Activation of AMP-activated Protein Kinase Suppresses Oxidized Low-density Lipoprotein-induced Macrophage Proliferation*

Received for publication, June 1, 2009, and in revised form, October 15, 2009. Published, JBC Papers in Press, October 20, 2009, DOI 10.1074/jbc.M109.028043

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Macrophage-derived foam cells play important roles in the progression of atherosclerosis. We reported previously that ERK1/2-dependent granulocyte/macrophage colony-stimulating factor (GM-CSF) expression, leading to p38 MAPK/Akt signaling, is important for oxidized low density lipoprotein (Ox-LDL)-induced macrophage proliferation. Here, we investigated whether activation of AMP-activated protein kinase (AMPK) could suppress macrophage proliferation. Ox-LDL-induced proliferation of mouse peritoneal macrophages was assessed by [3H]thymidine incorporation and cell counting assays. The proliferation was significantly inhibited by the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) and restored by dominant-negative AMPKαε, suggesting that AMPK activation suppressed macrophage proliferation. AICAR partially suppressed Ox-LDL-induced ERK1/2 phosphorylation and GM-CSF expression, suggesting that another mechanism is also involved in the AICAR-mediated suppression of macrophage proliferation. AICAR suppressed GM-CSF-induced macrophage proliferation without suppressing p38 MAPK/Akt signaling. GM-CSF suppressed p53 phosphorylation and expression and induced Rb phosphorylation. Overexpression of p53 or p27kip suppressed GM-CSF-induced macrophage proliferation. AICAR induced cell cycle arrest, increased p53 phosphorylation and expression, and suppressed GM-CSF-induced Rb phosphorylation via AMPK activation. Moreover, AICAR induced p21cip and p27kip expression via AMPK activation, and small interfering RNA (siRNA) of p21cip and p27kip restored AICAR-mediated suppression of macrophage proliferation. In conclusion, AMPK activation suppressed Ox-LDL-induced macrophage proliferation by suppressing GM-CSF expression and inducing cell cycle arrest. These effects of AMPK activation may represent therapeutic targets for atherosclerosis.

Macrophages are well known to be present in all stages of atherosclerosis and are considered to be fundamental to atherogenesis and the behavior of established plaques (1). Macrophages take up oxidized low density lipoprotein (Ox-LDL) through scavenger receptor pathways and transform into foam cells in vitro (2). Foam cells produce various bioactive molecules, such as cytokines and growth factors, which are believed to play important roles in the development and progression of atherosclerosis (1). One of the characteristic events in atherosclerotic lesions is the proliferation of cells, including vascular smooth muscle cells and macrophages, within arterial walls. Indeed, previous in vivo studies have reported that macrophages and macrophage-derived foam cells proliferate in atherosclerotic lesions (3–5). We (6–8) and others (9, 10) have shown that Ox-LDL enhances macrophage proliferation and survival in vitro. Therefore, it is possible that macrophage proliferation may promote the progression of atherosclerosis.

We previously reported that Ox-LDL-induced production of granulocyte/macrophage colony-stimulating factors (GM-CSFs) plays an important role in the growth signaling pathway for Ox-LDL-induced macrophage proliferation (11, 12). Moreover, we recently reported that the p38 MAPK/phosphatidylinositol 3-kinase/Akt signaling pathway is involved, at least in part, in the downstream signaling pathways after GM-CSF expression (8, 13).

AMP-activated protein kinase (AMPK) belongs to a protein kinase family that has been highly conserved throughout evolution in animals, plants, and yeast and plays major roles in cell responses to metabolic stress (14–16). AMPK is a heterotrimeric protein consisting of a catalytic α subunit and regulatory β and γ subunits (17). Each α and β subunit is encoded by two genes.

*This work was supported by grants-in-aid for scientific research from the Japan Society for the Promotion of Science, Japan (21591144 to T. M. and 20390259 to E. A.), a grant from the Takeda Science Foundation (to T. M.), and the Advanced Education Program for Integrated Clinical, Basic and Social Medicine, Graduate School of Medical Sciences, Kumamoto University (Support Program for Improving Graduate School Education, MEXT, Japan).

