THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 10, pp. 7917–7924, March 11, 2011

Hyaluronan Synthesis Is Inhibited by Adenosine Monophosphate-activated Protein Kinase through the Regulation of HAS2 Activity in Human Aortic Smooth Muscle Cells*[S]

Received for publication, October 12, 2010, and in revised form, December 3, 2010 Published, JBC Papers in Press, January 12, 2011, DOI 10.1074/jbc.M110.193656

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Hyaluronan (HA) is an extracellular matrix glycosaminoglycan (GAG) involved in cell motility, proliferation, tissue remodeling, development, differentiation, inflammation, tumor progression, and invasion and controls vessel thickening in cardiovascular diseases. Therefore, the control of HA synthesis could permit the fine-tuning of cell behavior, but the mechanisms that regulate HA synthesis are largely unknown. Recent studies suggest that the availability of the nucleotide-sugar precursors has a critical role. Because the formation of UDP-sugars is a highly energetically demanding process, we have analyzed whether the energy status of the cell could control GAG production. AMP-activated protein kinase (AMPK) is the main ATP/AMP sensor of mammalian cells, and we mimicked an energy stress by treating human aortic smooth muscle cells (AoSMCs) with the AMPK activators 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside and metformin. Under these conditions, HA synthesis, but not that of the other GAGs, was greatly reduced. We confirmed the inhibitory effect of AMPK using a specific inhibitor and knock-out cell lines. We found that AMPK phosphorylated Thr-110 of human HAS2, which inhibits its enzymatic activity. In contrast, the other two HAS isoenzymes (HAS1 and HAS3) were not modified by the kinase. The reduction of HA decreased the ability of AoSMCs to proliferate, migrate, and recruit immune cells, thereby reducing the pro-atherosclerotic AoSMC phenotype. Interestingly, such effects were not recovered by treatment with exogenous HA, suggesting that AMPK can block the pro-atherosclerotic signals driven by HA by interaction with its receptors.

Hyaluronan (HA)[3] is a ubiquitous and atypical glycosaminoglycan (GAG) that is neither sulfated nor linked to any core protein. It is composed of GlcUA and *N*-acetyl-D-glucosamine GlcNAc and is synthesized by three plasma membrane HA synthases (HAS1, HAS2, and HAS3) that generate linear polymers with molecular masses ranging from thousands to several millions of daltons (1). Many cellular effects of HA depend on its viscoelastic properties, molecular mass, and membrane receptors, including CD44, RHAMM (receptor for HA-mediated motility), Lyve-1 (lymphatic vessel endothelial receptor 1), HARE (HA receptor for endocytosis), and TLR4 (Toll-like receptor 4), which trigger several signaling events (1). During development, HA has a role in many critical events, including cell migration and differentiation, through spatiotemporal regulation of HAS expression in amphibians as well as in mammals (2–5).

In cardiovascular pathologies, HA has critical pro-atherosclerotic properties (6). In normal arteries, HA is typically located in the tunica adventitia, but following injuries, HA can be found in the media and neointima (7), where it can modulate the behavior of smooth muscle cells (SMCs) and contribute to inflammatory responses, including recruitment of immune cells (8, 9). SMCs have a critical role in vessel wall thickening due to their proliferation, migration, and secretion of extracellular matrix (ECM)-modifying enzymes (10). Moreover, SMCs are responsible for HA matrix deposition after vessel injury, and HA itself can modulate SMC motility (11) and dedifferentiation (12). Several experiments confirmed the critical pro-atherosclerotic role of HA using CD44 knock-out animals (12) or HAS2 transgenic mice (13), by blocking HA/CD44 interaction (14), or by inhibiting HA synthesis (15).

Therefore, regulation of HA synthesis could provide new methods to treat vascular pathologies. Growth factors, cytokines, prostaglandins, PKA, and PKC are known to modulate HA synthesis through HAS activity and HAS gene expression, although their physiological roles are not completely understood (1). Our research and that of others have revealed the critical role of the UDP-sugar precursors (UDP-GlcUA and UDP-GlcNAc) in the regulation of HA synthesis (16, 17). The ratio of NAD^+ to NADH, which is influenced mainly by oxygen

* This work was supported by the Fondazione Comunitaria del Varesotto-ONLUS, PRIN 2007 (to G. D. L.), Fondo di Ateneo per la Ricerca, and a Centro Insubre di Biotecnologie per la Salute Umana young researcher award (to D. V.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6.

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[3] The abbreviations used are: HA, hyaluronan; GAG, glycosaminoglycan; HAS, HA synthase; SMC, smooth muscle cell; ECM, extracellular matrix; AMPK, AMP-activated protein kinase; AoSMC, aortic SMC; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; 2-DG, 2-deoxyglucose; CS, chondroitin sulfate; HS, heparan sulfate; MEF, mouse embryonic fibroblast; CA-AMPK, constitutively active AMPK; DN-AMPK, dominant-negative AMPK.

availability, is known to control UDP-GlcUA content through the activity of UDP-glucose dehydrogenase in cartilage and cornea, which can lead to keratan sulfate synthesis (18).

The synthesis of HA precursors requires ATP, UTP, and other critical metabolic molecules, including glucose, glutamine, glucosamine, and acetyl-CoA (supplemental Fig. S1), which makes GAG production an energy-consuming process. As energy charge is critical for the regulation of all cellular metabolism, we tested whether the ATP/AMP ratio could control the synthesis of HA and other GAGs. We focused our attention on AMP-activated protein kinase (AMPK), which is the main energy sensor of the eukaryotic cell (19). AMPK is a heterotrimeric protein with a catalytic α subunit and two regulatory subunits (β and γ) that, when the ATP/AMP ratio decreases, inhibit anabolic processes and induce catabolic pathways to restore ATP levels (20). The mechanism for activating AMPK is complex and involves phosphorylation of the critical Thr-172 on the catalytic α subunit. By altering gene expression or by directly phosphorylating several pivotal enzymes, AMPK regulates cellular metabolic processes, including glycolysis, gluconeogenesis, lipid metabolism, and protein synthesis. However, the consequences for the metabolism of ECM components are not yet known. Therefore, the aim of this study was to determine how modulation of AMPK affects the metabolism of HA and other GAGs in primary human aortic SMCs (AoSMCs).

