Adenosine Monophosphate-Activated Protein Kinase Suppresses Vascular Smooth Muscle Cell Proliferation Through the Inhibition of Cell Cycle Progression

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Abstract—Vascular smooth muscle cell (VSMC) proliferation is a critical event in the development and progression of vascular diseases, including atherosclerosis. We investigated whether the activation of adenosine monophosphate-activated protein kinase (AMPK) could suppress VSMC proliferation and inhibit cell cycle progression. Treatment of human aortic smooth muscle cells (HASMCs) or isolated rabbit aortas with the AMPK activator 5-Aminooimidazole-4-carboxamide ribonucleoside (AICAR) induced phosphorylation of AMPK and acetyl Co-A carboxylase. AICAR significantly inhibited HASMC proliferation induced by both platelet-derived growth factor-BB (PDGF-BB) and fetal calf serum (FCS). Treatment with AICAR inhibited the phosphorylation of retinoblastoma gene product (Rb) induced by PDGF-BB or FCS, and increased the expression of cyclin-dependent kinase inhibitor p21<sup>cip</sup> but not that of p27<sup>kip</sup>. Pharmacological inhibition of AMPK or overexpression of dominant negative-AMPK inhibited both the suppressive effect of AICAR on cell proliferation and the phosphorylation of Rb, suggesting that the effect of AICAR is mediated through the activation of AMPK. Cell cycle analysis in HASMCs showed that AICAR significantly increased cell population in G0/G1-phase and reduced that in S- and G2/M-phase, suggesting AICAR induced cell cycle arrest. AICAR increased both p53 protein and Ser-15 phosphorylated p53 in HASMCs, which were blocked by inhibition of AMPK. In isolated rabbit aortas, AICAR also increased Ser-15 phosphorylation and protein expression of p53 and inhibited Rb phosphorylation induced by FCS. These data suggest for the first time that AMPK suppresses VSMC proliferation via cell cycle regulation by p53 upregulation. Therefore, AMPK activation in VSMCs may be a therapeutic target for the prevention of vascular diseases. (Circ Res. 2005;97:837-844.)

Key Words: AMP-activated protein kinase □ cell cycle arrest □ p53 □ p21 □ AICAR

Vascular smooth muscle cell (VSMC) proliferation is one of the critical events in the development and progression of various vascular diseases, including atherosclerosis and restenosis after coronary intervention. Mammalian cell proliferation is governed by the cell cycle. Cell cycle progression is a tightly controlled event regulated positively by cyclin-dependent kinases (CDKs) and their cyclin-regulatory subunits, and negatively by CDK inhibitors (CDKIs) and tumor suppressor genes. Mitogenic factors bind to their receptors and initiate a series of events resulting in the activation of CDKs, which in turn regulates cell cycle progression and mitosis.

The cell cycle entry of VSMCs is stimulated by a variety of growth factors produced from inflammatory cells, platelets, and the vascular cells where vascular injury occurs. Although these growth factors, including platelet-derived growth factor (PDGF), basic fibroblast growth factor, insulin-like growth factor, and angiotensin II (Ang II), use distinct signaling pathways to promote DNA synthesis in VSMC, these signaling pathways must converge on common regulators of the cell cycle such as CDKs and CDKIs. The final common pathway leading to G0/G1/S transition is the CDKs-induced hyperphosphorylation of the retinoblastoma gene product (Rb), which functions as a molecular switch dictating the cell to DNA replication. Hyperphosphorylation of Rb results in the release of the transcription factor E2F, which induces the expression of genes required for the progression through the S, G2, and M phases. CDKIs such as p21<sup>cip</sup> negatively regulate cell cycle progression by inhibiting cyclin/CDKs activity and phosphorylation of Rb, resulting in G1 arrest. Progression of the cell cycle is therefore regulated by the balance between the levels and activities of cyclin-
CDK complexes, CDKIs, and other growth suppressor proteins such as p53.

Tumor suppressor p53 is tightly regulated by its phosphorylation state. Cellular stresses such as γ-irradiation induce Ser-15 phosphorylation of p53. The phosphorylated p53 induces cell cycle arrest and/or apoptosis through the transcriptional regulation of p53 response genes such as p21<sup>3P</sup>. Adenosine monophosphate-activated protein kinase (AMPK) plays a key role in the regulation of energy homeostasis and monitors cellular energy charge, acting as a "metabolic master switch" to regulate adenosine triphosphate concentrations in the face of stresses that reduce cellular energy levels. AICAR (5-Aminoimidazole-4-carboxamide ribonucleoside) is a well-known activator of AMPK. AICAR is transported inside the cells through the adenosine transporter and phosphorylated by adenosine kinase to form zeatin riboside-5-monophosphate (ZMP), which mimics the stimulatory action of AMP on AMPK.

Previous studies reported that AICAR could inhibit apoptosis in primary astrocytes and endothelial cells. On the other hand, AICAR has been reported to cause apoptosis in neuroblastoma cell lines and B-cell chronic lymphocytic leukemia cells.