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3 The abbreviations used are: Ox-LDL, oxidized low density lipoprotein; ACC, acetyl-CoA carboxylase; Ad, adenovirus; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; DN, dominant-negative; CDKIs, cyclin-dependent kinase inhibitors; ERK1/2, extracellular signal-regulated kinase 1/2; ELISA, enzyme-linked immunosorbent assay; MAPK, mitogen-activated protein kinase; SMC, smooth muscle cell; siRNA, small interfering RNA; WT, wild-type.
AMPK Suppresses Ox-LDL-induced Macrophage Proliferation

genes (α1 and α2 or β1 and β2), whereas the γ subunit is encoded by three genes (γ1, γ2, and γ3). The protein is activated in response to increased ratios of AMP to ATP within the cell and therefore acts as an efficient sensor for the cellular energy state. AMP activates AMPK by direct allosteric activation and by protecting dephosphorylation of threonine residue (Thr-172) within the activation domain of the α subunit by inhibiting protein phosphatase 2Co (17, 18). One of the downstream targets of AMPK is the regulation of lipid metabolism. It is well known that AMPK directly phosphorylates and inactivates acetyl-CoA carboxylase (ACC), thereby suppressing malonyl-CoA production. The reduction in malonyl-CoA activates acetyl-CoA carboxylase (ACC), thereby suppressing malonyl-CoA production. The reduction in malonyl-CoA accelerates the entry of long-chain acyl-CoA into mitochondria for β-oxidation to restore the energy balance (17). 5-Aminooimidazole-4-carboxamide ribonucleoside (AICAR) is a well known activator of AMPK. AICAR is transported into cells through adenosine transporters and phosphorylated by adenosine kinase (19) to form 5- aminooimidazole-4-carboxamide-1-Ribofuranosyl-5′-monophosphate, which mimics the stimulatory action of AMP on AMPK (20). Recently, we (21) and others (22) reported that the proliferation of vascular smooth muscle cells (SMCs) is suppressed by activation of AMPK. On the other hand, Jhun et al. (23) reported that AICAR suppresses lipopolysaccharide-induced tumor necrosis factor-α expression in RAW264.7 murine macrophages. Therefore, AMPK activation may protect against the acceleration of atherosclerosis by suppressing SMC proliferation and inactivating macrophages. However, there is no further evidence regarding the issue of whether AMPK activation can suppress atherosclerotic events in macrophages, such as their proliferation.

In the present study, we investigated the effects of AMPK activation on Ox-LDL-induced macrophage proliferation. We found that AICAR-mediated AMPK activation suppressed Ox-LDL-induced macrophage proliferation by suppressing GM-CSF expression and inducing cell cycle arrest.

EXPERIMENTAL PROCEDURES

Materials—AICAR was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Antibodies against the total proteins of AMPKα, Rb, p53, and p21cip1 and phosphospecific antibodies against ACC (Ser-79), AMPKα1 (Thr-172), p53 (Ser-15), Rb (Ser-807/811), ERK1/2, p38 MAPK, and Akt were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against the total proteins of ERK1/2, p38 MAPK, Akt, p27kip1, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of the best grade available from commercial sources.

Lipoprotein Preparation—Human LDL (d = 1.019–1.063 g/ml) was isolated by ultracentrifugation from plasma samples obtained by consenting normolipidemic subjects after an overnight fast (7). LDL was dialyzed against 0.15 M NaCl and 1 mM EDTA (pH 7.4). Ox-LDL was prepared by incubation of LDL with 5 μM CuSO4 for 20 h at 37 °C, followed by the addition of 1 mM EDTA and cooling (7). The protein concentrations were determined using the BCA protein assay reagent (Pierce). The endotoxin level in the prepared Ox-LDL was <1 pg/μg protein, as measured using a Toxicolor System (Seikagaku Corp., Tokyo, Japan) (7).

Cell Cultures—The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Kumamoto University. Peritoneal macrophages were collected from anesthetized male C3H/He mice (25–30 g) by peritoneal lavage with 8 ml of phosphate-buffered saline, centrifuged at 200 × g for 5 min, resuspended in medium A (RPMI 1640 medium (Nissui Seiyaku Co., Tokyo, Japan)) supplemented with 10% fetal calf serum (Invitrogen), 0.1 mg/ml streptomycin, and 100 units/ml penicillin and incubated in appropriate tissue culture plates for 90 min (7). More than 98% of the adherent cells were considered to be macrophages based on four criteria, as described previously (24, 25).

