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Activation of AMP-activated Protein Kinase $\alpha 2$ by Nicotine Instigates Formation of Abdominal Aortic Aneurysms in Mice *in vivo*

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Abstract

Smoking is the only modifiable risk factor associated with development, expansion, and rupture of abdominal aortic aneurysm (AAA), a leading cause of death in the human population. However, the causative link between cigarette smoke and AAA remains to be established. Here we report that, like angiotensin II (AngII), acute infusion of nicotine, a major component of cigarette smoke, resulted in significantly increased elastin degradation, enlargement of the aorta, aberrant expression of matrix metalloproteinase 2 (MMP2), and an increased incidence of AAA in ApoE knockout (*ApoE*^{−/−}) and deficient in both ApoE and AMP-activated kinase (AMPK) $\alpha 1$ subunit (*ApoE*^{−/−}/*AMPK $\alpha 1$* ^{−/−}) mice. Importantly, genetic deletion of AMPK $\alpha 2$ (*ApoE*^{−/−}/*AMPK $\alpha 2$* ^{−/−}) markedly reduced the increase of maximal aortic diameter and incidence of AAA caused by nicotine or AngII *in vivo*. Transplantation of bone marrow cells from *ApoE*^{−/−} mice into *ApoE*^{−/−}/*AMPK $\alpha 2$* ^{−/−} mice or *vice versa* did not alter nicotine/AngII-induced AAA formation. Mechanistically, we found that both nicotine and AngII activated AMPK in cultured vascular smooth muscle cells (VSMC) and increased the nuclear co-localization of AMPK $\alpha 2$ and AP-2 α , a key transcriptional factor essential for MMP2 expression. Biochemical and biological analysis revealed that AMPK $\alpha 2$ directly phosphorylated AP-2 α at serine 219 and this phosphorylation increased AP-2 α -dependent MMP2 gene expression in VSMC. We conclude that nicotine increases the incidence of AAA *in vivo* through activation of AMPK $\alpha 2$ and AP-2 α . Moreover, our results provide the first demonstration of a causative link between nicotine and AAA *in vivo*.

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Author contributions

S.W. designed and conducted the experiments, analyzed data, and wrote the manuscript. C.Z. and J.Z. did the clinical experiments. B.L. partially performed the pathological experiments. M.Z. and L.X. did bone marrow transplantation. H.Z. and J.L. generated series mutants. B.V. provided the AMPK-deleted mice. M.-H.Z. conceived the project, designed the experiments, and wrote the manuscript.

Additional methods. Detailed methodology is described in the Supplementary Methods available online.

INTRODUCTION

Abdominal aortic aneurysm (AAA), characterized by permanent, localized dilatation (ballooning) of the abdominal aorta exceeding the normal diameter by more than 50%, is the most common form of aortic aneurysm. AAA is found in up to 8% of men aged >65 years, but usually remain asymptomatic until they rupture. Rupture of an AAA and its associated catastrophic physiological insult carries an overall mortality rate in excess of 80%; ruptured AAAs are the 13th leading cause of death in the United States¹. The exact cause of AAA is unknown. Among all identified risk factors, smoking, which increases the risk of AAA 7.6-fold, is the only modifiable factor associated with the development, expansion, and rupture of AAA. To date, however, no causative link has been proven between smoking and AAA formation.

Pathologically, AAAs are characterized by dilatation of all layers of the arterial wall as a result of loss of elastin, smooth muscle cell apoptosis, and compensatory collagen deposition^{2–4}. Inflammation and matrix degradation in the vasculature are crucial for AAA formation. Reactive oxygen species (ROS) and reactive nitrogen species are reported to be a common link between inflammation, matrix degradation, and AAA pathogenesis. For example, angiotensin II (AngII) increases NAD(P)H oxidase-derived ROS⁵, activates matrix metalloproteinase (MMPs) protein and mRNA expressions⁶, and induces AAA formation in animal experiments via inflammation⁷. Although increased ROS in AAA is well documented, the molecular mechanisms by which ROS induce AAA remain poorly characterized.

AMPK is a serine/threonine kinase consisting of α , β , and γ subunits. The α subunit (isoforms $\alpha 1$ and $\alpha 2$) contains the catalytic activity, while the β and γ regulatory subunits maintain the stability of the heterotrimer complex. As indicated by its name, AMPK is activated by increased intracellular AMP; thus, AMPK is considered to be an energy sensor in the regulation of whole body energy homeostasis and dysfunctional AMPK has been reported in multiple diseases, including obesity, diabetes, and cardiovascular dysfunction. Recently, Birnbaum MJ's group^{8,9} and ours^{10,11} have found that AMPK can be activated by oxidants or hypoxia including hydrogen peroxide and peroxynitrite, which activate AMPK via LKB1-dependent or -independent pathways^{11,12}. Accordingly, oxidant-mediated AMPK activation in vascular smooth muscle cells (VSMCs) inhibits cell proliferation and restenosis induced by AngII or wire injury^{12,13}. However, whether AMPK is involved in AAA formation remains unknown. The aim of the present study was to determine the roles and underlying molecular mechanisms of AMPK in smoking-enhanced AAA formation. We report that nicotine, a major component of cigarette smoke, activates AMPK in VSMCs and that AMPK $\alpha 2$ phosphorylates AP-2 α at serine 219 resulting in aberrant expression of MMP2 and consequent AAA formation.

RESULTS

Deletion of AMPK $\alpha 2$ suppresses nicotine-induced AAA formation *in vivo*

Nicotine is a major component of cigarette smoke and is rapidly absorbed into the bloodstream, with blood concentrations ranging from 40–100 ng/ml after each cigarette¹⁴. In regular smokers, nicotine is present in the blood at 90–100 nM over the first 6 to 8 h of the day¹⁵. Although there is overwhelming epidemiological evidence linking smoking and AAA¹⁶, there is no evidence that smoking or nicotine causes AAA formation in humans or in animals. We firstly investigated whether nicotine infusion (1, 5 mg/kg/day, 6 weeks) increased the incidence of AAA in *ApoE*^{-/-}, *ApoE*^{-/-}/*AMPK $\alpha 1$* ^{-/-} or *ApoE*^{-/-}/*AMPK $\alpha 2$* ^{-/-} mice. None of the animals died during this time, and nicotine infusion did not affect hemodynamic parameters (heart rate, systolic blood pressure, and diastolic blood

pressures) or metabolic indexes (serum cholesterol, triglyceride, and glucose levels) in mice (Supplementary Tab. 1,2, online).