Thus far, only 2 studies have reported the role of AMPK activation in VSMCs. Whereas Rubin et al reported the activation of AMPK with 2-deoxyglucose plus N<sub>2</sub>, but not with AICAR, in rat carotid artery smooth muscle, Nagata et al reported that AICAR activated AMPK in rat aortic SMCs and further inhibited Ang II-induced SMC proliferation. No information is available, however, on the effect of AICAR in human aortic SMCs (HASMCs). Therefore, in the present work, we determined whether AMPK activation by AICAR could suppress proliferation or induce apoptosis in HASMCs, and further investigated the mechanisms of AICAR-induced suppression of VSMC proliferation. We have found that AICAR exerts an antiproliferative effect through the activation of AMPK in HASMCs, and that the mechanism seems to involve cell cycle arrest through the upregulation of p53 and p21<sup>3P</sup>.

### Materials and Methods

#### Cell Culture and Reagents

HASMCs were purchased from Clonetics (Walkersville, Md). For all experiments, early passaged (passages 4 to 7) SMCs were used. AICAR was obtained from Toronto Research Chemicals. We purchased 5'-amino-5'-deoxyadenosine (AMDA), dipyridamole, diethylymaleate (DEM), and PDGF-BB from Sigma.

#### Cell Proliferation Assay

We used 2 different methods, cell counting assay and Alamar Blue assay as described previously (see also expanded Materials and Methods available online at http://circres.ahajournals.org).

#### Determination of DNA Content Using Hoechst 33258 Dye

DNA content in SMCs was determined as an index for cell proliferation or for cytoxicity according to the instruction supplied by Thermo Labsystems (see also expanded Materials and Methods).

### Experiments Using AdenoviralVectors

An adenoviral vector expressing dominant negative (DN)-AMPK (Ad-DN-AMPK), which serves as a nonphosphorylatable T172A mutant of AMPK α-subunit and contains a c-myc tag at the NH<sub>2</sub> terminus, was used to inhibit AMPK activity as described previously. SMCs were infected with the indicated adenoviral vectors at 100 multiplicity of infection (100 MOI) for 2 hours. The medium was then changed to Dulbecco’s modified eagle medium (DMEM) containing 0.2% fetal calf serum (FCS). After the incubation for 2 days, infected cells were stimulated with 10 ng/mL PDGF-BB or 15% FCS in the presence or absence of AICAR. In some experiments, cells were pretreated with AICAR for 4 hours. In experiments using inhibitors, inhibitors were added 30 minutes before AICAR treatment.

### Cell Cycle Analysis

The fraction of cells present in each cell cycle phase (G0/G1, S, and G2/M) was determined by flow cytometry using a BD FACStar flow cytometer and ModFit software from Verity House.

### Detection of Apoptosis

A sandwich ELISA method was used to assess apoptosis using the Cell Death ELISA plus kit (Roche) as described previously.

### Western Blots

Western blotting was performed essentially as previously reported.

### Ex Vivo Experiments

Male Japanese white rabbits (Kyudo Co Ltd, Saga, Japan) were euthanized by overdose of Inactin. The descending thoracic aorta was rapidly excised and cleaned of connective tissues. The endothelium was removed by gently rubbing the vessel with wet cotton swab. The aorta was cut into 5-mm rings and the rings were cut open into strips. The aortic strips were stimulated without or with 15% FCS in the presence or absence of AICAR. After the stimulation, strips were homogenized in the lysis buffer. Western Blot analyses were performed as described above.

Experimental procedures for a real-time reverse transcription polymerase chain reaction (RT-PCR) analysis, a dual-luciferase assay for p53-dependent transcription, trypan blue exclusion assay, and detailed information for procedures described above are available in an expanded Materials and Methods section at http://circres.ahajournals.org.

### Results

**AICAR Suppresses Proliferation and DNA Synthesis of HASMCs Stimulated by PDGF-BB or 15% FCS**

To determine the roles of AMPK on SMC proliferation, we first investigated the effect of AICAR on proliferation by the cell count assay. Treatment of HASMCs with PDGF-BB (10 ng/mL) or 15% FCS increased cell proliferation by ~2.6-fold and ~3.6-fold, respectively, compared with control cells incubated with 0.2% FCS. AICAR decreased the number of cells induced by PDGF-BB or 15% FCS in a dose-dependent manner (Figure 1A and 1B). Similar results were obtained in primary rabbit aortic SMCs (RASMCs) (supplemental Figure S1A and S1B).

We further investigated the inhibitory effect of AICAR on proliferation using Alamar Blue assay. Treatment with AICAR significantly reduced Alamar Blue fluorescence intensity in HASMCs stimulated with PDGF-BB or 15% FCS (Figure 1C). Microscopic observation after Alamar Blue assay confirmed that the decreased fluorescence intensity in AICAR-treated cells was due to the reduced cell number.
Furthermore, treatment with 15% FCS increased DNA synthesis in HASMCs, and AICAR significantly suppressed the increase in DNA synthesis in a dose-dependent manner (Figure 1D). Notably, AICAR treatment did not reduce DNA amounts in cells compared with the control cells treated with 0.2% FCS, suggesting the inhibitory effect of AICAR on DNA synthesis rather than the loss of cellular DNA due to a cytotoxic effect.