Infection with Adenoviral Vectors—Adenoviruses expressing wild-type (WT)-AMPKα1 (Ad-AMPKα1) and dominant-negative (DN)-AMPKα1 (Ad-DN-AMPKα1), which serves as a nonphosphorylatable T172A mutant of the AMPKα1-subunit (26) and contains a c-myc tag at the NH2 terminus, were used as described previously (27). An adenovirus vector that expresses WT p53 (Ad-p53) was kindly gifted from Dr. Shinji Ishikawa (Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan). Mouse peritoneal macrophages were infected with the indicated adenoviral vectors at a multiplicity of infection of ~100, as described previously (28) and allowed to recover in medium A for 2 h. These conditions conferred expression of LacZ using adenoviruses expressing LacZ (Ad-LacZ) as a marker gene in nearly 100% of the transfected cells.

Transfection of Plasmid—An expression plasmid of human p27kip1 (pcDNA3FLAG-hp27) was kindly gifted from Dr. Masaki Matsumoto (Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan). Mouse peritoneal macrophages (2 × 106 cells/well) were transfected with pcDNA3FLAG-hp27 using Lipofectamine 2000 (Invitrogen, Japan, K.K. Tokyo, Japan) and incubated for 4 h. Then, the medium was removed, and cells were resuspended with medium A (29). After a 24-h incubation, cells were treated with recombinant GM-CSF, and Western blot assay and tritiated thymidine incorporation assay were performed as described below.

Transfection of siRNA—The siRNAs against p21cip1 and p27kip1 and an irrelevant 21-nucleotide siRNA duplex as a control were purchased from Santa Cruz Biotechnology. Mouse peritoneal macrophages (2 × 106 cells/well) were transfected with the siRNA of p21cip1, p27kip1, or control using Lipofectamine 2000 (Invitrogen) as described above.

Tritiated Thymidine Incorporation and Cell Counting Assays—Macrophage monolayers (2 × 106 cells/well) were cultured in 24-well tissue culture plates (15.5 mm in diameter; Corning Glass Works, Corning, NY) in the presence of various effectors for 6 days. For thymidine incorporation assays, the cells were incubated with 1 μCi/ml [3H]thymidine for 18 h before the termination of the experiments. Tritiated thymidine incorporation assays were performed as described previously (7). For cell counting assays, cultured cells were lysed in 1% (w/v) Triton X-100, and naphthol blue-black-stained nuclei were counted in a hemocytometer as described previously (11, 13).
Western Blot Analysis—Macrophages (2 × 10^6 cells/well) were incubated with various effectors, and whole cell lysates were purified as described previously (8). The protein concentrations were determined using the Micro BCA protein assay reagent (Pierce). Samples were separated by electrophoresis in 10% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). The membranes were incubated with appropriate primary antibodies at a dilution of 1:1,000 for 2 h. After washing, the membranes were stained with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) (8). Immunoreactive bands were quantified using National Institutes of Health Image analysis software (8).

Enzyme-linked Immunosorbent Assay (ELISA) for GM-CSF—Macrophages (5 × 10^6 cells/plate) were cultured with various effectors, followed by the addition of 20 μg/ml Ox-LDL. After 4 h of incubation, the media were collected, and the GM-CSF protein concentrations were determined as described previously (10).

Cell Cycle Analysis by Flow Cytometry—Macrophages (1 × 10^6 cells/well) were incubated with various effectors for 48 h (apoptosis assays) or 96 h (cell cycle analyses). The cells were then fixed with 70% ethanol, treated with RNase A (0.25 mg/ml), and stained with propidium iodide (0.02 mg/ml). The fractions of cells present in the different cell cycle phases (G0/G1, S, and G2/M) were determined by flow cytometry using a FACStar flow cytometer (BD Biosciences) and the ModFit software (Verity House, Topsham, ME) (21).

Apoptosis Assay—Macrophages (1 × 10^5 cells/well) were incubated with various effectors for 48 h and then subjected to quantification of cytoplasmic histone-associated DNA fragments using a cell death detection ELISA kit (Roche Applied Science). In addition, the percentages of sub-G0/G1 cells were analyzed by flow cytometry as described above.

Statistical Analysis—All data were expressed as the mean ± S.E. Differences between groups were examined for statistical significance by one-factor analysis of variance. Values of \( p < 0.01 \) were considered to indicate a statistically significant difference.