EXPERIMENTAL PROCEDURES

Cell Cultures and Treatments—Primary human AoSMCs were purchased from Lonza and were grown for four to five passages in complete SmGm2 culture medium (Lonza) supplemented with 5% FBS as described previously (15). 2.5×10^5 cells were seeded in 35-mm dishes, and after 6 h, SmGm2 medium was replaced with high glucose DMEM supplemented with 0.2% FBS to induce quiescence. After 48 h, DMEM was maintained in the positive control (quiescent cells), whereas it was replaced with SmGm2 medium supplemented with 15% FBS in the negative control (growth cells). In the treated cells, DMEM was substituted with SmGm2 medium enriched with 15% FBS and supplemented with increasing concentrations of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), metformin, or 2-deoxyglucose (2-DG) (all from Sigma).

Western Blotting—Western blot experiments were performed as reported previously (14) using antibodies against AMPKα, phospho-AMPKα (Thr-172), or phospho-acetyl-CoA carboxylase (Ser-79) (all from Cell Signaling). To investigate the phosphorylation of HAS2 at the putative AMPK target residue Thr-110, we used a affinity-purified phospho-specific polyclonal antibody prepared in rabbit against the synthetic peptide RKCLQSVKRLpTYPGIKV, which resembles residues 100–116 of the human HAS2 sequence (Davids Biotechnologie, Regensburg, Germany). As a control, we also used a polyclonal antibody against the same peptide without threonine phosphorylation (generated by the same company).

GAG Determinations—HA, chondroitin sulfate (CS), and heparan sulfate (HS) released into the culture medium were purified by proteinase K (Finnzymes) and ethanol precipitation. *Streptomyces dysgalactiae* hyaluronidase, chondroitinase ABC, and heparinases I–III (all from Seikagaku) were used to obtain the unsaturated (Δ) disaccharides, which were quantified by HPLC analyses (21, 22). Pericellular HA matrices were visualized using a particle exclusion assay (15).

Gene Expression Determinations by Quantitative RT-PCR—Total RNAs were extracted from AoSMCs with TRIzol (Invitrogen), retrotranscribed using the High Capacity cDNA synthesis kit (Applied Biosystems), and amplified on an ABI Prism 7000 instrument (Applied Biosystems). The following human TaqMan gene expression assays were used: HAS1 (Hs00155410_m1), HAS2 (Hs00193435_m1), HAS3 (Hs00193436_m1), UDP-glucose dehydrogenase (Hs00163365_m1), UDP-glucose pyrophosphorylase (Hs00198879_m1), and β-actin (Hs99999903_m1). The relative gene expression was determined by comparing ΔC_t (14).

Cell Mobility and Adhesion Assays—Confluent AoSMCs were growth-arrested by culture in DMEM with 0.2% FBS for 2 days. The cells were scratched by blue tip and then cultured under different conditions for 24 h. Migrated cells were quantified as described previously (15). To assess the adhesiveness of AoSMCs under different conditions, we performed monocyte adhesion assays using the U937 monocyte cell line as reported previously (15).

Cell Transfections—Primary AoSMCs were nucleofected with 5 μg of plasmid DNA using a Nucleofector apparatus (Amaxa) and the human AoSMC Nucleofector kit as described previously (17) to have high efficiency and low mortality. Wild-type and knock-out mouse embryonic fibroblasts (MEF) and COS-1 and COS-7 cells were efficiently transfected using ExGen 500 (Fermentas) following the manufacturer's protocol.

Microsome Purification and HAS Activity Assay—Control or transfected COS-7 cells were lysed in 10 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.4) supplemented with protease and phosphatase inhibitors (Roche Applied Science) by scraping and sonication. The lysate was centrifuged at $5000 \times g$ for 5 min at 4 °C, and the supernatant was centrifuged at $100{,}000 \times g$ for 40 min at 4 °C to obtain microsomes containing vesiculated fragments of the plasma membrane (23). The pellet was resuspended in the lysis buffer, and protein content was determined by Bradford assay. Twenty μg of proteins were used to determine HAS activity as described previously (8).

Statistical Analyses—Statistical analyses of the data were performed by analysis of variance, followed by Bonferroni post hoc tests using Origin 7.5 software (OriginLab). Probability values of $p < 0.01$ or 0.05 were considered statistically significant. Experiments were repeated three times, each time in duplicate, and data are expressed as means ± S.E.

RESULTS AND DISCUSSION

Effects of AMPK Activation on GAG Synthesis—To induce AMPK activation, AoSMCs were incubated for 2 days with 0.2% FBS to induce quiescence (positive control) and then treated for 48 h with 15% FBS (negative control) or with 15% FBS plus AICAR, a pharmacological activator of AMPK that mimics AMP, as previously reported in AoSMCs (24). By Western blotting, we demonstrated that AICAR induced phosphorylation of Thr-172 on the AMPK α subunit and that AICAR induced AMPK activation by detecting phospho-acetyl-CoA carboxyl-