AICAR Activates AMPK in HASMCs, RASMCs, and Isolated Aortic Strips

Next, we investigated the effect of AICAR on the phosphorylation of AMPK in HASMCs and RASMCs by Western blot analyses using an antibody specific for the Thr-172 phosphorylation of \( \alpha \)-subunit of AMPK (\( \alpha \)-AMPK). Treatment with AICAR for 2 hours markedly increased the phosphorylation of \( \alpha \)-AMPK compared with the vehicle-treated control in HASMCs and RASMCs (Figure 2A and 2B). Although the increased Thr-172 phosphorylation of \( \alpha \)-AMPK is indicative of the activation of this kinase, we also immunoblotted with anti-pan-\( \alpha \)-AMPK antibody. Representative blots of 3 independent experiments are shown.

Inhibition of AMPK Activity by Inhibitors of AICAR Function or DN-AMPK Blocks the Growth-Suppressive Effect of AICAR

To exclude the possibility that the inhibitory effect of AICAR on SMC proliferation was caused by mechanisms other than AMPK activation, we investigated the effects of 2 different inhibitors of AICAR function, dipyridamole and AMDA. Dipyridamole inhibits transport of AICAR into cells by inhibiting an adenosine transporter, and AMDA inhibits the phosphorylation of AICAR by blocking the adenosine kinase in the cells.\(^19,29,30\) Pretreatment with dipyridamole completely blocked the inhibitory effect of AICAR on proliferation.
AMDA partially but significantly blocked the inhibitory effect of AICAR on proliferation (Figure 3B).

Pretreatment with these inhibitors completely inhibited AICAR-induced phosphorylation of AMPK and ACC (Figure 3C). These results indicated that ZMP formation through both transport and phosphorylation of AICAR is required for the suppression of growth by AICAR, suggesting that AMPK activation is a key process for an inhibitory effect of AICAR on SMC proliferation.

To further confirm the involvement of AMPK on growth-suppressive effect of AICAR, we performed the experiments using an adenoviral vector expressing DN-AMPK, which has been reported to inhibit AMPK activation as a nonphosphorylatable T172A mutant. Overexpression of DN-AMPK, but not of green fluorescent protein (GFP) (control), suppressed AICAR-induced phosphorylation of AMPK and ACC (Figure 3D). Overexpression of DN-AMPK was confirmed by Western blotting using both anti-c-myc and antipan-α-AMPK antibodies. In HASMCs infected with Ad-GFP, AICAR completely suppressed proliferation (Figure 3E) as observed in Figure 1A. DN-AMPK significantly inhibited the suppressive effect of AICAR on proliferation. These results indicate the involvement of AMPK on AICAR-induced suppression of SMC proliferation.

AICAR Inhibits Phosphorylation of Rb

Next, we examined the effect of AICAR on the phosphorylation of Rb stimulated with 15% FCS or PDGF, as phosphorylation of Rb has been reported to be a critical and common event during cell proliferation process. Increased phosphorylation of Rb was detected 12 hours after stimulation with FCS. Rb phosphorylation was further in-
increased in a time-dependent manner, indicating the cell cycle progression induced by FCS. AICAR significantly inhibited FCS-induced Rb phosphorylation (Figure 4A and 4B). AICAR also strongly suppressed PDGF-induced Rb phosphorylation (supplemental Figure S2A). These results suggest that AICAR suppresses a G1 event in cell cycle progression. This suppressive effect of AICAR on Rb phosphorylation was inhibited by overexpression of DN-AMPK but not by that of the control GFP (Figure 4C and 4D and supplemental Figure S2B).

**AMPK Induces G1 Cell Cycle Arrest but Not Apoptosis**

Reduction in cell number induced by AMPK activation could be the result of the inhibition of proliferation or increased cell death. To distinguish these possibilities, we first investigated the effect of AMPK on cell cycle progression using a flow cytometry analysis. Compared with control cells treated with 15% FCS, AICAR significantly increased the cells in the G0/G1 phase (from 76.0±2.2% to 88.6±1.9%) and decreased those in S (from 14.6±0.8% to 5.7±1.4%) and G2/M phase (from 9.4±1.5% to 5.7±1.5%) (Figure 5A). This effect of AICAR was statistically significant (P<0.01, n=5) and was almost completely inhibited either by coincubation with dipyridamole or by overexpression of DN-AMPK (supplemental Figure S3). These data suggest AMPK activation causes G1 arrest in HASMCs.

To examine the second possibility, we investigated whether AICAR could induce cell death. In trypan blue exclusion assay, DEM significantly decreased viable cell number. In contrast, no difference was observed in the rate of appearance for dead cells between AICAR-treated and vehicle-treated HASMCs (Figure 5B). Using a cell death ELISA quantitative assay, no differences were observed in the rates of cytoplasmic DNA-histone complex formation between HASMCs treated with AICAR and those with 15% FCS alone (Figure 5C). In addition, increased population in sub-G1 was not observed even after 72 hours in AICAR-treated HASMCs (data not shown). These data indicate that AMPK-induced cell number reduction in HASMCs is due to the inhibition of cell proliferation rather than cell death.