RESULTS

AICAR Inhibits Ox-LDL-induced Macrophage Proliferation—It has been reported that mouse peritoneal macrophages express high levels of AMPKα1 but express low levels of
AMPK Suppresses Ox-LDL-induced Macrophage Proliferation

AMPKα2 (23). Therefore, we focused on AMPKα1 and investigated whether AICAR could induce AMPKα1 activation in mouse peritoneal macrophages. AICAR phosphorylated AMPKα1 and ACC, one of the target molecules of AMPK, in dose-dependent manners (Fig. 1A). Moreover, our time course experiments revealed that AICAR-induced phosphorylation of AMPKα1 and ACC was observed as early as 1 h, which sustained until 7 days (Fig. 1, B and C). These results confirmed that AICAR activated AMPKα1 and its downstream signals in macrophages.

Next, we examined the effects of AICAR on OX-LDL-induced macrophage proliferation. Ox-LDL (20 μg/ml) significantly increased [3H]thymidine incorporation into macrophages (Fig. 1D), as previously reported (6–8, 12). Pretreatment with AICAR at concentrations of 50 μM or higher suppressed the Ox-LDL-induced increase in [3H]thymidine incorporation in a dose-dependent manner (Fig. 1D). Cell counting assays confirmed that the Ox-LDL-induced increase in cell number was suppressed by AICAR (Fig. 1E).

To clarify the involvement of AMPK activation in AICAR-mediated suppression of macrophage proliferation, we examined the effects of WT-AMPKα1 and DN-AMPKα1 on macrophage proliferation. Expression of WT-AMPKα1 by an adenovirus vector caused ~2-fold increases in total AMPK expression and AMPKα1 phosphorylation (Fig. 2A) and suppressed Ox-LDL-induced macrophage proliferation (Fig. 2B). Moreover, overexpression of DN-AMPKα1 suppressed the phosphorylation of AMPKα1 and ACC (Fig. 2C) and restored AICAR-mediated suppression of macrophage proliferation (Fig. 2D), suggesting that AICAR-mediated suppression of macrophage proliferation was caused by activation of AMPK.

AICAR-induced Macrophage Apoptosis Is Not the Main Mechanism for the Suppression of Macrophage Proliferation—It has been reported that activation of AMPK induces apoptosis in human B-cell chronic lymphocytic leukemia cells (30) and human neuroblastoma cells (31). Therefore, we examined the effects of AICAR on macrophage apoptosis. At 1,000 μM, AICAR induced macrophage apoptosis (Fig. 3A). However, AICAR at concentrations of 500 μM or lower, which can suppress macrophage proliferation, did not induce macrophage apoptosis (Fig. 3A). To obtain further evidence, the percentages of sub-G0/G1 cells were quantified by fluorescence-activated cell sorter analysis using propidium iodide staining (Fig. 3, B and C). Neither 10 μM nor 100 μM AICAR increased the percentage of sub-G0/G1 cells in both 48 h and 7 days incubation,
AMPK Suppresses Ox-LDL-induced Macrophage Proliferation

AMPK Suppresses Ox-LDL-induced Macrophage Proliferation

AICAR Partially Suppresses Ox-LDL-induced GM-CSF Expression—We previously reported that ERK1/2-dependent GM-CSF expression is mainly involved in Ox-LDL-induced macrophage proliferation (8, 11). Therefore, we investigated the effects of AICAR on GM-CSF-induced macrophage proliferation. AICAR at 100 µM, which can suppress macrophage proliferation by 85% based on the cell counting assay, slightly but significantly suppressed Ox-LDL-induced GM-CSF protein (Fig. 4A) and mRNA (Fig. 4B) expressions by 29 and 25%, respectively. Moreover, 100 µM AICAR markedly increased the percentage of sub-G0/G1 cells (Fig. 3, B and C). Therefore, mechanisms other than apoptosis are involved in the suppression of macrophage proliferation by low concentrations of AICAR.