All $ApoE^{-/-}$, $ApoE^{-/-}/AMPK\alpha1^{-/-}$ or $ApoE^{-/-}/AMPK\alpha2^{-/-}$ mice did not exhibit AAA when infused with vehicle (Fig. 1a–d). However, both low-dose and high-dose of nicotine infusions for 6 weeks significantly increased the incidence of AAA, the maximal aortic diameter, and total aortic weight in $ApoE^{-/-}$ and $ApoE^{-/-}/AMPK\alpha1^{-/-}$ mice (Fig. 1a–d and Supplementary Fig. 1a–c, Online). Compared with mice infused with vehicle, nicotine markedly increased the size of the aortic lumen and the wall thickness of $ApoE^{-/-}$ and $ApoE^{-/-}/AMPK\alpha1^{-/-}$ mice, but not $ApoE^{-/-}/AMPK\alpha2^{-/-}$ mice (Fig. 1e). As expected, the elastic lamina was typically disrupted and degraded in nicotine-infused $ApoE^{-/-}$ and $ApoE^{-/-}/AMPK\alpha1^{-/-}$ mice.

Deletion of AMPK $\alpha2$ markedly reduced the incidence of nicotine-induced AAA, compared to nicotine-infused $ApoE^{-/-}$ mice (AAA incidence: 20% for $ApoE^{-/-}$ vs. 0% for $ApoE^{-/-}/AMPK\alpha2^{-/-}$, $P<0.05$, Fig. 1b). The maximal aortic diameter and total aortic weight in nicotine-infused $ApoE^{-/-}/AMPK\alpha2^{-/-}$ mice was also significantly smaller than that in nicotine-infused $ApoE^{-/-}$ mice (Fig. 1c,d), and nicotine infusion failed to cause significant elastic lamina degradation and aortic expansion in $ApoE^{-/-}/AMPK\alpha2^{-/-}$ mice (Fig. 1e,f). Positive staining for α -actin indicated that VSMCs were the major cellular component in the area of the AAA (Fig. 1e). Overall, these results suggest that deletion of AMPK $\alpha2$ protects against nicotine-enhanced AAA formation in $ApoE^{-/-}$ mice *in vivo*.

AMPK $\alpha2$ deficiency attenuates AngII-induced AAA formation *in vivo*

AAA induced by AngII infusion is a well-characterized mouse model^{17,18}. To further validate the role of AMPK $\alpha2$ in AAA, we examined the effects of 4-week AngII infusion in $ApoE^{-/-}$, $ApoE^{-/-}/AMPK\alpha1^{-/-}$, and $ApoE^{-/-}/AMPK\alpha2^{-/-}$ mice. As shown in Online Supplementary Tab. 3,4, AngII infusion had no effects on heart rate, serum cholesterol, triglyceride, and glucose levels. AngII infusion markedly increased both systolic and diastolic blood pressure in $ApoE^{-/-}$, $ApoE^{-/-}/AMPK\alpha1^{-/-}$, and $ApoE^{-/-}/AMPK\alpha2^{-/-}$ mice. In saline-infused mice, there was no difference in the gross morphology of aortas among control $ApoE^{-/-}$, $ApoE^{-/-}/AMPK\alpha1^{-/-}$, and $ApoE^{-/-}/AMPK\alpha2^{-/-}$ mice (Supplementary Fig. 2a, Online).

Consistent with previous reports^{2,7,19}, the incidence of AAA induced by AngII infusion was 85% and 80% in $ApoE^{-/-}$ mice and $ApoE^{-/-}/AMPK\alpha1^{-/-}$ mice (Supplementary Fig. 2a,b). Concomitantly, both the maximal abdominal aortic diameter (Supplementary Fig. 2c) and total aortic weight (Supplementary Fig. 2d) were markedly increased in AngII-treated $ApoE^{-/-}$ mice and $ApoE^{-/-}/AMPK\alpha1^{-/-}$ mice, similar to Daugherty's reports which AngII induced AAA formation as well as increased maximal abdominal aortic diameter in $ApoE^{-/-}$ or $LDLr^{-/-}$ mice^{20–22}. Histological analysis showed that $ApoE^{-/-}$ and $ApoE^{-/-}/AMPK\alpha1^{-/-}$ mice infused with AngII exhibited the increased size of the aortic lumen and wall thickness (Supplementary Fig. 2e) and the elastic lamina was frequently disrupted and degraded (Supplementary Fig. 2f).

In contrast, only 17% of the AngII-infused $ApoE^{-/-}/AMPK\alpha2^{-/-}$ mice developed AAA (Supplementary Fig. 2b). The maximal abdominal aortic diameter (Supplementary Fig. 2c) and total aortic weight (Supplementary Fig. 2d) were markedly reduced in AngII-treated $ApoE^{-/-}/AMPK\alpha2^{-/-}$ mice, compared to AngII-treated $ApoE^{-/-}$ mice or $ApoE^{-/-}/AMPK\alpha1^{-/-}$ mice. The aortas of AngII-infused $ApoE^{-/-}/AMPK\alpha2^{-/-}$ mice showed no changes in aortic wall thickness/aortic expansion or elastic lamina degradation (Supplementary Fig. 2e,f). These results suggest that deletion of AMPK $\alpha2$ reduces AngII-induced AAA formation *in vivo*.

AMPK α 2 deficiency abolishes nicotine- or AngII-induced MMP2 upregulation *in vivo*

MMPs are important in the initiation and progression of AAA²³ because they degrade the extracellular matrix (ECM), thus decreasing the resistance of the vessel wall to blood flow²⁴. In particular, VSMC-derived MMP2 and macrophage-derived MMP9 are key enzymes in AAA formation^{25,26}. Thus, we next determined the relative contributions of MMP2 and MMP9 in nicotine-enhanced AAA formation. The expression of MMP2 was significantly increased in nicotine-infused *ApoE*^{-/-} and *ApoE*^{-/-}/*AMPK α 1*^{-/-} mice (Fig. 2a). The expression of MMP9 protein was also increased by nicotine, but to a lesser extent than that of MMP2. In contrast, *ApoE*^{-/-}/*AMPK α 2*^{-/-} mice displayed reduced MMP2 protein expression after nicotine infusion.