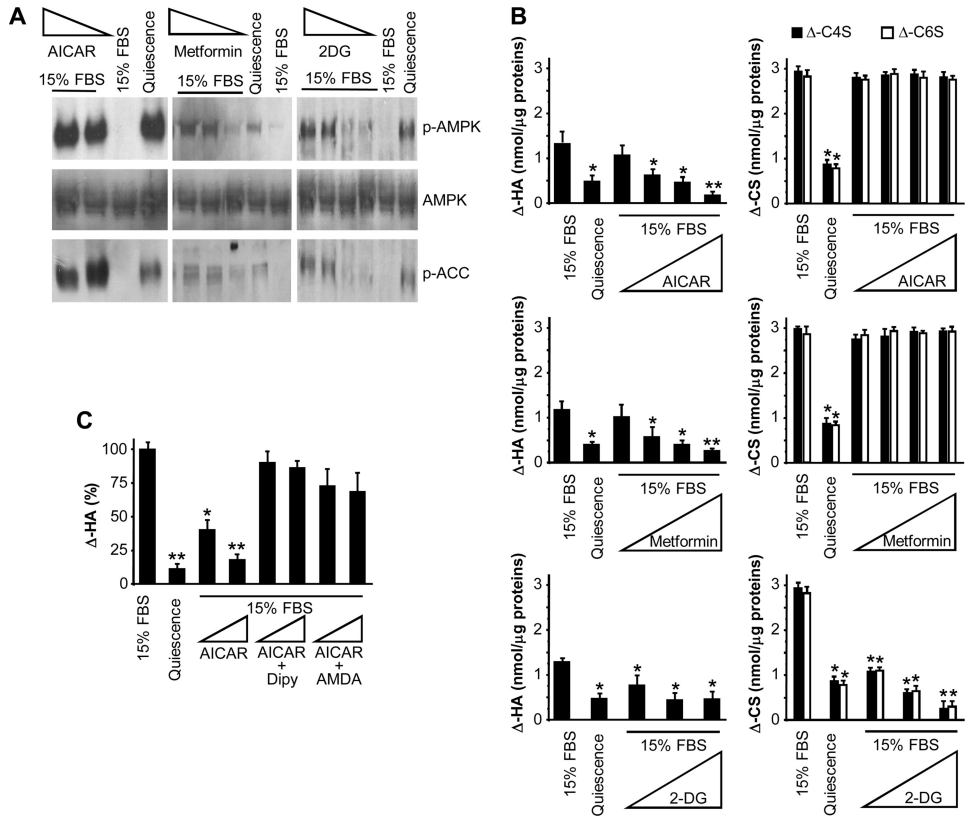

FIGURE 1. **AICAR, metformin, and 2-DG inhibit HA synthesis but not CS synthesis in AoSMCs.** *A*, Western blot analysis of 30 μg of protein extracts prepared from quiescent AoSMCs (0.2% FBS, 48 h) or quiescent cells treated with 15% FBS or 15% FBS plus AICAR (1 and 2 mM; 48 h), with 15% FBS plus metformin (2, 3, and 4 mM; 48 h), or with 15% FBS plus 2-DG (15, 25, 50, and 75 mM; 48 h; the high 2-DG concentrations were needed to compete with the 25 mM glucose contained in the AoSMCs growth medium) using specific anti-phospho-AMPKα (*p-AMPK*), anti-total AMPKα (*AMPK*), or anti-phospho-acetyl-CoA carboxylase (*p-ACC*) antibodies. In the 2-DG experiment, the 15% FBS sample also contained 50 mM sorbitol as an osmotic control. The images are representative of three independent experiments. *B*, quantification of Δ-HA, Δ-C4S, and Δ-C6S prepared from GAGs released into the culture medium of quiescent AoSMCs treated for 48 h with 15% FBS, with 15% FBS plus AICAR (0.5, 1, 2, and 4 mM), with metformin (0.5, 1, 2, and 4 mM), or with 2-DG (12, 25, and 50 mM) as described for *A*. *C*, relative quantification of Δ-HA prepared from GAGs released into the culture medium of quiescent AoSMCs treated with 15% FBS, with AICAR (1 and 2 mM), with AICAR plus 10 μM dipyridamole (*Dipy*), or with AICAR plus 10 μM 5′-amino-5′-deoxyadenosine (*AMDA*). Results are expressed as means ± S.E. in three different determinations. *, $p < 0.5$; **, $p < 0.01$, 15% FBS *versus* treatments, respectively.

ase, an AMPK target (Fig. 1*A*). In other experiments, quiescent AoSMCs were treated for 48 h with metformin and 2-DG (Fig. 1*A*), which induce AMPK phosphorylation and activation driven by different mechanisms from AICAR, *i.e.* through the upstream kinase complex LKB1-STRAD-MO25 (25) and by ATP depletion, respectively. These results showed that AICAR, metformin, and 2-DG sustained phospho-AMPK and phospho-acetyl-CoA carboxylase activities in 15% FBS in dose-dependent responses.

At this time point, we collected aliquots of cell medium and purified the secreted GAGs. Unsaturated (Δ) disaccharides from HA, CS, and HS were obtained by digestion with specific enzyme eliminases. The resulting Δ-disaccharides were then derivatized with 2-aminoacridone and quantified by HPLC (21, 22). The treatment of quiescent AoSMCs with 15% FBS induced an ~2.5-fold increase in HA, whereas the addition of 15% FBS plus AICAR or metformin caused a dose-dependent and statistically significant inhibition of HA synthesis (Fig. 1*B*). In contrast, the synthesis of CS (both 6- and 4-sulfated isomers) (Fig. 1*B*) as well as HS (supplemental Fig. S1) did not change after the treatments, and only minor and not statistically significant modification of sulfation patterns were observed. These results clearly demonstrate that HA synthesis, but not CS or HS synthesis, is inhibited by both AICAR and metformin. Previous studies showed that metformin does not decrease sulfate incorporation into GAGs (26). However, because HA is not sulfated, our results are novel and particularly interesting because of the critical functions of HA in the vasculature. The "conventional" GAG synthesis inhibitor 2-DG (27) was used as a control and indeed reduced synthesis of both HA and CS (Fig. 1*B*). Moreover, HA, which remains associated with cell membranes to form pericellular coats, also decreased after the treatments as evaluated by particle exclusion assays (supplemental Fig. S2). To confirm the results obtained with AICAR, AoSMCs were also treated with dipyridamole, which inhibits AICAR transport into cells by blocking an adenosine transporter, and with 5′-amino-5′-deoxyadenosine, which inhibits the cytosolic phosphorylation of AICAR by blocking the adenosine kinase (24). Both these treatments avoided the HA reduction induced by AICAR (Fig. 1*C*).