**AMPK Increases the Expression of p21\(^{\text{CIP}}\)**

We further investigated the effect of AMPK on the protein expression of CDKIs p21\(^{\text{CIP}}\) and p27\(^{\text{KIP}}\). Increased expression of p21\(^{\text{CIP}}\) protein but not of p27\(^{\text{KIP}}\) was observed in AICAR-treated HASMCs compared with those treated with 15% FCS alone from 6 hours to 24 hours after stimulation with FCS (Figure 6A and 6B). FCS stimulation decreased p27\(^{\text{KIP}}\) expression. AICAR did not block the FCS-induced reduction of p27\(^{\text{KIP}}\) (Figure 6A, middle panel). Expression of p21\(^{\text{CIP}}\) has been reported to be regulated both in p53-dependent and -independent manners. To test whether AMPK increases the p21\(^{\text{CIP}}\) expression through the activation of p53, we examined the effect of AICAR on expression and Ser-15 phosphorylation of p53. AICAR increased phosphorylated p53 and its protein expression, and also increased the expression of p21\(^{\text{CIP}}\) (Figure 6C) in a dose-dependent manner. The increase in p53 protein was associated with an increased p21\(^{\text{CIP}}\) level.

We further investigated the effect of AICAR on the mRNA expression of p21\(^{\text{CIP}}\) and p53 using a real-time RT-PCR analysis. AICAR increased the expression of p21\(^{\text{CIP}}\) mRNA, whereas no significant change was observed in the mRNA expression of p53 (supplemental Figure S4A), indicating the transcriptional and post-transcriptional mechanisms for p21\(^{\text{CIP}}\) and p53 upregulation, respectively. We further investigated whether p53 is functionally activated in HASMCs treated with AICAR using a reporter assay system. This dual-luciferase assay revealed that p53-dependent transcription in AICAR-treated cells significantly increased compared with both control cells and those treated with 15% FCS alone (supplemental Figure S4B).
Finally, we investigated whether AMPK activation could exhibit several effects in isolated rabbit aortas, as observed in cultured SMCs. As observed in Figure 2C, AICAR significantly increased the phosphorylation of AMPK and ACC in our ex vivo experimental system. Furthermore, increased p53 phosphorylation and p53 protein expression were accompanied with the decreased Rb phosphorylation in AICAR-treated aortic strips (Figure 6D). The increased levels of phosphorylated p53 and p53 protein were dependent on the activation of AMPK, as either dipyridamole or AMDA completely blocked these AICAR-induced changes in p53 (supplemental Figure S4C). These findings indicate that AMPK exhibits the growth-inhibitory effect in aortic SMCs in vivo, as observed in cultured SMCs and in aortic strips.

**Discussion**

In the present study, we have demonstrated for the first time that AMPK inhibited FCS- and PDGF-induced proliferation in human aortic SMCs. Similar results were also obtained in rabbit aortic SMCs. The mechanism of growth suppression induced by AMPK turned out to be a cell cycle arrest at G1 phase but not by an apoptosis. We further investigated how AMPK induces cell cycle arrest in SMCs, and found that activation of AMPK increased the CDKI p21^{CIP} protein through the upregulation of p53, which in turn inhibited the Rb phosphorylation required for cell cycle progression.

Transport of AICAR into cells has been studied previously.20,29,32 Lopez et al reported that adenosine inhibited the accumulation of ZMP in AICAR-treated Jurkat cells via competition with AICAR transport into the cells.32 Dipyridamole and AMDA were reported to block adenosine-induced apoptosis by inhibiting AMPK activation.29 Further, adenosine and 5-iodotubercidine, another adenosine kinase inhibitor, were reported to inhibit apoptosis and AMPK phosphorylation in B-cell chronic lymphocytic leukemia cells treated with AICAR.29 Because of these reports, we investigated the effect of dipyridamole and AMDA on AICAR-induced growth suppression. As expected, both inhibitors blocked the inhibitory effect of AICAR on SMC proliferation, as well as AICAR-induced phosphorylation of AMPK and ACC. These observations indicate that both uptake and phosphorylation of AICAR are necessary for the activation of AMPK by AICAR and AICAR-induced suppression of SMC proliferation, suggesting that AMPK activation is required for the growth suppression of AICAR. Adenosine was reported to induce apoptosis in HASMCs via A2b adenosine receptor and cAMP-dependent pathways.33 Although AICAR is an adenosine analogue, treatment of HASMCs with adenosine or AICAR seems to induce totally different cellular events, apoptosis or growth-suppression without apoptosis, respectively. We also demonstrated that DN-AMPK inhibited the growth suppression by AICAR, which further supports the involvement of AMPK in AICAR-induced suppression of SMC proliferation.