We next assayed MMP activity, mRNA and protein levels in isolated aortas from *ApoE*^{-/-}, *ApoE*^{-/-}/*AMPK α 1*^{-/-} and *ApoE*^{-/-}/*AMPK α 2*^{-/-} mice. Both MMP2 and MMP9 activities were detectable, although MMP2 appeared to be the major MMP in vehicle- or nicotine-treated *ApoE*^{-/-} and *ApoE*^{-/-}/*AMPK α 2*^{-/-} mice (Fig. 2b). Nicotine infusion increased the activity of MMP2 markedly in *ApoE*^{-/-} and *ApoE*^{-/-}/*AMPK α 1*^{-/-} mice and to a lesser extent in *ApoE*^{-/-}/*AMPK α 2*^{-/-} mice, but appeared to have minimal effects on MMP9 in *ApoE*^{-/-}, *ApoE*^{-/-}/*AMPK α 1*^{-/-} or *ApoE*^{-/-}/*AMPK α 2*^{-/-} mice. In addition, nicotine infusion significantly increased MMP2 protein and mRNA levels in *ApoE*^{-/-}, *ApoE*^{-/-}/*AMPK α 1*^{-/-} or *ApoE*^{-/-}/*AMPK α 2*^{-/-} mice (Fig. 2c,d and Supplementary Fig. 3a, Online). Similarly, AngII infusion markedly increased the protein and mRNA expressions, and activity (Supplementary Fig. 4a,b, Online) of both MMP2 and MMP9 in *ApoE*^{-/-} and *ApoE*^{-/-}/*AMPK α 1*^{-/-} mice but not in *ApoE*^{-/-}/*AMPK α 2*^{-/-} mice.

Vascular AMPK α 2-deficiency is essential for AAA Inhibition

Both VSMC-derived MMP2 and macrophage-derived MMP-9 use elastin as their substrate. Because AMPK α 1, rather than α 2, is the predominant isoform in macrophages and deletion of AMPK α 1 did not alter Nicotine/AngII-induced AAA formation, we hypothesized that AMPK α 2 in VSMCs plays a major role in AAA formation. To test this, we transplanted *ApoE*^{-/-}/*AMPK α 2*^{-/-} bone marrow (BM) cells into irradiated *ApoE*^{-/-} mice or *ApoE*^{-/-}/*AMPK α 2*^{+/-} BM cells into irradiated *ApoE*^{-/-}/*AMPK α 2*^{-/-} mice. After 42 days of engraftment, flow cytometric analysis indicated that >90% of circulating cells in recipient mice were donor-derived (Data not shown). We then treated the mice with nicotine (1 mg/kg/day, 42 days). As expected, formation of AAA was observed in *ApoE*^{-/-} mice transplanted with either *AMPK α 2*^{-/-} or *AMPK α 2*^{+/-} BM cells, but not in *ApoE*^{-/-}/*AMPK α 2*^{-/-} mice transplanted with either *AMPK α 2*^{-/-} or *AMPK α 2*^{+/-} BM cells (Fig. 3a). The incidence of AAA was identical in *ApoE*^{-/-} mice transplanted with *AMPK α 2*^{-/-} or *AMPK α 2*^{+/-} BM cells (appropriately 20%). Importantly, no AAA (AAA incidence: 0%) was seen in *ApoE*^{-/-}/*AMPK α 2*^{-/-} mice transplanted with either *AMPK α 2*^{-/-} or *AMPK α 2*^{+/-} BM cells (Fig. 3a) and there was no difference in the maximum abdominal aortic diameter (Fig. 3b) and total aortic weight (Fig. 3c). Similar to nicotine, AngII infusion caused AAA formation, increased AAA incidence (Fig. 3d), maximum abdominal aortic diameter (Fig. 3e), and total aortic weight (Fig. 3f) in *ApoE*^{-/-} mice transplanted with either *AMPK α 2*^{-/-} or *AMPK α 2*^{+/-} BM cells, but not in *ApoE*^{-/-}/*AMPK α 2*^{-/-} mice transplanted with either *AMPK α 2*^{-/-} or *AMPK α 2*^{+/-} BM cells (Fig. 3d-f). Taken together, these data suggest that loss of AMPK α 2 expression in vascular cells, rather than BM-derived cells, is crucial for the development of AAA.

MMP2 is crucial for nicotine/AngII-induced elastin degradation

In order to further support the key role of VSMC-derived MMP2 in AAA formation, we generated acute MMP2-deficient mice by *in vivo* siRNA delivery. As depicted in Online Supplementary Fig. 5a, MMP2 siRNA significantly silenced MMP2 protein expression in

mice aortas and both AngII and nicotine enhanced Pro-MMP2 protein levels in control siRNA-transfected mice. The content of collagen IV, a direct substrate of MMP2, was reduced by AngII or nicotine in control siRNA mice but not in MMP2 siRNA mice (Supplementary Fig. 5a,b). Accordingly, MMP2 siRNA but not control siRNA blocked AngII or nicotine-induced elastin degradation (Supplementary Fig. 5b,c), indicating MMP2, rather than MMP9, mediates AngII/nicotine-induced elastin degradation.

Infusion with nicotine or AngII increases ROS *in vivo*

Increased ROS and the associated vascular inflammation are considered as key mediators of AngII-induced AAA formation⁷. We first measured the levels of malondialdehyde (MDA) and 3-nitrotyrosine (3-NT), two markers of oxidative stress²⁷, in *ApoE*^{-/-}, *ApoE*^{-/-}/*AMPKα1*^{-/-} and *ApoE*^{-/-}/*AMPKα2*^{-/-} mice. As shown in Fig. 2a and Supplementary Fig. 4a, infusion with nicotine or AngII significantly increased the levels of MDA and 3-NT in *ApoE*^{-/-}, *ApoE*^{-/-}/*AMPKα1*^{-/-} and *ApoE*^{-/-}/*AMPKα2*^{-/-} mice. Serum levels of cytokines (IL-6, TNFα, IFNγ, and CypA) were also elevated in AngII/nicotine-infused mice (Supplementary Tab. 5 and 6, Online). The increased ROS levels were further confirmed by assaying superoxide with dihydroethidium (DHE) by HPLC. As depicted in Fig. 2e and Supplementary Fig. 4c, either nicotine or AngII increased DHE fluorescence in *ApoE*^{-/-}, *ApoE*^{-/-}/*AMPKα1*^{-/-} and *ApoE*^{-/-}/*AMPKα2*^{-/-} mice. These data suggest that both AngII and nicotine trigger oxidative stress *in vivo* and ROS may be the upstream activator of AMPK.