To confirm the involvement of AMPK in HA synthesis inhibition, we used Compound C, a specific AMPK inhibitor (25). As shown in Fig. 2*A*, Compound C in the presence of AICAR increased the HA level to near control levels. Therefore, these data substantiate the critical involvement of AMPK in regulating HA synthesis. Furthermore, AoSMCs were nucleofected

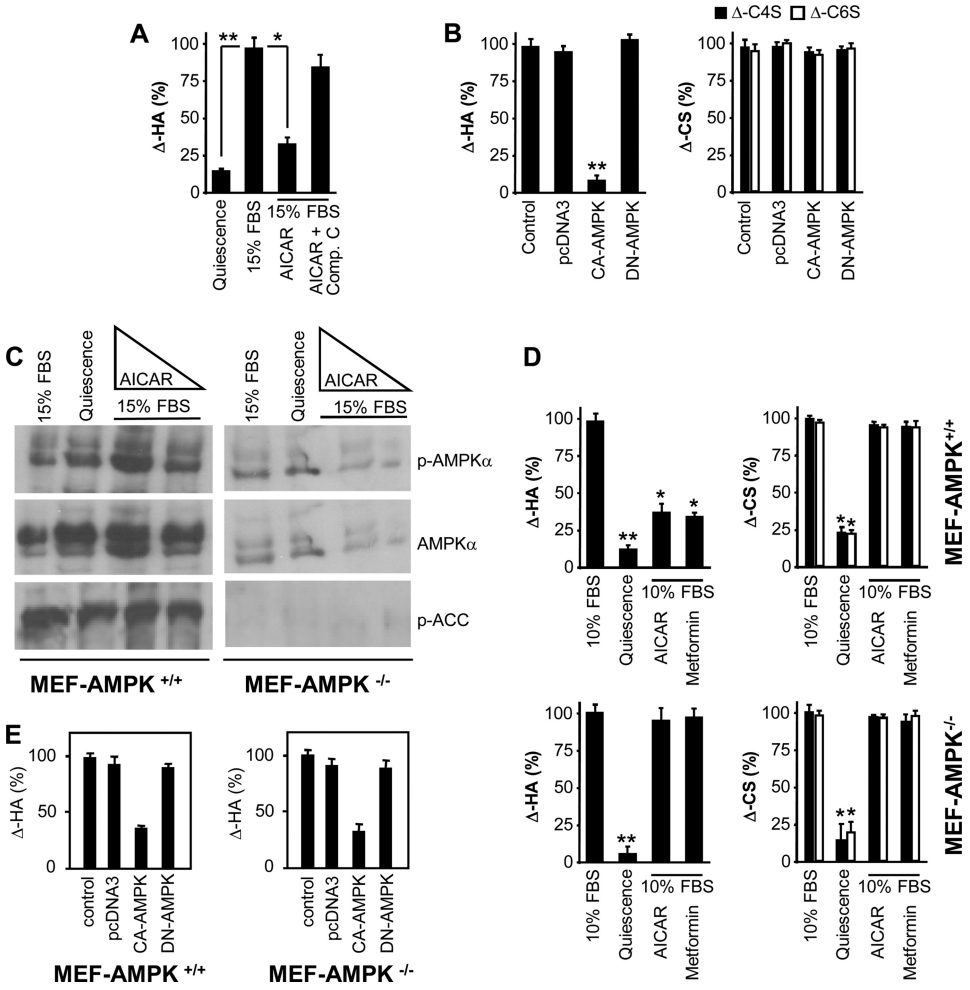

FIGURE 2. **AMPK is involved in AICAR- and metformin-dependent HA synthesis inhibition.** *A*, relative HPLC quantification of Δ-HA prepared from GAGs released into the culture medium of quiescent AoSMCs treated with 15% FBS (set as 100%), with 15% FBS plus 1 mM AICAR, or with 15% FBS plus 1 mM AICAR plus 100 μM Compound C (*Comp. C*). *B*, relative HPLC quantification of Δ-HA and Δ-CS prepared from GAGs released into the culture medium of AoSMCs nucleofected with an expression vector coding for CA-AMPK or DN-AMPK or with an empty vector (pcDNA3). The analyses were performed 48 h after the nucleofections. The content of Δ-HA, Δ-C4S, or Δ-C6S in control cells (*i.e.* subjected to the electrical protocol without DNA) was set to 100%. *C*, Western blot analysis of 30 μg of protein extracts prepared from quiescent (0.2% FBS, 48 h) wild-type ($AMPK^{+/+}$) MEFs, from $AMPK^{-/-}$ MEFs, or from quiescent cells treated with 15% FBS or with 15% FBS plus AICAR (1 and 2 mM; 48 h) using specific anti-phospho-AMPKα (*p-AMPK*), anti-total AMPKα, or anti-phospho-acetyl-CoA carboxylase (*p-ACC*) antibodies. The absence of phospho-acetyl-CoA carboxylase bands in $AMPK^{-/-}$ MEFs indicates the absence of AMPK activity. *D*, relative HPLC quantification of Δ-HA and Δ-CS prepared from GAGs released into the culture medium of $AMPK^{+/+}$ MEFs or $AMPK^{-/-}$ MEFs incubated for 48 h with 0.2% FBS (quiescent), with 10% FBS (set as 100%), with 10% FBS plus 2 mM AICAR, or with 10% FBS plus 4 mM metformin. *, $p < 0.5$; **, $p < 0.01$, 15% FBS (or control in *B* or 10% FBS in *C*) *versus* treatments, respectively. *E*, relative HPLC quantification of Δ-HA and Δ-CS prepared from GAGs released into the culture medium of $AMPK^{+/+}$ MEFs, untransfected $AMPK^{-/-}$ MEFs (control), or cells transfected with plasmid coding for CA-AMPK or DN-AMPK or with an empty vector (pcDNA3).

with a recombinant pcDNA3 plasmid coding for constitutively active AMPK (CA-AMPK) or for dominant-negative AMPK (DN-AMPK). The amount of HA secreted was dramatically decreased after CA-AMPK nucleofection but did not change after DN-AMPK nucleofection (Fig. 2*B*). Similarly, HA pericellular coats were abolished by CA-AMPK but not DN-AMPK treatment (supplemental Fig. S2). Interestingly, CS synthesis was not altered by either of the treatments (Fig. 2*B*), indicating a specific role of AMPK in HA synthesis.