Recently, Nagata et al reported that AICAR-induced AMPK activation inhibited Ang II-induced proliferation of VSMCs derived from rat aortas.22 Their observations are consistent with our present study, although the mitotic stimulations used were different. To confirm their observations, we performed several preliminary experiments. Indeed, AICAR inhibited proliferation induced by Ang II in HASMCs, and we also found that AICAR induced cell cycle arrest in Ang II-treated HASMCs (unpublished data, 2004). These findings indicate activation of AMPK by AICAR induces cell cycle arrest and suppresses proliferation in SMCs treated with either PDGF-BB, Ang II, or FCS.

As mentioned above, AICAR has been reported to be able to either induce or inhibit apoptosis depending on the cell types.17–20 In HASMCs, AICAR did not induce apoptosis. AICAR may have an anti-apoptotic effect in HASMCs, as AICAR treatment significantly reduced sub-G1 fraction in SMCs growing without rendering quiescent (10.6±2.2% of control cells or 4.7±0.9% of those treated with AICAR were located in sub-G1 fraction, respectively; P<0.01, n=5 each; unpublished data, 2004). Further intensive investigations are required to elucidate for the anti-apoptotic effect of AICAR in SMCs.
In the present study, AICAR increased p53 and p21 protein expression, as well as Ser-15 phosphorylated p53, in HASMCs and isolated rabbit aortas. Our results are consistent with a report by Imamura et al. They reported that AICAR suppressed proliferation via p53 phosphorylation and its accumulation in HepG2 cells. It has also been reported that Ser-15 modification of p53 results in decreased binding affinity between mdm2 and p53, thereby suppressing the degradation of p53 protein, resulting in p53 accumulation. In response to stresses such as hypoxia or DNA damage, p53 is activated by several mechanisms, including the phosphorylation, and the activated p53 induces either cell cycle arrest or apoptosis. In our present study, activation of p53 signaling in HASMCs by AICAR was confirmed using both reporter-gene analysis and RT-PCR analysis for p21(CIP).

It has been reported that adenovirus-mediated overexpression of p21 or p53 inhibits vascular SMC proliferation and suppresses neointima formation in the rat carotid artery, suggesting that p21 or p53 functions to suppress SMC proliferation and neointima formation.

The most striking effect of AICAR was the induction of a CDKI p21(CIP). Inactivation of CDKs by CDKIs maintains Rb at hypophosphorylated state, which keeps E2F inactive. E2F is required for induction of several factors essential for S phase progression; thus, maintaining E2F in an inactive state leads to G1 arrest. In our present study, upregulation of p21(CIP) and decline in Rb phosphorylation by AICAR were observed, indicating that p21(CIP) plays a major role in AICAR-induced G1 arrest in HASMCs.

In the present study, we have demonstrated that AICAR treatment increased Ser-15 phosphorylation and protein expression of p53 in HASMCs and isolated rabbit aortas. Because either dipryridamole or overexpression of DN-AMPK completely blocked these events, it is suggested that either AMPK itself or downstream kinase is involved in AICAR-induced p53 upregulation. Although we have not tested whether Ser-15 phosphorylation of p53 is necessary for the AICAR-induced cell cycle arrest, during the preparation of this article, Jones et al reported that activation of AMPK either by glucose limitation, treatment with AICAR, or overexpression of constitutive active AMPK induced a G1 cell cycle arrest via AMPK-dependent Ser-15 phosphorylation of p53 in primary mouse embryonic fibroblasts. In addition, they demonstrated that Ser-15 phosphorylation of p53 was required for AMPK-induced cell cycle arrest using mouse embryonic fibroblasts derived from p53Ser15Ala mice, in which Ser-18 of mouse p53 corresponding to Ser-15 of human p53 was mutated. Therefore, we speculated that AICAR phosphorylated p53 protein and subsequently increased the amount of both p53 protein and phosphorylated p53 protein because the phosphorylation of p53 protein has been reported to increase the stability of this protein. The Ser-15 phosphorylation of p53 has been reported to be mediated by 3 distinct protein kinases, Ataxia-Telangiectasia Mutated (ATM), and ATM and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK) in response to DNA damage. Jones et al demonstrated that AICAR-induced Ser-15 phosphorylation of p53 was ATM-independent but AMPK-dependent, suggesting the possibility that AMPK may directly phosphorylate Ser-15 of p53. In our present study, it was not tested whether AMPK activation by AICAR could activate ATM, ATR, or DNA-PK and whether these kinases could phosphorylate p53 under our experimental conditions. Further investigation is required to understand the mechanisms of AICAR-induced p53 phosphorylation and accumulation in HASMCs.

In conclusion, this is the first study to show that activation of AMPK by AICAR effectively suppressed cell cycle progression in primary human VSMCs and isolated rabbit aortas, suggesting that AMPK could be a target for the prevention of vascular proliferative disorders such as atherosclerosis.

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Expanded Materials and Methods

Materials

AICAR was obtained from Toronto Research Chemicals (Toronto, Canada). 5'-amino-5'-deoxyadenosine (AMDA; adenosine kinase inhibitor), dipyridamole (adenosine transporter inhibitor), diethylmaleate (DEM) and PDGF-BB were from Sigma Chemical Co. (St. Louis, MO). Antibodies for the total proteins of Rb, p53, p21 and for phosphospecific antibodies for ACC (Ser-79), AMPK (Thr-172), p53 (Ser-15), Rb (Ser-807/811) were obtained from Cell Signaling Technology (Beverly, MA). Antibodies for p27 and actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pan-α-AMPK antibody was from Upstate Biotechnology (Lake Placid, NY). RNase and propidium iodide were from Nakarai Chemicals (Kyoto, Japan).