Activation of AMPK by nicotine or AngII increases the expression of pro-MMP2 in VSMCs

Since VSMCs are the major cell type in AngII- and nicotine-triggered AAA in mice and AMPK expression in VSMCs appears to be essential in MMP2 expression, it seemed highly likely that nicotine and AngII would activate AMPK and that this activation would modulate MMP2 expression and activity in VSMCs. Indeed, AMPK was activated by exposure of cells to low concentrations (0.01–0.1 μM) of nicotine that are equivalent to the blood concentrations in habitual smokers (Supplementary Fig. 6a,b, Online). The degree of AMPK activation was similar to that caused by the potent AMPK activators 5-amino-4-imidazole carboxamide riboside (AICAR, 2 mM) or peroxynitrite (50 μM). Nicotine also increased phosphorylation of AMPK Thr172 in a time-dependent manner (Supplementary Fig. 6c). Likewise, AngII also activated AMPK and its substrate ACC in VSMCs in a time-dependent manner (Supplementary Fig. 6d,e), consistent with previously published data¹². Treatment with AngII or nicotine did not alter the total level of AMPKα, suggesting that changes in AMPK Thr172 phosphorylation were not due to altered expression of the protein.

To further establish the role of AMPKα2 in MMP2 expression in the vascular wall, we tested the effects of nicotine or AngII on the protein and mRNA expressions and activity of MMP2 in cultured human VSMCs. As indicated in Supplementary Fig. 3c,d and Supplementary Fig. 7a,b, exposure of VSMCs to AngII or nicotine markedly increased MMP2 mRNA and protein expressions in whole cell lysates and the activity of MMP2 in cultured medium. Compound C, a known AMPK inhibitor, abolished the effects of AngII or nicotine on MMP2, indicating that AMPK mediates MMP2 expression induced by AngII or nicotine.

AMPKα2, but not AMPKα1, mediates upregulation of MMP2 by AngII in VSMCs

The observation that deletion of AMPKα2, but not AMPKα1, provided protection from Nicotine-induced AAA formation suggests that only AMPKα2 regulates MMP2 expression (Fig. 1a–c). To further investigate AMPK isoform-specific MMP2 expression, we silenced AMPKα1 or AMPKα2 in VSMC by transfection with AMPKα1-specific or AMPKα2-specific siRNAs. As depicted in Fig. 4a,c and Supplementary Fig. 3e,g, transfection with

AMPK α 2 siRNA, but not AMPK α 1 siRNA, abolished nicotine, metformin, AngII, or AICAR-enhanced MMP2 protein and mRNA expressions and activity in cultured medium of human VSMCs.

We next performed these experiments in VSMCs isolated from WT, *AMPK α 1*^{-/-}, or *AMPK α 2*^{-/-} mice. As expected, AngII up-regulated MMP2 levels in *AMPK α 2*^{-/-} VSMCs, but not in WT and *AMPK α 1*^{-/-} cells (Fig. 4b,d and Supplementary Fig. 3f,h). These data are in agreement with those obtained with AMPK α 2-specific siRNA and *ApoE*^{-/-}/*AMPK α 2*^{-/-} mice. Overall, our results support the notion that AMPK α 2, but not AMPK α 1, is required for MMP2 expression in VSMCs.

Upregulation of MMP2 by nicotine or AngII is AP-2 α -dependent

Activator protein 2 α (AP-2 α) is a transcription factor that is required for MMP2 expression in endothelial cells²⁸. *AP-2 α* ^{-/-} mice die after birth due to abnormal vascular development, suggesting an important role of AP-2 α in regulating vascular angiogenesis^{29,30}. Currently, there is no evidence that AP-2 α can regulate MMP2 gene expression in VSMCs. We hypothesized that nicotine- or AngII-induced MMP2 upregulation occurs through AP-2 α -dependent gene transcription and expression of MMP2. Indeed, transfection of VSMCs with AP-2 α -specific or AMPK α 2-specific siRNAs, but not control siRNA, inhibited nicotine-induced pro-MMP2 protein expression and MMP2 activity (Fig. 5a), implying that the upregulation of MMP2 by nicotine in VSMCs requires AP-2 α . Similarly transfection of VSMCs with AP-2 α siRNA, but not control siRNA, also abolished AngII-induced MMP2 upregulation (Fig. 5b).

Nicotine and AngII increase AP-2 α -binding to the MMP2 promoter

Since AP-2 α is required for nicotine- and AngII-induced MMP2 gene transcription, we reasoned that AngII and nicotine might activate AP-2 α to increase its binding affinity for the MMP2 gene promoter. As shown in Fig. 5c–f, an increased DNA shift and activity of AP-2 α detected by EMSA and luciferase reporter gene was observed in AngII- or nicotine-stimulated VSMC, suggesting that AngII and nicotine increased AP-2 α activity and the affinity to DNA. Inhibition of AMPK α 2 with either siRNA or compound C significantly suppressed the AngII- or nicotine-enhanced AP-2 α activity and the binding of AP-2 α to DNA. Conversely, metformin, a well-characterized AMPK activator, increased AP-2 α /DNA binding affinity (Fig. 5c). Finally, binding was inhibited by AMPK α 2 siRNA (Fig. 5c). Overall, these data suggest that AMPK mediates nicotine/AngII-induced activation of AP-2 α and its binding to DNA.

We next investigated whether AP-2 α specially binds to the MMP2 gene promoter in VSMCs using ChIP analysis. In human VSMCs, the MMP2 gene promoter was positively amplified in samples from AngII-treated cells following immunoprecipitation with AP-2 α primary antibody but not with control IgG (Fig. 5g), suggesting that the positive amplification of the MMP2 gene promoter is specific to AP-2 α . Further, compound C attenuated the AngII-induced binding of AP-2 α to the MMP2 gene promoter, implying a requirement for AMPK activation in this binding. Similar effects were observed in nicotine-treated VSMCs (Fig. 5g–i).