To definitely demonstrate AMPK effects on HA synthesis, we used MEFs from animals null in the α1 and α2 subunits of AMPK ($AMPK^{-/-}$) or from wild-type ($AMPK^{+/+}$) mice, both kindly provided by Benoît Viollet (28). In preliminary control experiments, we verified the lack of AMPK activity in $AMPK^{-/-}$ MEFs by demonstrating the absence of the phospho-acetyl-CoA carboxylase band in Western blotting as well as the lack of cell cycle inhibition after AICAR addition (Fig. 2*C* and supplemental Fig. S3). $AMPK^{+/+}$ and $AMPK^{-/-}$ MEF cultures were treated with 15% FBS or with 15% FBS plus AICAR or metformin. After 48 h, AICAR and metformin reduced HA released into the medium by ~60% in $AMPK^{+/+}$ MEF cultures (Fig. 2*D*) and caused a near absence of cell-associated HA (supplemental Fig. S4). As expected, CS was not affected by the treatments (Fig. 2*D*). In contrast, HA in $AMPK^{-/-}$ MEF cultures was not changed by these treatments (Fig. 2*D*). These results demonstrate that cells without AMPK activity are not able to reduce HA synthesis in response to AICAR or metformin. The transfection of the CA-AMPK-coding plasmid into $AMPK^{-/-}$ MEFs restored their ability to inhibit HA synthesis (Fig. 2*E*). This clearly shows that the energy charge, through AMPK activity, regulates HA synthesis and therefore affects ECM composition.

HA Does Not Influence AMPK-mediated Reduction of Cell Proliferation, Migration, and Adhesiveness—SMCs are involved in vascular diseases through their proliferation, intima invasion, and recruitment of immune cells, and HA has a central role to control SMC behavior. Therefore, we investigated whether AICAR and metformin are able to modulate AoSMC cell cycle responses. Using cytofluorometric analyses, we confirmed the previously published data (24) indicating that AICAR and metformin, through AMPK, can block the cell cycle in G_0/G_1 phase without inducing cell death or apoptosis (data not shown). Furthermore, the exogenous addition of high molecular mass HA (average of $\sim 4 \times 10^6$ Da; Healon) and the exogenous addition of low molecular weight HA (prepared by digesting Healon with testicular hyaluronidase for 90 min, which generates oligosaccharides <500 kDa) to AICAR- or metformin-treated cells maintained AoSMCs arrested in G_0/G_1 (supplemental Fig. S5), suggesting that the HA-activated signaling by specific cell-surface receptors cannot bypass the block of the cell cycle induced by AMPK. These data confirm the finding that high molecular mass HA antagonizes mitogen-induced S phase entry in vascular SMCs by modulating cyclin D_1 and $p27^{kip1}$ (12, 29). On the other hand, by means of scratch tests, we found that AMPK activity reduced cell migration by ~70%, whereas AMPK inhibition by Compound C maintained cell motility (Fig. 3*A*). The exogenous addition of high molecular mass HA was not able to bypass the blocking effect of AMPK on cell migration (supplemental Fig. S5). This issue is interesting, as high molecular mass HA is generally known to greatly induce cell mobility, and by decreasing HA synthesis, AICAR or metformin could be useful to limit HA-dependent cell invasion that is critical in several pathologies, such as cancer metastasis or neointima formation. Interestingly, low molecular mass HA, which is known to strongly induce cell motility, was able to partially overcome the effects AICAR and metformin (supplemental Fig. S5). As *in vivo* HA can be fragmented during neointima formation due to an inflammatory response (30), the migration inhibitory effect of AMPK could be maintained also in the presence of HA oligosaccharides that physiologically are a strong activator of cell proliferation or motility.

AICAR and metformin treatments also strongly reduced adhesion of fluorescent U937 monocytes on AoSMCs, whereas AMPK inhibition with Compound C maintained the number of adherent monocytes comparable with the control (Fig. 3, *B* and *C*). Interestingly, the monocyte binding in the Compound C-treated samples confirmed the role of HA in cell adhesion because enzymatic removal of HA and the exogenous high molecular mass HA added to treated cells reduced monocyte adhesion to near control levels. It is known that HA can modulate inflammation triggering TLR4 signaling (1) or can mediate immune cell adhesion on the endothelium or SMCs via CD44 (9, 31, 32). The results of these experiments demonstrate that activation of AMPK *in vitro* inhibits proliferation, migration, and monocyte adhesion, thereby reducing AoSMC proatherosclerotic behaviors.

Vessel thickening involves a plethora of causes partially still unknown, but ECM remodeling is one of the main events affecting vascular cells (33). Previous studies have shown that administration of AICAR *in vivo* suppressed neointima forma-