AICAR Suppresses Ox-LDL-induced GM-CSF Production and ERK1/2 Phosphorylation—To clarify possible mechanisms other than GM-CSF production for the AICAR-mediated suppression of macrophage proliferation, we examined the effects of AICAR on GM-CSF-induced macrophage proliferation. At 100 µM, GM-CSF significantly increased [3H]thymidine incorporation into macrophages (Fig. 5A), as previously reported (8). Pretreatment with AICAR at concentrations of 50 µM or higher dose-dependently suppressed the GM-CSF-induced increase in [3H]thymidine incorporation (Fig. 5A). Cell counting assays confirmed that the GM-CSF-induced increase in cell number was suppressed by AICAR (Fig. 5B). Overexpression of WT-AMPKα1 suppressed GM-CSF-induced macrophage proliferation (Fig. 5C). Moreover, overexpression of DN-AMPKα1 restored AICAR-mediated suppression of macrophage proliferation (Fig. 5D). These results suggested that AICAR-mediated AMPK activation suppressed GM-CSF-induced macrophage proliferation.

AICAR Induces Cell Cycle Arrest without Suppression of p38MAPK/Akt Signal—We previously reported that GM-CSF expression is involved in Ox-LDL-induced macrophage proliferation (8, 11). Moreover, among the downstream signals of GM-CSF release, p38 MAPK and its subsequent signaling molecules phosphatidylinositol 3-kinase and Akt are mainly involved in Ox-LDL-induced macrophage proliferation. Therefore, we investigated the effects of AICAR on GM-CSF-induced phosphorylation of p38 MAPK and Akt. GM-CSF increased the phosphorylation of p38 MAPK and Akt (Fig. 6A), as reported previously (8). Surprisingly, treatment with AICAR alone also induced phosphorylation of p38 MAPK and Akt, and AICAR enhanced the GM-CSF-induced phosphorylation of p38 MAPK and Akt (Fig. 6A), suggesting that p38 MAPK/Akt signaling was not the main target for AICAR-mediated suppression of macrophage proliferation.

Next, we investigated the effects of AICAR on GM-CSF-induced cell cycle progression by flow cytometry. Compared with control cells treated with GM-CSF, AICAR significantly increased the percentage of cells in G0/G1 phase (from 80.3 ± 1.5% to 93.4 ± 1.8%) and decreased the percentages in S phase (from 13.2 ± 0.9% to 6.1 ± 0.5%) and G2/M phase (from 7.2 ± 0.7% to 2.5 ± 0.3%) (Fig. 6B), suggesting that AICAR induced G1 arrest in the proliferating macrophages.

AICAR Increases the Expression of p21cip and p27kip, Thereby Suppressing Macrophage Proliferation—Based on the above findings, we investigated the effects of AICAR on the phosphorylation and expression of p53, which is a suppressor of cell cycle progression. Treatment with GM-CSF suppressed the phosphorylation and expression of p53 (Fig. 6, C and D). Pretreatment with AICAR restored the GM-CSF-induced suppression of phosphorylation and expression of p53, and these effects by AICAR were abrogated by the treatment with DN-AMPKα1 (Fig. 6, C and D). Interestingly, treatment with AICAR alone increased the phosphorylation and expression of p53 (Fig. 6, C and D). Moreover, treatment with GM-CSF increased the phosphorylation of Rb (Fig. 6, C and E), which is a regulator of cell cycle progression, and pretreatment with AICAR suppressed this effect (C and E).

We further investigated the effects of AICAR on the expression of the cyclin-dependent kinase inhibitors (CDKIs) p21cip and p27kip. Treatment with GM-CSF decreased p21cip expression (Fig. 6, C and D). Pretreatment with AICAR rescued the GM-CSF-mediated suppression of p21cip expression, and these effects by AICAR were abrogated by the treatment with DN-AMPKα1 (Fig. 6, C and D). On the other hand, treatment with GM-CSF did not affect p27kip expression (Fig. 6, C and E), whereas pretreatment with AICAR increased p27kip expression.
Moreover, treatment with DN-AMPK/H9251 attenuated these effects by AICAR (Fig. 6, C and E). Interestingly, treatment with AICAR alone increased the expression of p21cip and p27kip (Fig. 6, C–E).