Cell Culture and Adenoviral Infection of SMCs

Human aortic SMCs (HASMCs) were purchased from Clonetics, Inc (Walkersville, MD). Primary rabbit aortic SMCs (RASMCs) were obtained by the explant method as previously described (1). The cells were cultured and
propagated in Smooth Muscle Cell Growth Medium (Cell Applications Inc, San Diego, CA). For all experiments, early passaged (passages 4-7) SMCs were used. Immunofluorescence for SMC α-actin confirmed SMC identity. A replication-defective adenoviral vector expressing dominant negative (DN)-AMPK (Ad-DN-AMPK) was used to inhibit AMPK activation as described previously (2). An adenoviral vector expressing green fluorescence protein (Ad-GFP) was used for the infection-control experiments. All adenoviral vectors were amplified and titered in 293 cells using standard methodologies. SMCs were infected with or without the indicated adenoviral vectors at a multiplicity of infection of 100 plaque forming units (100 MOI) for 2 h in serum-free DMEM. The medium was then changed to DMEM containing 0.2% FCS. After the incubation for 2 days, infected cells were stimulated with 10 ng/mL PDGF-BB or 15% FCS in the presence or absence of the indicated concentrations of AICAR. In some experiments, cells were pretreated with AICAR for 4 hours. In experiments used inhibitors, inhibitors were added 30 minutes before AICAR treatment.

**Cell proliferation assay**
For cell proliferation assay, we used two different methods, cell counting assay and Alamar Blue assay. For cell counting assay, HASMCs or RASMCs were seeded at 2 X 10^4 cells on 6-well dish. Six hours after plating, cells were serum starved by replacing with DMEM with 0.2% FCS for 2 days to render quiescent. Quiescent SMCs were stimulated with PDGF-BB (10 ng/mL) or FCS (15%) in the presence or absence of indicated concentrations of AICAR for indicated periods. Then cell number was determined with the hemocytometer measurement method described earlier (3).

Alamar Blue assay was performed as described previously (4) with slight modifications. Quiescent cells on 96-well plate were treated as described in cell counting assay. After incubation for indicated period, culture medium was replaced with DMEM containing 10% Alamar Blue (Serotec Inc, Raleigh, NC). After 3 hours, fluorescence was determined by use of excitation/emission wavelengths of 544/ 590 nm in a cytofluorometer (Fluoroskan Asent FL, Thermo Labsystems Co, Boston MA). Cell number was estimated using linear regression analysis of fluorescence readings obtained from predetermined cell numbers.
Control were incubated with DMEM containing 0.2% FCS alone. Our unpublished data demonstrated that quiescent SMCs were able to survive without significant change in cell number for 5 days in this medium.

**Determination of DNA content using Hoechst 33258 dye**

DNA content in SMCs was determined, as an index for cell proliferation or for cytotoxicity, according to the instruction supplied by Thermo Labsystems. Briefly, cells on a 96-well plate were treated for indicated periods as described above. After removing culture media from the wells, cells were washed with PBS twice and frozen. After adding 50 µL of distilled water, followed by two cycles of freezing-thawing, 50 µL of Hoechst 33258 (Nakarai Chemicals) in distilled water (final dye concentration 1.25 µg/mL) were added to each well. Hoechst 33258 reagent produces fluorescence only when bound to double-stranded DNA. Fluorescence was measured with excitation set at 360 nm and emission at 460 nm. Genomic DNA purified from HASMCs was used as a standard to determine DNA concentration. DNA standard curve was prepared using a series of dilutions of the genomic DNA in triplicate and cell number was estimated using linear
regression analysis of fluorescence readings obtained from predetermined cell numbers.

**Cell Cycle Analysis (FACS)**

Cells were harvested, fixed in 70% ethanol and stored at –20 °C. Cells were washed twice with ice-cold PBS, treated with RNase, and then stained for DNA with propidium iodide. The fraction of cells present in each cell cycle phase (G0/G1, S, G2/M) was determined by flow cytometry using a BD Biosciences FACStar flow cytometer (San Jose, CA) and ModiFit software from Verity House (Topsham, ME).

**Trypan Blue Exclusion**

Quiescent SMCs in 35-mm dishes were treated without or with 1 mM AICAR, or 1 mM DEM for 24 or 48 hours in DMEM containing 15% FCS, and were then incubated with 0.4% trypan blue after the removal of the medium. After 5 minutes, the cells in the dishes were counted. Cells with blue staining were counted as nonviable cells.