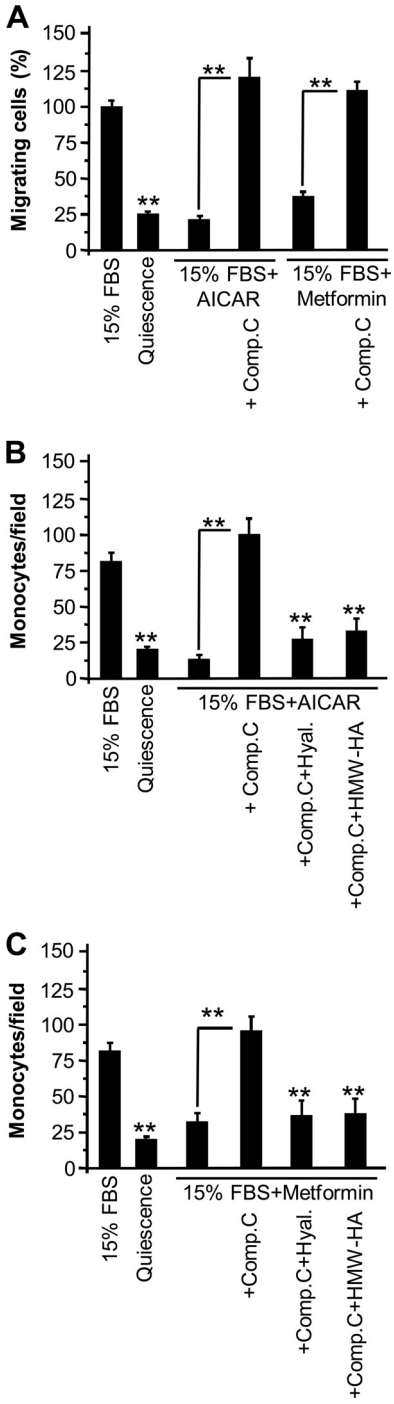

FIGURE 3. **AICAR and metformin modulate AoSMC motility and adhesive properties through AMPK.** *A*, relative quantification of AoSMC mobility. Quiescent confluent AoSMCs were treated with 15% FBS, with 15% FBS plus AICAR (1 mM), or with 15% FBS plus metformin (2 mM) with or without 100 μM Compound C (*Comp.C*). After 24 h, cells were scratched using a pipette tip, and after 24 h, migrated cells were counted. *B* and *C*, U937 monocyte adhesion assays performed on AoSMCs treated with AICAR (*B*) or metformin (*C*). Seventy percent quiescent confluent cells were treated with 15% FBS, with 15% FBS plus AICAR (1 mM), or with 15% FBS plus metformin (2 mM) with or without 100 μM Compound C. After 48 h, fluorescent U937 cells were added for 20 min at 37 °C, washed, and counted under a fluorescence microscope. Control experiments were done by treating AoSMCs with hyaluronidase (*Hyal*) or by competing for cell-associated HA adding high molecular weight HA (*HMW-HA*). Results are expressed as the number of adherent U937 cells per field and are represented as means ± S.E. **, $p < 0.01$, untreated *versus* treated samples.

tion after mechanical injury in the rat femoral artery, although no information was provided on ECM composition (34). Furthermore, it has been known for decades that metformin can inhibit vascular complications of type 2 diabetes patients (35), and AMPK has also been indicated as a therapeutic target for atherosclerosis and cancer (36). Therefore, in addition to its multiple effects on cell cycle progression and on protein, fatty acid, and cholesterol synthesis (36), our results indicate that AMPK can modulate ECM composition by inhibiting HA synthesis, suggesting its pivotal role in all the processes that contribute to matrix remodeling.

Molecular Mechanism of AMPK-mediated Inhibition of HA Synthesis—To elucidate the critical effect of AMPK activation only on HA synthesis, we determined whether AMPK can modulate the expression of genes involved in HA metabolism. In fact, it is known that AMPK controls the expression of several genes, including the critical glucose metabolism transcription factor FOXO (37). Therefore, by means of quantitative RT-PCR, we quantified the relative content of mRNAs coding for HA-metabolizing enzymes, including UDP-glucose pyrophosphorylase, UDP-glucose dehydrogenase, HASs, and hyaluronidases after AoSMCs treatments with AICAR and metformin, and we did not find any significant gene expression differences (supplemental Fig. S6). The finding that UDP-glucose pyrophosphorylase and UDP-glucose dehydrogenase genes are not modulated by AMPK could be reasonable considering that UDP-glucose and UDP-GlcUA are critical not only for HA synthesis but also for all other sulfated GAGs, glycogen, and other glycoconjugates that are necessary for cell survival. However, a proteomic study revealed that UDP-glucose pyrophosphorylase can be a target of AMPK in pigs, enhancing the complexity of this issue (38).

Although HASs are known to be regulated at the transcriptional level by several growth factors and cytokines (1), this seems not to be the case for AMPK. As HASs are transmembrane proteins, their activity can be phospholipid-dependent (39). Interestingly, AMPK can regulate mitochondrial cardiolipin content (40), and this lipid is known to regulate bacterial HAS (39). As cardiolipin is typically in the mitochondria, this issue could be not critical for eukaryotic HASs, although very recently, it was reported that the AMPK γ1 subunit can control erythrocyte membrane elasticity (41). Therefore, AMPK could modify membrane lipid composition indirectly by modulating HAS activity or stability.

On the other hand, AMPK could act on HASs at a post-translational level (*i.e.* phosphorylation), which would assure a rapid response. Interestingly, the AMPK β1 subunit has a myristoylation signal that localizes the AMPK complex in the plasma membrane (42), where AMPK could interact with HASs. Bioinformatic analyses have shown that HASs conserve several AMPK consensus sequences in the intracytoplasmic loop, which makes such enzymes hypothetical AMPK targets. To study this issue, we transiently transfected COS-7 cells, which are known to synthesize a limited amount of HA, with plasmid coding for FLAG-tagged human HAS1 (FLAG-HAS1), HAS2 (FLAG-HAS2), or HAS3 (FLAG-HAS3) and measured the HA released into the culture medium (Fig. 4*A*). Each construct increased HA synthesis significantly. However, only the