We next investigated the effects of overexpression of p21cip and p27kip on GM-CSF-induced macrophage proliferation. Introduction of Ad-p53 increased the expression and phosphorylation of p53, increased the expression of p21cip, and suppressed GM-CSF-induced increase in [3H]thymidine incorporation into macrophages (Fig. 7, A and B). Moreover, introduction of pcDNA3FLAG-hp27 increased the expression of p27kip and suppressed GM-CSF-induced increase in [3H]thymidine incorporation (Fig. 7, C and D). Finally, we investigated the effects of siRNA for p21cip and/or p27kip on GM-CSF-induced macrophage proliferation. In comparison with the control siRNA, siRNA for p21cip and p27kip suppressed the AICAR-induced expression of p21cip and p27kip, respectively (Fig. 7, E and F). Moreover, treatment with p21cip siRNA and p27kip siRNA restored AICAR-mediated suppression of macrophage proliferation, and additive effect of the siRNAs was observed (Fig. 7G), suggesting that AICAR-mediated increase in the expression of p21cip and p27kip is involved in AICAR-mediated suppression of macrophage proliferation.

**DISCUSSION**

AMPK is a serine/threonine protein kinase that serves as an energy sensor in all eukaryotic cells (14). Several studies have indicated that AMPK activation by AICAR strongly suppresses cell proliferation in hepatoma HepG2 cells (32) and mouse embryonic fibroblasts (33). Moreover, we recently reported that AMPK activation suppresses proliferation in human aortic SMCs and rabbit aortic strips (21). In the present study, we have demonstrated that AICAR-mediated AMPK activation also suppresses Ox-LDL-induced macrophage proliferation.

Activation of AMPK induces apoptosis in human B-cell chronic lymphocytic leukemia cells (30) and human neuroblastoma cells (31). Consequently, we speculated that the inhibitory effects of AMPK activation would be mediated by macrophage apoptosis. In fact, we found for the first time that 1 mM AICAR induced macrophage apoptosis. However, suppression of macrophage proliferation was still observed at low concentrations of AICAR (<1 mM). Therefore,
mechanisms other than apoptosis are involved in the suppression of macrophage proliferation.

Our previous studies indicated that Ox-LDL-induced GM-CSF production is mainly involved in macrophage proliferation (8, 11). Therefore, the mechanisms of Ox-LDL-induced macrophage proliferation can be divided into two parts: (i) intracellular signaling pathway before GM-CSF release, and (ii) proliferation signaling pathway through GM-CSF receptors. We demonstrated previously that Ox-LDL induces ERK1/2 activation and subsequently leads to GM-CSF expression in macrophages (8, 11). Hence, ERK1/2-dependent GM-CSF expression is one of the key phenomena for Ox-LDL-induced macrophage proliferation. AMPK activation suppresses angiotensin II-induced ERK1/2 activation in SMCs (22). On the other hand, AMPK activation does not suppress lipopolysaccharide-induced ERK1/2 activation in RAW264.7 cells (23). Therefore, the effects of AMPK activation on ERK1/2 activation may depend on the cell types involved or its inducers. In the present study, we found that AMPK activation suppressed Ox-LDL-induced ERK1/2 activation by 22% and GM-CSF protein and mRNA expressions by 29 and 25%, respectively, in mouse peritoneal macrophages. These results suggest that suppression of the GM-CSF expression pathway is only partially involved and that other mechanisms, such as modification of the downstream signaling pathway of GM-CSF release, are involved in AMPK-mediated suppression of macrophage proliferation.

As expected, we found that AMPK activation suppressed the macrophage proliferation induced by GM-CSF, suggesting that AMPK activation inhibits Ox-LDL-induced macrophage proliferation by suppressing the subsequent signaling of GM-CSF release. We further found that AMPK activation did not suppress p38 MAPK/Akt signaling, which is involved in GM-CSF-induced macrophage proliferation. Therefore, we speculated that the suppressive effects of AMPK on Ox-LDL-induced macrophage proliferation depended on cell cycle arrest, and subsequently found that AICAR significantly increased the percentage of cells in G0/G1 phase and decreased the percentages in S phase and G2/M phase. Therefore, AMPK-induced cell cycle arrest should be the main cause of the AMPK-mediated suppression of macrophage proliferation.