**Detection of apoptosis**
A sandwich ELISA method was used to assess apoptosis using Cell Death ELISA plus kit (Roche, Palo Alto, CA). This method is based on the quantification of the enrichment of mono- and oligonucleosomes in the cytoplasm. Quiescent subconfluent cell monolayers on 24-well dish were incubated without or with the indicated concentrations of AICAR for 48 hours, or 1 mM DEM for 24 hours in DMEM containing 15% FCS. Cells were lysed and centrifuged. The supernatant containing cytoplasmic histone-associated DNA fragments was transferred to a microplate coated with streptavidin and then reacted with a mixture of anti-histone antibodies labeled with biotin and anti-DNA antibodies coupled to peroxidase. The peroxidase substrate was then added, and color development was read photometrically at 405 nm against 490 nm as the background. In addition, apoptotic cells containing sub G1 (apoptotic) nuclei were also identified and counted by flow cytometry analysis as described previously (5).

**Real-time RT-PCR**

Total RNA was extracted from HASMCs treated without or with AICAR in the absence or presence of 15% FCS and then used for reverse transcription to
amplify cDNAs using Revertra Ace (TOYOBO, Japan). Real-time PCR with SYBR green dye was carried out using a LightCycler instrument (Roche Diagnostics, Meylan, France). Primers for human p21\textsuperscript{CIP}, p53 and GAPDH were prepared as described previously (6). The PCR conditions were also performed according to this report.

**Reporter assay (Luciferase assay)**

For assessment of the effect of AICAR on p53-dependent transcription, analytical plasmid pp53-TA-Luc (BD Biosciences), which contains a p53 response element, located upstream of the firefly luciferase reporter gene, was used. HASMCs were co-transfected with 500 ng of pp53-TA-Luc and 25 ng of the internal control plasmid pHRL-CMV (Promega, Madison, WI) with the use of FuGENE6 transfection reagent (Roche). Cells were serum-deprived for 24 hours and then incubated without or with 1mM AICAR in the presence of 15% FCS for 12 hours. The cell lysates were assayed sequentially for firefly and renilla luciferase activities with the Dual-Luciferase Reporter Assay System (Promega) as described previously (7).
Western blots

Western blotting was performed essentially as previously reported (8,9). 30 μg of protein was resolved by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes and immunoblotting was performed with the indicated monoclonal or polyclonal antibodies. Following incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were detected with ECL plus kit (Amersham, Piscataway, NJ) according to the instructions provided by the manufacturer. Immunoreactive bands were quantified by NIH image analysis software.

Ex Vivo Experiments

Male Japanese white rabbits (2.5 to 3.5 kg body weight) purchased from Kyudo Co Ltd. (Saga, Japan) were used in the present study. The rabbits were euthanized by overdose of Inactin. The descending thoracic aorta was rapidly excised under sterile condition, transferred to a petri dish containing serum-free DMEM with the addition of antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) and cleaned of connective tissues. The endothelium was removed
by gently rubbing the vessel with wet cotton swab, and removal of the endothelium was confirmed by the lack of endothelial nitric oxide synthase (eNOS) proteins detected by western blot (data not shown). The aorta was cut into 5-mm-wide rings and the rings were cut open into strips. Strips were washed gently with serum-free DMEM. Each strip was pretreated without or with 1 mM AICAR in serum-free DMEM for 4 hours and stimulated with 15% FCS in the presence or absence of the same concentrations of AICAR for indicated periods. After the incubation for indicated periods, strips were washed with PBS, frozen in liquid N$_2$ and stored at −80 °C until analysis. The frozen strips were pulverized and then homogenized in the lysis buffer on ice using a homogenizer. Equal amounts of protein samples were subjected to SDS-PAGE and Western Blots were carried out as described above.

**Statistical Analyses**

Quantitative data are presented as mean ± SEM of at least three independent experiments. Statistical analysis was based on Student's t-test for paired or unpaired data as appropriate. A $p$ value less than 0.05 was used to
determine statistical significance.

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Figure Legends for Supplemental Figures

Supplemental Figure 1

AICAR inhibited RASMC proliferation

A-B, Dose-dependent effect of AICAR on cell number of RASMCs stimulated by PDGF-BB (A) or FCS (B), respectively. Quiescent cells on 6-well dish were stimulated with PDGF-BB (10 ng/mL) or FCS (15%) in the presence (solid bars) or absence (open bars) of indicated concentrations of AICAR for 4 days. Cell number was determined by the cell counting. Data are shown as mean ± SEM from three separate experiments. *p < 0.05, **p < 0.01 as compared with PDGF or 15% FCS alone.

Treatment of RASMCs with AICAR inhibited PDGF- and FCS-induced proliferation in a dose-dependent manner as similar to those observed in HASMCs (Figure 1A-B).

Supplemental Figure 2

AMPK activation by AICAR inhibited Rb phosphorylation in HASMCs

A, Quiescent HASMCs were pre-treated without or with 1 mM AICAR in
DMEM containing 0.2% FCS for 4 hours. Then cells were stimulated without or with 10 ng/mL PDGF-BB for the indicated periods.