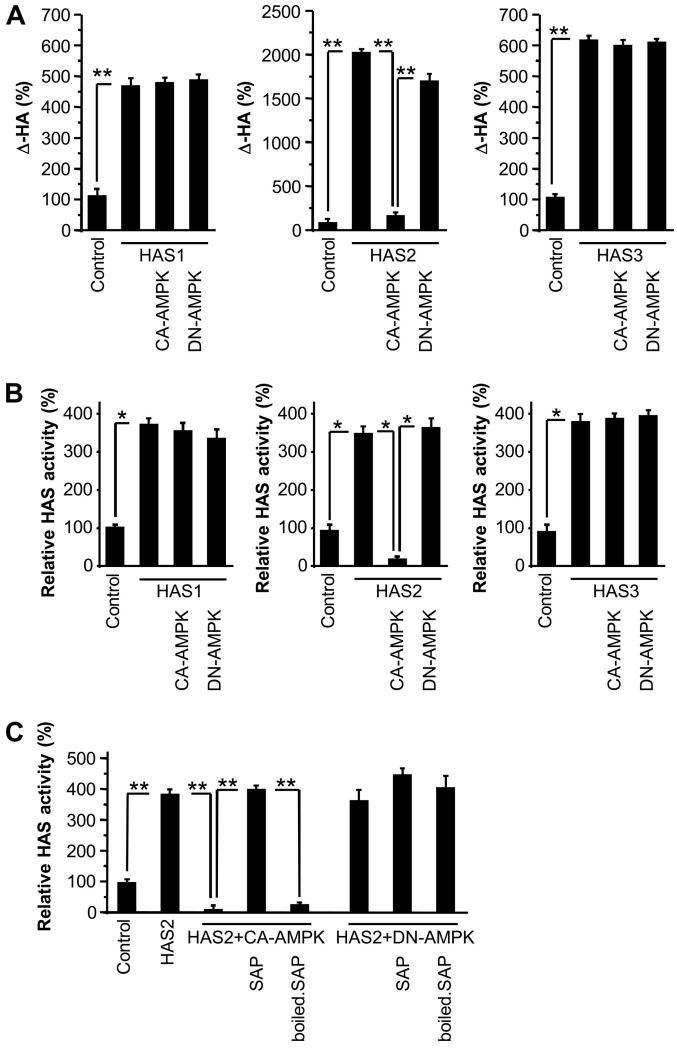

FIGURE 4. **AMPK inhibits HAS2 activity.** *A*, relative HPLC quantification of Δ-HA prepared from GAGs released into the culture medium 48 h after transfection of COS-7 cells with the FLAG-HAS1-, FLAG-HAS2-, or FLAG-HAS3-expressing vector alone, cotransfection with the CA-AMPK- or DN-AMPK-expressing vector, or transfection with the empty pcDNA3 vector alone (control). *B*, relative quantification of HAS enzymatic activity in microsomes prepared from COS-7 cells transfected as described for *A*. *C*, relative quantification of HAS activity in microsome proteins extracted from COS-7 cells transfected with FLAG-HAS2 or cotransfected with the FLAG-HAS2+CA-AMPK- or FLAG-HAS2+DN-AMPK-expressing plasmid. In some experiments, membrane fraction proteins were preincubated with shrimp alkaline phosphatase (*SAP*) to hydrolyze phosphate groups. Control experiments were done by preincubating the samples with heat-inactivated SAP (*boiled.SAP*). Results are represented as means ± S.E. *, $p < 0.05$; **, $p < 0.01$, untreated *versus* treated samples.

FLAG-HAS2-transfected cells decreased HA synthesis to near control levels when cotransfected with CA-AMPK but not with DN-AMPK (Fig. 4*A*). This strongly suggests that only HAS2 can be targeted by AMPK. To evaluate whether AMPK can modulate HAS functionality, we quantified the HAS enzymatic activity in microsomes because HAS enzymes are transmembrane proteins, and their activity can be recovered only in crude cell membranes. We found a strong reduction of HAS activity only when FLAG-HAS2 was cotransfected with CA-AMPK, whereas the functionality of other HAS enzymes (*i.e.* HAS1 and HAS3) was not affected by CA-AMPK (Fig. 4*B*). Although HASs share a high degree of amino acidic identity, the inhibi-

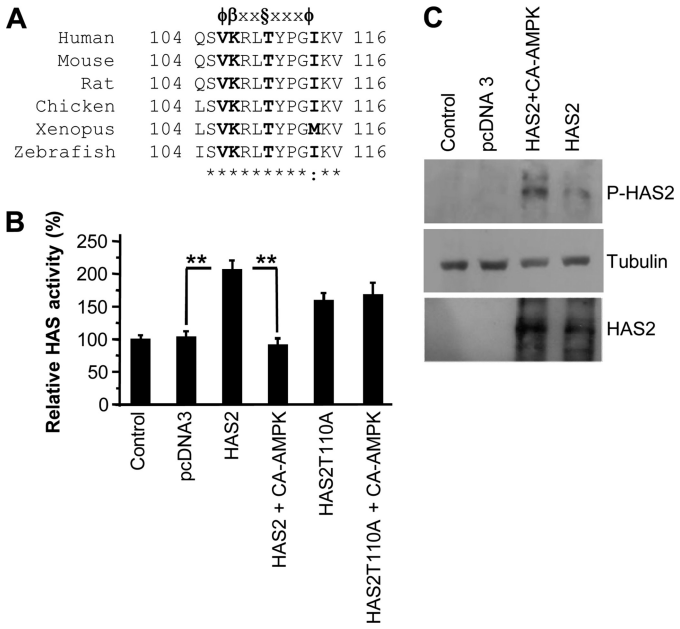

FIGURE 5. **Identification of the critical residue involved in AMPK-dependent HAS2 inhibition.** *A*, alignment of HAS2 sequences around Thr-110 from different organisms showing the conservation of the AMPK consensus site (with critical residues shown in *boldface*). ϕ, hydrophobic residue; β, basic residue; *x*, any residue; §, serine or threonine. *B*, relative quantification of HAS activity in microsome fractions prepared from COS-7 cells transfected with the wild-type 6-Myc-HAS2 (*HAS2*)- or 6-Myc-HAS2 T110A (*HAS2T100A*)-expressing plasmid or with the empty pcDNA3 vector alone (control) or cotransfected with the CA-AMPK-expressing vector. Results are expressed as means ± S.E. **, $p < 0.01$, pcDNA3 or HAS2+CA-AMPK *versus* HAS2 samples. *C*, Western blot analysis of 30 μg of protein extracts prepared from untransfected COS-7 cells (control) or from COS-7 cells transfected with plasmid coding for 6-Myc-HAS2 or an empty vector (pcDNA3) or cotransfected with plasmid coding for 6-Myc-HAS2 and CA-AMPK (HAS2+CA-AMPK) using the polyclonal antibody that recognizes phospho-Thr-110 (*P-HAS2*), tubulin (as a loading control), or HAS2 (as a transfection efficiency control).

