorylation.

To verify the specificity of AICAR on the activation of AMPK, we have confirmed, using the conventional peptide substrate assay, that dnAMPK overexpression augmented 3H-thymidine incorporation induced by Ang II. Valsartan but not PD123319 inhibited 3H-thymidine incorporation, showing that this VSMCs culture system worked appropriately. Same experiments were repeated 3 times. Each bar represents mean±SEM (n=5). *P<0.001; †P<0.01.

Figure 4. AMPK inhibits VSMC proliferation induced by Ang II. AMPK activated by AICAR significantly inhibited 3H-thymidine incorporation by Ang II–stimulated VSMCs. dnAMPK overexpression augmented 3H-thymidine incorporation induced by Ang II. Valsartan but not PD123319 inhibited 3H-thymidine incorporation, showing that this VSMCs culture system worked appropriately. Same experiments were repeated 3 times. Each bar represents mean±SEM (n=5). *P<0.001; †P<0.01.
stimulated VSMCs. The reasons for these discrepancies are unknown, but the function of AMPK might be cell type dependent.

Although Ang II activated AMPK, this activation was significantly inhibited by catalase, NAC, and DPI. Furthermore, AMPK was activated by 
\[ \text{H}_2 \text{O}_2 \] suggesting that AMPK is redox sensitive. In the present study, the AT1R antagonist valsartan but not the AT2R antagonist PD123319 significantly inhibited Ang II-induced AMPK activation. These results strongly suggest that Ang II-induced AMPK activation was specifically AT1R-dependent. Our data are compatible with those of previous reports that showed that AT1R mediates NADPH oxidase activation and upregulates ROS production.5

AICAR pretreatment significantly inhibited Ang II-induced \(^{3}H\)-thymidine incorporation. On the other hand, inhibition of AMPK by dnAMPK overexpression augmented Ang II–induced proliferation. These results suggest that AMPK inhibits the proliferation signal induced by Ang II. Furthermore, because the augmented \(^{3}H\)-thymidine incorporation in dnAMPK-overexpressed VSMCs decreased to the control level when treated with U0126 (data not shown), we speculate that the AMPK target for inhibiting the MAPK/ERK pathway might exist between AT1R and MEK.

AICAR suppressed neointimal formation after transluminal mechanical injury of the rat femoral artery when administered by subcutaneous injection for 2 weeks. VSMCs apoptosis is a major modulator of restenosis induced by acute balloon injury.26 Although some investigators have shown that AMPK has antiapoptotic effects in some cell lines,25,27–29 others have shown that AMPK has proapoptotic effects in B lymphocytes,30 hepatocytes,31 and a \( \beta \)-cell line.32 Details of this mechanism remain unknown. Because we observed that the viability of VSMCs observed by WST-1 assay did not change within 24 hours after treatment with AICAR (control:AICAR=0.450±0.014;0.433±0.008; absorbance, 450 nm; mean±SEM; \( P=0.36 \)) and that the TUNEL-positive fraction did not increase in AICAR-treated rat femoral arteries compared with controls (control:AICAR=11.9±3.2%;9.2±1.3%; mean±SEM; \( P=0.46 \)), apoptosis was supposed to be a minor modulator at least during the early and late apoptotic phase. Imamura et al33 showed that, based on p53 phosphorylation and p21\(^{Cip1}\) expression, AICAR-induced AMPK activation inhibits cell cycle progression in hepatocellular carcinoma cell lines. On the other hand, Campos et al30 recently reported that AICAR stimulation has no effect on p53 levels or phosphorylation in B-cell chronic lymphocytic leukemia cells. Further studies are needed to clarify the involvement of cyclin-dependent kinase inhibitor expression in the inhibitory regulation of VSMCs proliferation by AMPK.

In the present study, we showed that Ang II-induced AMPK activation is synchronized with ERK signaling and that AMPK suppresses the Ang II–induced proliferative pathway (Figure 7). Recently, mutations of the \( \gamma_2 \) regulatory subunit of AMPK (PRKAG2), 1 missense or 1 in-frame single codon insertion, have been reported to cause familial...
hypertrophic cardiomyopathy associated with aberrant conduction from the atria to the ventricles (pre-excitation or Wolff-Parkinson-White syndrome). Moreover, several investigators showed that antidiabetic adipocytokine adiponectin has antiatherosclerotic effects. Yamauchi et al recently reported that adiponectin regulates glucose use and fatty acid oxidation by activating AMPK in C2C12 myocytes and skeletal muscle. These findings suggest that some unknown mutations of AMPK components might be responsible for the susceptibility to develop cardiovascular diseases, including atherosclerosis. Combining these data with the results of the present study shows that AMPK might work as an important regulator of the atherogenic pathway in vascular tissues and that AMPK signaling might serve as a new therapeutic target of vascular remodeling in patients with cardiovascular diseases.

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References

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