Nicotine and AngII promote the co-localization and association of AMPK α 2 and AP-2 α in VSMCs

To characterize the mechanisms by which AMPK participates in the activation of AP-2 α by AngII or nicotine and AP-2 α is located in the nucleus, we first determined the subcellular location of AMPK α . As depicted in Fig. 6a, the nicotine/AngII-induced nuclear translocation of AMPK α 2, but not AMPK α 1, was observed by western blot analysis of

separated cytoplasmic and nuclear fractions. Treatment of cells with compound C inhibited the nicotine/AngII-promoted AMPK α 2 nuclear translocation.

The interaction of AMPK α 2 and AP-2 α after nicotine/AngII treatment was confirmed by immunoprecipitation experiments. As indicated in Fig. 6b, binding of AMPK α 2/AP-2 α was evident in the nuclear cell fraction pulled down by either AP-2 α or AMPK α 2. However, no binding was detected between AMPK α 1 and AP-2 α .

The nuclear import and co-localization of AMPK α 2 and AP2 α were further verified by immunofluorescence experiments (Supplementary Fig. 8, online). The protein-protein interaction of AMPK α 2 and AP2 α was also further confirmed by *in vitro* co-incubation and pull-down experiments (Fig. 6c). Together, these data suggest that AMPK α 2 directly affects AP-2 α through protein/protein interaction.

AMPK increases phosphorylation of AP-2 α at serine 219

Based on our findings that AngII triggers nuclear import of AMPK α 2 and its co-localization with AP-2 α , we proposed a mechanism by which AMPK might directly regulate AP-2 α function. Post-translational modifications of AP-2 α , including serine phosphorylation, are known to regulate its transcriptional activity³¹. As AMPK is a Thr/Ser protein kinase, we reasoned that AMPK might directly phosphorylate AP-2 α serine(s). To test this, we first measured total serine phosphorylation of AP-2 α in VSMCs after AngII/nicotine treatment. As depicted in Fig. 6d, exposure of VSMC to AngII or nicotine increased the serine phosphorylation of AP-2 α . Conversely, inhibition of AMPK by compound C dramatically inhibited AngII- or nicotine-induced AP-2 α serine phosphorylation, implying that activation of AMPK may increase AP-2 α serine phosphorylation.

Direct phosphorylation of AP-2 α by AMPK α 2 was demonstrated by measuring *in vitro* AMPK kinase activity (Fig. 6e). Wild type AMPK α 2 (WT-AMPK α 2), but not dominant negative AMPK α 2 (DN-AMPK α 2), significantly phosphorylated AP-2 α recombinant protein as detected by P³²-ATP radio-autography, indicating that AP-2 α is a substrate of direct phosphorylation by AMPK α 2.

Next, we determined which serine residue(s) in AP-2 α protein are phosphorylated by AMPK α 2 by comparison with an optimal AMPK substrate motif³². Analysis of AP-2 α (Supplementary Fig 9a–c, Online) revealed potential AMPK target motifs at serines 181, 219, 233, 252, and 320 in the regulatory and helix-span-helix domains, that are similar to previously identified AMPK substrates (the SAMS peptide, eNOS, ACC, ULK1, H2B, and TSC2)^{33–35}. When we compared *in vitro* phosphorylation of peptides corresponding to residues 176–185, 214–223, 228–237, 247–256, or 315–324 of AP-2 α with that of SAMS peptide, a canonical AMPK target sequence, only peptide 214–223 of AP-2 α was phosphorylated by AMPK α 2 protein (Fig. 6f). Furthermore, mutation of serine to alanine at Ser6 of SAMS and Ser219 of AP-2 α peptide 214–233 abolished AMPK α 2-induced phosphorylation (Fig. 6g).

To further confirm S219 of AP-2 α as the phosphorylation target of AMPK α 2, we generated five site-directed mutant constructs of AP-2 α (Serine to Alanine at S181A, S219A, S233A, S252A, and S320A). In HEK293 cells transfected with WT AP-2 α , treatment with AICAR, which phosphorylates AMPK at Thr172, dramatically increased AP-2 α serine phosphorylation (Fig. 6h). Among the AP-2 α mutant constructs, AICAR-induced AP-2 α serine phosphorylation was abolished only in cells transfected with AP-2 α S219A, confirming that serine 219 is the target of AMPK-induced AP-2 α serine phosphorylation.

Mutation of AP-2 α Serine 219 to alanine abolishes AngII- or nicotine-induced upregulation of MMP2 in VSMCs

Since AMPK α 2 directly phosphorylates AP-2 α serine 219 in cells, we next tested whether serine 219 phosphorylation of AP-2 α determines AP-2 α activity and function. As shown in Online Supplementary Fig. 10a–c, transfection of human VSMCs with the AP-2 α S219A mutant dramatically abrogated AngII/nicotine-enhanced upregulation of MMP2 (mRNA level, protein expression, and activity), AP-2 α -DNA binding affinity, and binding of AP-2 α to the MMP2 gene promoter. These results indicate that serine 219 phosphorylation of AP-2 α by AMPK α 2 promotes its binding to the MMP2 promoter resulting in aberrant MMP2 expression in VSMCs.

Increased AMPK α 2 activity, AMPK Thr172 phosphorylation, and ACC Ser 172 phosphorylation in human AAAs

To establish human relevance, we further examined AMPK α 2 activity, AMPK Thr172 phosphorylation, and Ser79 phosphorylation of acetyl CoA carboxynase (ACC), a well characterized downstream enzyme of AMPK. As shown in Supplemental Figure 11, human AAA samples exhibited higher AMPK α 2 activity, AMPK Thr172 phosphorylation, and ACC Ser79 phosphorylation in aortic tissues from AAA than those from non-AAA control patients.

Cigarette smoke increases AMPK α 2 activity, AMPK Thr172 phosphorylation, and ACC Ser 172 phosphorylation in humans

Finally, to address if cigarette smokers altered AMPK activity, we assayed AMPK α 2 activity, p-AMPK, and p-ACC in the plasma of both active smokers and non-smokers. In acute smokers, AMPK α 2 activity is higher than in non-smokers (Supplemental Figure 11). Taken together, our data suggest that smoke-activated AMPK is important in the initiation and progression of AAA.