B: HASMCs were not infected ((Infection (-)) or infected with Ad-GFP or Ad-DN-AMPK, and were made quiescent. Then cells were treated without or with 1 mM AICAR for 4 hours and then stimulated with 10 ng/mL PDGF-BB for 24 hours. Western blot analyses were performed to detect total Rb and phosphorylated Rb (p-Rb). The expression of DN-AMPK was confirmed by blotting with anti-c-myc antibody. Representative blots of 3 independent experiments are shown.

Our preliminary experiments revealed that stimulation with 10 ng/mL PDGF-BB for 24 hours could phosphorylate Rb at similar levels in non-infected or infected HASMCs with either Ad-GFP or Ad-DN-AMPK. As shown in Supplemental Figure 2A, Rb phosphorylation could be detected 12 hours after PDGF-stimulation in cells treated without AICAR. AICAR significantly inhibited PDGF-induced Rb phosphorylation at both time points (12 and 24 hours after PDGF-stimulation). As shown in Supplemental Figure 2B, AICAR strongly
suppressed Rb phosphorylation induced by PDGF-BB in non-infected and GFP-infected HASMCs. However, HASMCs overexpressing DN-AMPK were highly resistant to AICAR-induced inhibition of Rb phosphorylation.

Supplemental Figure 3

Overexpression of DN-AMPK but not of control GFP inhibited cell cycle arrest induced by AICAR

The distribution of cells in G0/G1, S or G2/M phase of cell cycle was measured by flow cytometer. Quiescent HASMCs infected with indicated adenoviral vector were treated without or with 1 mM AICAR in the presence of 15% FCS for 24 hours. Dipyridamole was added to cells infected with Ad-GFP 30 min before treatment with AICAR. Data are expressed as a percentage of total cells. Each value represents mean ± SEM from 4 experiments. *p < 0.01 as compared with AICAR-treated in Ad-GFP-infected cells.

AICAR induced cell cycle arrest in GFP-infected HASMCs as shown in Figure 5A. Overexpression of DN-AMPK significantly blocked AICAR-induced cell cycle arrest. Dipyridamole also significantly inhibited AICAR-induced cell cycle arrest.
We had confirmed that vehicle (DMSO) did not influence the cell cycle distribution at 0.02%, which we used in the present study (data not shown).

Supplemental Figure 4

AICAR increased mRNA expression of p21$^{CIP}$ and increased p53-dependent transcription in HASMC

A, Real-time RT-PCR analyses for mRNA expression of p21$^{CIP}$ (solid bars) or p53 (open bars) versus GAPDH, respectively. HASMCs were treated without or with 1 mM AICAR in the absence or presence of 15% FCS for 12 hours. Real-time RT-PCR analyses were performed as described in Expanded Materials and Methods. Data are expressed as mean ± SEM (n = 4). *p < 0.05 as compared with the cells incubated with 15% FCS alone and #p < 0.05 as compared with the cells incubated without FCS nor AICAR.

The expression of p21$^{CIP}$ mRNA in AICAR-treated HASMCs significantly increased compared with that in either non-treated or HASMCs treated with FCS alone. On the other hand, p53 mRNA expression did not change by FCS nor AICAR treatment. These results indicate that increased p21$^{CIP}$ protein
expression is, at least in part, due to the increased mRNA expression of p21<sub>CIP</sub> and that increased p53 protein expression is not mediated by transcriptional mechanism suggesting the involvement of post-transcriptional mechanisms including protein modification such as phosphorylation.

B. Reporter assay for p53-dependent transcription. Cells co-transfected with pp53-TA-Luc and phRL-CMV were serum-deprived for 24 hours and then incubated without or with 1mM AICAR in the absence or presence of 15% FCS for 12 hours. Reporter assay were performed as described in Expanded Materials and Methods. Promoter activity is expressed as the ratio of firefly to renilla luciferase activity (relative light units (RLU)). *p < 0.01 as compared with HASMCs treated with 15% FCS alone and #p < 0.01 as compared with non-treated cells.

AICAR treatment significantly increased p53-dependent transcription compared with either non-treated or FCS-treated HASMCs without AICAR.
Supplemental Figure S1

S1A

Cell number (x 10^4 / well)

Control

0.1 0.25 0.5 1.0

AICAR (mM)

10 ng/mL PDGF-BB

S1B

Cell number (x 10^4 / well)

Control

0.1 0.25 0.5 1.0

AICAR (mM)

15% FCS
Supplemental Figure S2

S2A

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S2B

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PDGF-BB / 24h
Supplemental Figure S3

% of total cells

- GFP + AIC (−)
- GFP + AIC (+)
- DN-AMPK + AIC (+)
- GFP + AIC (+) + Dipyridamole

× × × × ×
Supplemental Figure S4

S4A

![Graph showing mRNA/GAPDH expression levels for p21 and p53 under different conditions.]

S4B

![Graph showing RLU (%) under different conditions.]

S4C

![Western blot images showing expression levels of p-ACC, p-p53, p53, Actin, and AICAR treatment conditions.]

Legend:
- AICAR
- 15% FCS
- DMSO
- Dipyri
- AMDA
- p21
- p53
- p-ACC
- p-p53
- p53
- Actin

[Statistical symbols: * and # indicate significance levels.]