Mammalian cell proliferation is controlled by the cell cycle machinery. Cell cycle progression is managed positively by CDKs and their cyclin-regulatory subunits (34) and managed negatively by CDKIs and tumor suppressor genes (35). Mito-
AMPK Suppresses Ox-LDL-induced Macrophage Proliferation

FIGURE 7. AICAR-induced expression of p21\textsuperscript{cip} and p27\textsuperscript{kip} is involved in AICAR-mediated suppression of macrophage proliferation. Macrophages were infected with adenoviral vectors containing LacZ (Ad-LacZ) or wild-type p53 (Ad-p53) (A and B), transfected with empty plasmid (mock) or plasmid containing p27\textsuperscript{kip} (C and D), or transfected with siRNA for control (cont), p21\textsuperscript{cip} (p21), or p27\textsuperscript{kip} (p27) (E–G), and the cells were incubated for 24 h (C–G) or 48 h (A and B) Then the cells were treated with 10 psu GM-CSF in the absence or presence of 100 μM AICAR for 24 h (A, C, E, and F) or 5 days (B, D, and G). A, C, E, and F, protein samples were immunoblotted with anti-phospho-p53 (p-p53), anti-p53, anti-p21\textsuperscript{cip}, anti-p27\textsuperscript{kip}, or anti-β-actin antibodies. B, D, and G, \(^{[3}H\)thymidine incorporation assays were performed. Data represent the means ± S.E. of four separate experiments. *, \(p < 0.01\), compared with untreated cells infected with Ad-LacZ. **, \(p < 0.01\), compared with Ad-LacZ-infected cells incubated with GM-CSF alone. †, \(p < 0.01\), compared with untreated cells transfected with mock. ††, \(p < 0.01\), compared with transfected with mock and incubated with GM-CSF alone. #, \(p < 0.01\), compared with untreated cells transfected with control siRNA. ##, \(p < 0.01\), compared with cells transfected with control siRNA and incubated with GM-CSF alone. $, \(p < 0.01\), compared with cells transfected with control siRNA and incubated with GM-CSF plus AICAR.

CDKIs, such as p21\textsuperscript{cip} and p27\textsuperscript{kip}, negatively regulate the cell cycle by inhibiting cyclin/CDK activities and Rb phosphorylation, resulting in G\textsubscript{1} arrest (36). On the other hand, the expression and functions of the tumor suppressor p53 are tightly regulated by its phosphorylation state. Cellular stresses, such as \(γ\)-irradiation and glucose deprivation, induce phosphorylation of p53 at Ser-15 (37, 33). The phosphorylated p53 induces cell-growth arrest and/or apoptosis through transcriptional regulation of p53 response genes such as p21\textsuperscript{cip} and p53AIP1 (39). It has been reported that AMPK activation by AICAR inhibits the proliferation of various cancer cell lines in vitro and in vivo by increasing p21\textsuperscript{cip}, p27\textsuperscript{kip}, and p53 (38). Our group previously reported that AMPK activation suppresses the proliferation of vascular SMCs by increasing the phosphorylation of p53 and subsequent expression of p21\textsuperscript{cip} (21). In macrophages, we newly found that GM-CSF suppressed the phosphorylation of p53 and expression of p21\textsuperscript{cip} and that AICAR restored these effects. Interestingly, expression of p27\textsuperscript{kip} was not abundant in unstimulated macrophages and remained unaffected by GM-CSF. However, AICAR drastically increased the expression of p27\textsuperscript{kip}. Moreover, we found that AICAR alone increased the phosphorylation and expression of p53 and the expression of p21\textsuperscript{cip} and p27\textsuperscript{kip}. Furthermore, the overexpression of p53, p21\textsuperscript{cip} and p27\textsuperscript{kip} suppressed GM-CSF-induced macrophage proliferation, and the knockdown of p21\textsuperscript{cip} and p27\textsuperscript{kip} attenuated AICAR-mediated suppression of macrophage proliferation. These results suggest that the suppressive effects of AMPK activation on macrophage proliferation were mediated not by the interruption of GM-CSF-mediated intracellular signal pathway but by direct cell cycle arrest through the induction of p53 phosphorylation, p21\textsuperscript{cip} expression, and p27\textsuperscript{kip} expression.

In conclusion, we have revealed for the first time that activation of AMPK suppresses Ox-LDL-induced macrophage proliferation by inhibiting the expression of GM-CSF and inducing cell cycle arrest. Because the proliferation of vascular cells including macrophages is a key event in the development and progression of atherosclerosis (1, 3–5), the suppressive effect of AMPK activation on cell proliferation may be a therapeutic target for atherosclerosis.

Acknowledgments—We greatly appreciate the technical assistance from members of the Gene Technology Center (Kumamoto University), as well as Kenshi Ichinose.

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