DISCUSSION

In the present study, we provide the first evidence that nicotine increases AAA in *ApoE*^{-/-} mice *in vivo* through activation of AMPK α 2 (Supplementary Fig. 12). Mechanistically, we found that nicotine- or AngII-derived ROS trigger nuclear translocation of AMPK α 2, which binds and phosphorylates AP-2 α at serine 219 resulting in aberrant expression of MMP2 and consequent ECM degradation. This novel mechanism not only provides a causative link between nicotine and AAA, but also explains how pro-inflammatory factors such as ROS promote AAA formation.

One of our most important findings is that activation of AMPK α 2 by nicotine and AngII triggers AAA formation in *ApoE*^{-/-} mice. This conclusion is supported by several observations. First, gene deletion of AMPK α 2 suppressed AngII/nicotine-induced MMP2 protein expression in cells or MMP2 activity in culture medium. Inhibition of AMPK with the pharmacological inhibitor compound C reproduced this effect. Second, activation of AMPK by AICAR produced similar effects to those induced by AngII or nicotine. Third, the formation of AAA following infusion with AngII or nicotine was reduced in *ApoE*^{-/-}/*AMPK α 2*^{-/-} mice compared with *ApoE*^{-/-} mice. We thus demonstrated that ablating AMPK activity by deleting the AMPK gene has a marked protective effect against aortic aneurismal disease. In fact, a clinical investigation has indicated that diabetes is negatively associated with AAA formation³⁶, and there is evidence that hyperglycemia suppresses AMPK activity^{37,38} whereas insulin treatment promotes the enlargement of aortic diameter^{39,40} through the effect of lowering blood glucose. These findings suggest an important role for AMPK in the pathogenesis of AAA.

Another important finding is that AP-2 α is a direct substrate for phosphorylation by AMPK α 2 at serine 219. AP-2 α is also phosphorylated at Ser233 by protein kinase A (PKA)³¹ and at both Ser252 and Ser320 by protein kinase D (PKD)⁴¹, based on the phosphorylation motifs (RRXS/T and LXRXXS/T) in its basic transactivation domain. By comparing the amino acid sequence of AP-2 α with known substrates of AMPK, we identified serine 219 as a novel target of AMPK α 2. Phosphorylation of S219 by AMPK α 2 was demonstrated by the following evidence: (1) AP-2 α is phosphorylated only in the presence of AMPK α 2 in a cell-free system; (2) AMPK α 2 specifically phosphorylated an AP-2 α peptide containing S219; (3) Mutation of serine 219 to alanine abolished AMPK-induced AP-2 α serine phosphorylation, increased DNA binding affinity, and MMP2 levels. In fact, AP-2 α is very important for mouse embryonic development and mice lacking AP-2 die at or before birth as a result of severe defects²⁹. *Combined with our observations, we think defects of cardiovascular development by loss of MMP2 in AP-2a deletion mice may be one of factors to lethal. Our data also indicate that AP-2a may have a broader role in regulating genes critical to normal embryogenesis.*

Our studies are in line with earlier reports^{8,34,35} that oxidants-mediated AMPK activation by concentrations of nicotine relevant to smokers in adipocytes as well as AngII in VSMC. In addition, our study has extended these observations by showing that AMPK activation might be detrimental in the initiation and progression of AAA, which contradicts the idea that AMPK activation is beneficial in cardiovascular diseases. For example, AMPK α 2 deletion is reported to cause accelerated atherosclerosis³⁶ and pharmacological activation of AMPK has been shown to ablate wire injury-induced vascular remodeling by inhibiting VSMC proliferation⁸. Although atherosclerosis, restenosis, and AAA are associated with vascular inflammation and have many of the same predisposing risk factors, there is an important difference between the pathways mediating atherogenesis and AAA formation. For example, early atherosclerosis is primarily characterized by pro-inflammatory factor-mediated foam cell accumulation in the intima³⁷. In contrast, AAA involves degradation of the tunica media in which enhanced activation of the proteolytic system, such as MMPs, plays a crucial role³⁸. In addition, reduced VSMC proliferation has also been found within fragmented media in human AAA biopsy specimens. Thus, VSMC proliferation, which leads to acceleration of atherosclerosis and restenotic vascular remodeling, might be paradoxically beneficial for AAA⁴⁰. Therefore the inhibitory effects of AMPK activation on VSMC proliferation might accentuate the development of AAA. Furthermore, in a recent review paper, Golledge et al⁴¹ concluded that a plurality of studies indicate an independent effect of medications on aneurysms and atherosclerosis in animal models, further suggesting distinct pathogenetic mechanisms.

Another interesting issue of this study is the different effects of AngII and nicotine in AAA formation. From our observations, although both AngII and nicotine have a common pathway (ROS/AMPK/AP-2 α /MMP2) in VSMC to induced AAA in ApoE^{-/-} mice, the incidence and size of AAA induced by AngII is significantly larger than nicotine (Fig. 1a–c and Supplementary Fig. 2a–c). One might expect that the lower incidence of AAA in nicotine infused mice would correlate with lower ROS generation (Fig. 2e and Supplementary Fig. 4c). In addition, it is reported that AngII has other pathways to induce AAA, such as TGF- β and other growth factors, which can be transcriptionally activated by MMP2^{23,42}. Whether or not these pathways contribute to nicotine-enhanced AAA warrants further investigation.

Smoking increases the risk of AAA 7.6-fold. Moreover, smoking is the only modifiable factor associated with the development, expansion, and rupture of AAA. The results presented in this work have for the first time established a causal effect of nicotine, a major component of cigarettes, on the formation, progression, and rupture of AAA *in vivo*. This

finding is highly important, not only because it has established a causative link between cigarette smoke and AAA, but also because it has further identified nicotine as an active agent in smoke-triggered AAA. The finding that nicotine increases AAA formation might be clinically important. One common approach to the cessation of smoking is nicotine supplementation, such as with gum or patches. The rapid absorption of nicotine can increase plasma nicotine concentrations to as high as 5×10^{-7} M within 15 min⁴³, a concentration that, based on our findings, might increase AAA formation. Thus, we suggest that nicotine supplementation might be cautiously reconsidered and that the duration of nicotine supplementation should be minimized in populations with advanced age.