tory effect only on HAS2 activity seems to be very specific. From a pathophysiological point of view, HAS2 transgenic mice exacerbate neointima formation after pro-atherosclerotic stimuli (13); therefore, the specific inhibitory effect of AMPK on the main HAS responsible of vessel thickening could be of crucial pharmacological importance. Interestingly, the enzymatic hydrolysis of protein phosphate groups in FLAG-HAS2+CA-AMPK microsomes, by treatment with shrimp alkaline phosphatase, restored HAS activity to the control level (Fig. 4*C*), which strongly suggests that one or more phosphorylation sites in the HAS2 enzyme or in an accessory protein present in the membrane can reduce HAS2 activity.

To identify critical phosphorylation residue(s), we searched the Human Protein Reference Database, which revealed the presence of a putative AMPK phosphorylation consensus motif in HAS2 protein at Thr-110. The sequence around this amino acid is very well conserved in HAS2 proteins from zebrafish to mouse (Fig. 5*A*), suggesting its importance. Moreover, this site is not shared with HAS1 or HAS3, which would explain why HAS1 and HAS3 are not regulated by AMPK. Furthermore, this residue is on a cytoplasmic loop of HAS2 that was previously shown to be important for glycosyltransferase activity (39). Therefore, we mutated Thr-110 to alanine (T110A) in the 6-Myc-HAS2 plasmid to prevent phosphorylation and transfected the mutant construct into COS-7 cells with or without CA-AMPK. After 48 h, we measured the HAS-specific activity in microsomes and found that the HA synthetic activity of the T110A-transfected COS-7 cells was comparable with wild-type HAS2 and that CA-AMPK was not able to inhibit HA synthesis (Fig. 5*B*). To determine whether Thr-110 could be phosphorylated, we used two polyclonal antibodies prepared against two peptides that represent the HAS2 sequence from amino acids 100 to 116 in which Thr-110 was phosphorylated or not. As shown in Fig. 5*C*, in Western blot experiments, the antibody generated against the phosphopeptide (*P-HAS2 panel*) was able to detect a band corresponding to HAS2 in protein extracts prepared in COS-7 cells cotransfected with plasmids coding for 6-Myc-HAS2 and CA-AMPK. A faint band was also visible in extracts prepared from transfection with only 6-Myc-HAS2, where a small amount of HAS2 phosphorylation could be due to endogenous COS-7 AMPK activity. The antibody generated against the unphosphorylated peptide (*HAS2 panel*) was able to detect HAS2 protein in both treatments, revealing similar transfection efficiency. These results strongly suggest that Thr-110 can be the target of AMPK and that the phosphorylation of this amino acid drastically reduces the HA synthetic activity. A previous study reported the phosphorylation of HAS3 without describing the effect on enzyme functionality (43), whereas another work clearly showed that ERK-mediated serine phosphorylation of all three HASs increased their specific activity (44). This finding suggests that HAS proteins could have different regulatory mechanisms in which the same post-translational modification (*i.e.* phosphorylation) in different sites could increase or decrease the enzymatic activity. Moreover, a recent work demonstrated that HAS2 and HAS3 can make homo- and heterodimers and that HAS2 activity can be modulated by ubiquitination at Lys-190 (45). Such results suggest that HASs (and HAS2 in particular) can be complex proteins with several possibilities for post-translational modifications capable of modulating enzymatic activity.

Our findings demonstrate that at low ATP/AMP ratios, AMPK activity can block HA synthesis without altering the synthesis of other GAGs. The mechanism of such regulation does not involve a change in gene expression but involves the phosphorylation of HAS2, which decreases its HA synthetic activity. When AMPK is active, cells are already known to be growth-arrested, and for this reason, AMPK was indicated as a therapeutic marker for cancer and vascular pathologies (36). Our results highlight this point, demonstrating that AMPK can deeply modify the ECM environment, thereby preventing cell mobility and reducing pro-inflammatory cell recruitment, which, *in vivo*, could be associated with reduced disease development or progression. The ATP/AMP ratio depends on nutrient availability, and glucose has a pivotal role in physiology. In hypoglycemic conditions, cells have a low energy charge, and by switching off non-vital anabolic processes (*i.e.* HA synthesis) and concomitantly switching on lipid catabolism, AMPK can thereby increase the ATP/AMP ratio and overcome stress periods. On the other hand, in hyperglycemic conditions, cells have a high energy charge and inactive AMPK but can initiate several intracellular stress responses that can lead to autophagy (46). In this condition, HA synthesis is known to be dramatically elevated, and HA cable-like structures can be produced extracel-

lularly that are strongly adhesive for inflammatory cells (47). In this context, a deeper knowledge of the mechanisms that control cell microenvironment metabolism (*i.e.* synthesis, degradation, and modifications) could identify critical factors involved in pathologies, such as the role of AMPK in cardiovascular diseases.

Acknowledgments—We are grateful to Benoît Viollet (Institut Cochin, University Paris 5, Paris, France) for knock-out and wild-type MEFs, Dave Carling (Medical Research Council Clinical Sciences Centre, Imperial College, London, UK) for the CA-AMPK vector, Morris J. Birnbaum (Howard Hughes Medical Institute, Philadelphia, PA) for the DN-AMPK vector, Paul H. Weigel (University of Oklahoma) for the HAS1 and HAS3 vectors, and Federica Cossu and Martino Bolognesi (University of Milan, Milan, Italy) for bioinformatic studies on HAS2. We acknowledge the Centro Grandi Attrezzature per la Ricerca Biomedica at the Università degli Studi dell'Insubria for instrument availability.

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