Methods

Animals

Male wild-type (C57BL6) and *ApoE*^{-/-} mice (12–16 weeks of age; 20–25 g body weight) were obtained from Jackson Laboratories (Bar Harbor, ME). *AMPKα1*^{-/-} and *AMPKα2*^{-/-} mice were generated as previously described⁴⁴. Western blot indicates over 90% of AMPKα1 or α2 is knockdown. *AMPKα1*^{-/-} and *AMPKα2*^{-/-} mice previously backcrossed to a C57BL6 background were crossed with *ApoE*^{-/-} mice of C57BL6 background to generate *ApoE*^{-/-}/*AMPKα1*^{-/-} and *ApoE*^{-/-}/*AMPKα2*^{-/-} mice. *ApoE*^{-/-}/*AMPKα*^{+/+} mice served as controls. Mice were housed in temperature-controlled cages with a 12-h light-dark cycle and given free access to water and food. The animal protocol was reviewed and approved by the University of Oklahoma Institute Animal Care and Use Committee.

Statistical analyses

Quantitative results are expressed as mean ± SEM. Comparisons of parameters among more than two groups were made by one-way analysis of variance, and comparisons of different parameters between each group were made by a post hoc analysis using the Bonferroni test. Chi-Square test was applied to comparisons of AAA incidence and survival rate. Statistical significance was evaluated with GraphPad Prism4. A value of *P* < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

AMPKα2 deficiency prevents nicotine-induced AAA formation in ApoE^{-/-} mice. ApoE^{-/-}, ApoE^{-/-}/AMPKα1^{-/-} and ApoE^{-/-}/AMPKα2^{-/-} mice were infused with nicotine (1 mg/kg/day) for 6 weeks using an osmotic pump. N=10–15 in each group. **(a)** Representative photographs showing macroscopic features of aneurysms induced by nicotine. The arrow indicates a typical AAA. **(b)** The incidence of nicotine-induced AAA, **(c)** Maximal abdominal aortic diameter, and **(d)** Total aortic weights in ApoE^{-/-}, ApoE^{-/-}/AMPKα1^{-/-} and ApoE^{-/-}/AMPKα2^{-/-} mice after nicotine infusion. **(e)** Representative staining with HE, elastin, and α-actin in suprarenal aortas of ApoE^{-/-}, ApoE^{-/-}/AMPKα1^{-/-} and ApoE^{-/-}/AMPKα2^{-/-} mice. **(f)** Grade of elastin degradation in aorta wall. *P<0.05 vs. vehicle-infused ApoE^{-/-} mice. #P<0.05 vs. vehicle-infused ApoE^{-/-}/AMPKα1^{-/-} mice. \$P<0.05 vs. nicotine-infused ApoE^{-/-} mice.

Figure 2.

Nicotine infusion results in oxidative stress and enhances MMP2/9 expressions via AMPKα2 in vivo. *ApoE*^{-/-}, *ApoE*^{-/-}/*AMPKα1*^{-/-} and *ApoE*^{-/-}/*AMPKα2*^{-/-} mice were infused with nicotine (1 mg/kg/day) for 6 weeks. N is 10–15 in each group. (a) Representative stainings of MMP2, MMP9, MDA, and 3-NT in supracranial aortas in nicotine-infused mice. (b, c and d) MMPs activity, protein expressions, and mRNA levels assayed by Zymography, western blot and RT-PCR in mice aortas. (e) ROS productions detected by DHE/HPLC in mice aortas. *P* < 0.05 vs. control *ApoE*^{-/-} mice. **P* < 0.05 vs. vehicle-infused *ApoE*^{-/-} mice. #*P* < 0.05 vs. vehicle-infused *ApoE*^{-/-}/*AMPKα1*^{-/-} mice. \$*P* < 0.05 vs. nicotine-infused *ApoE*^{-/-} mice.

Figure 3.

Bone marrow (BM) reconstitution shows a key role for vascular-specific AMPKα2 deficiency in AAA formation. After irradiation, *ApoE*^{-/-} and *ApoE*^{-/-}/*AMPKα2*^{-/-} mice were immediately injected with BM-derived cells from *ApoE*^{-/-} and *ApoE*^{-/-}/*AMPKα2*^{-/-} mice. (a–c) 6 weeks later, mice were infused with nicotine (1 mg/kg/day) for 6 weeks. (a) The incidence of AAA, (b) Maximal abdominal aortic diameters, and (c) Total aortic weights in nicotine-infused mice. N=10–15 in each group. **P*<0.05 vs. nicotine-infused *ApoE*^{-/-} plus *ApoE*^{-/-}/*AMPKα2*^{+/+} BM-derived cells. NS indicates no significant difference. (d–f) 1 week later, mice were infused with AngII (1.44 mg/kg/day) for 4 weeks by using an osmotic pump. (d) The incidence of AAA, (e) Maximal abdominal aortic diameters, and (f) Total aortic weights in AngII-infused mice. N=10–15 in each group. **P*<0.05 vs. AngII-infused *ApoE*^{-/-} plus *ApoE*^{-/-}/*AMPKα2*^{+/+} BM-derived cells. NS indicates no significant difference.

Figure 4.

Inhibition of AMPK α 2, but not AMPK α 1, abolished nicotine/AngII-induced pro-MMP2 mRNA, protein expression, and MMP2 activity in VSMCs. **(a)** Cultured human VSMCs were transfected with control siRNA, AMPK α 1 siRNA, or AMPK α 2 siRNA for 48 h and then incubated with nicotine (0.1 μ M) or metformin (2 mM) for 24 h. pro-MMP2 protein and MMP2 mRNA level in cell lysates, and MMP2 activity in culture medium were detected by Western blot, RT-PCR, and zymography, respectively. N=3. * P <0.05 vs. control siRNA. # P <0.05 vs. AMPK α 1 siRNA. \$ P <0.05 vs. control siRNA plus nicotine or metformin. **(b)** Cultured mouse VSMCs (WT, AMPK α 1 $^{-/-}$, and AMPK α 2 $^{-/-}$) from the descending aorta were incubated with nicotine (0.1 μ M) for 24 h. pro-MMP2 protein and mRNA levels in cell lysates and MMP2 activity in culture medium were assayed. N=3. * P <0.05 vs. WT. # P <0.05 vs. AMPK α 1 $^{-/-}$. \$ P <0.05 vs. WT plus nicotine. **(c)** Cultured human VSMCs were transfected with control siRNA, AMPK α 1 siRNA, or AMPK α 2 siRNA for 48 h and then incubated with AngII (1 μ M) or AICAR (2 mM) for 24 h. pro-MMP2 protein and MMP2 mRNA level in cell lysates, and MMP2 activity in culture medium were detected by Western blot, RT-PCR, and zymography, respectively. N=3. * P <0.05 vs. control siRNA. # P <0.05 vs. AMPK α 1 siRNA. \$ P <0.05 vs. control siRNA plus AngII or AICAR. **(d)** Cultured mouse VSMCs (WT, AMPK α 1 $^{-/-}$, and AMPK α 2 $^{-/-}$) from the descending aorta were incubated with AngII (1 μ M) for 24 h. pro-MMP2 protein and mRNA levels in cell lysates and MMP2 activity in culture medium were assayed. N=3. * P <0.05 vs. WT. # P <0.05 vs. AMPK α 1 $^{-/-}$. \$ P <0.05 vs. WT plus AngII.

Figure 5.

AP-2α mediates nicotine/AngII-induced AMPKα2-dependent MMP2 protein expression in VSMCs. **(a)** Cultured human VSMCs were transfected with control siRNA, AMPKα2 siRNA, or AP-2α siRNA for 48 h and then incubated with nicotine (0.1 μM) for 24 h. MMP2 activity in culture medium and pro-MMP2 protein expression in cell lysates were detected by Zymography and western blot, respectively. Representative results from three independent experiments are shown. **P*<0.05 vs. Vehicle, #*P*<0.05 vs. Nicotine alone. **(b)** Cultured human VSMCs were transfected with control siRNA and AP-2α siRNA for 48 h and then incubated with AngII (1 μM) for 24 h. pro-MMP2 and AP-2α protein expression in cell lysates, mRNA level in cells, and MMP2 activity in culture medium were detected by western blot, RT-PCR, and zymography, respectively. N=3. **P*<0.05 vs. control siRNA, #*P*<0.05 vs. control siRNA plus AngII. **(c)** Cultured human VSMCs were transfected with control siRNA or AMPKα2 siRNA for 48 h and then incubated with AngII (1 μM) or metformin (2 mM) for 2 h. Nuclear extract was subjected to EMSA to measure AP-2α DNA affinity. Negative control (–) consists of biotin-labeled DNA probe without extract. Positive control (+) consists of biotin-labeled DNA probe with nuclear extract provided by the kit. N=3. **P*<0.05 vs. control siRNA, #*P*<0.05 vs. control siRNA plus metformin or AngII. **(d–h)** Cultured human VSMCs were pre-incubated with or without compound C (10 μM) for 30 min and then treated with nicotine (0.1 μM) or AngII (1 μM) for 2 h. AP-2α activity was detected by EMSA **(d, e)** or luciferase reporter gene **(f)** and binding of AP-2α to the MMP2 gene promoter were detected by ChIP **(g, h)**, respectively. **(d, e)** N=3, **P*<0.05 vs. control or vehicle, #*P*<0.05 vs. AngII or Nicotine. **(f)** N is 3. **P*<0.05 vs. control, #*P*<0.05 vs. AngII alone, \$*P*<0.05 vs. Nicotine alone. For ChIP experiments, the complex of chromatin/protein was pulled down by AP-2α primary antibody and the MMP2 promoter was amplified by PCR. Positive control, 10% of the total chromatin in the absence of immunoprecipitation (lane 1–4, input). Negative control, chromatin immunoprecipitated with IgG and amplified with MMP2 promoter primers (IgG, lane 5–8). The expected PCR product is 200 bp. Representative results from three independent experiments are shown. **(i)** Quantifications of ChIP. N is 3. **P*<0.05 vs. control, #*P*<0.05 vs. AngII alone, \$*P*<0.05 vs. Nicotine alone.

Figure 6.

Nicotine/AngII promotes the binding of AMPKα2 to AP-2α and AMPKα2-dependent AP-2α serine 219 phosphorylation. **(a, b)** Cultured human VSMCs were incubated with nicotine (0.1 μM) or AngII (1 μM) for 2 h in the presence of compound C (10 μM). **(a)** Cell fractions were subjected to western blot analysis to determine the subcellular localization of AMPKα1/2 and AP-2α. LDH, cytoplasmic marker; H2AX, nuclear marker. N=3. **(b)** AMPKα1, AMPKα2, or AP-2α proteins in total cell lysates were pulled down by the appropriate primary antibody and subjected to western blot analysis to detect the binding of AP-2α to AMPKα1/2. The blot is representative of three independent blots. **(c)** GST-AMPKα2 and GST-AP-2α protein were co-incubated in reaction buffer for 30'. Mixture was subjected to run native gel or pulled down by primary antibody followed by western blot. 3 independent experiments were performed. **(d)** Serine phosphorylation of AP-2α was assayed by IP with primary AP-2α antibody followed by western blotting with anti-phospho-serine antibody. The experiment was repeated three times. **(e)** Cultured HEK293 cells were transfected with wild type AMPKα2 (WT-AMPKα2) or dominant negative (DN-AMPKα2) for 24 h. WT-AMPKα2 and DN-AMPKα2 protein were purified by IP with primary AMPKα2 antibody. AP-2α protein and IP-derived AMPKα2 protein were co-incubated in kinase buffer in the presence of P³²-labeled ATP and phosphorylation of AP-2α was visualized by radioautography. N=3. **(f)** Peptides (SAMS, AP-2α 176-185/214-233/228-237/247-226/315-324) were co-incubated with AMPKα2 protein in reaction buffer in the presence of P³²-ATP. Phosphorylation of AP-2α and SAMS peptides was visualized by radioautography. N=3. **(g)** Peptides (SAMS, SAMS S6A, AP-2α 214-223, AP-2α 214-233 S219A) were co-incubated with AMPKα2 protein in reaction buffer in the presence of P³²-ATP. Phosphorylation of AP-2α and SAMS peptides was visualized by radioautography. N=3. **(h)** HA-tagged WT and site-directed mutants of full-length AP-2α were transfected into HEK293 cells for 24 h and then treated with AICAR (2 mM) for 2 h. Serine phosphorylation of AP-2α was determined by pull-down with anti-HA and western blot analysis with anti-phosphor-serine antibody. N=3.