Original Article

α1AMP-Activated Protein Kinase Mediates Vascular Protective Effects of Exercise

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Objective—We investigated whether AMP-activated protein kinase (AMPK) may be involved in the signaling processes leading to exercise-mediated vascular protection.

Methods and Results—The effects of voluntary exercise on AMPK activity, endothelial NO synthase expression and phosphorylation, vascular reactive oxygen species formation, and cell senescence were tested in α1AMPK knockout and corresponding wild-type mice. Exercise significantly improved endothelial function, and increased plasma nitrite production in wild-type mice, associated with an activation of aortic AMPK assessed by its phosphorylation at threonine 172. In addition, regular physical activity resulted in an upregulation of endothelial NO synthase protein, serine 1177 endothelial NO synthase phosphorylation, and an increase of circulating Tie-2^Sca-1^Flk-1^ myeloid progenitor cells. All these changes were absent after α1AMPK deletion. In addition, exercise increased the expression of important regulators of the antioxidative defense including heme oxygenase-1 and peroxisome proliferator-activated receptor γ coactivator 1α, decreased aortic reactive oxygen species levels, and prevented endothelial cell senescence in an α1AMPK-dependent manner.

Conclusions—Intact α1AMPK signaling is required for the signaling events leading to the manifestation of vascular protective effects during exercise. Pharmacological AMPK activation might be a novel approach in the near future to simulate the beneficial vascular effects of physical activity. (Arterioscler Thromb Vasc Biol. 2012;32:0–0)

Key Words: AMP-activated protein kinase ■ cell senescence ■ endothelial function ■ exercise ■ NO synthase ■ oxidative stress

AMP-activated protein kinase (AMPK) is considered to be a metabolic master switch, which governs energy expenditure and energy production according to demand. It is activated by decreased ATP levels and a concomitant rise in cellular AMP, which allows cellular survival during metabolic stress. Emerging data suggest that AMPK has also distinct functions in the vasculature because it activates and phosphorylates endothelial NO synthase (eNOS),1 protects endothelial cells against oxidative stress,2 prevents vascular smooth muscle proliferation,3 and mediates angiogenesis.4 All these effects suggest a protective role of AMPK in the vascular system.

Sedentary lifestyle is a major cause for the increase of vascular diseases in Western societies, and exercise is a simple and effective way to prevent cardiovascular risk factors such as obesity, diabetes mellitus, and hypertension in the long term.5 On physical activity, AMPK is activated in skeletal muscle, and recent data suggest that its activation is required for the metabolic response to exercise in vivo.6 Besides the modulation of cardiovascular risk factors, exercise may also have direct effects on the vasculature leading to an improvement of endothelial function.7 The mechanisms how exercise may directly affect vascular function are rather multifactorial, and include an upregulation of eNOS expression,8 serine 1177 eNOS phosphorylation,9 decreased oxidative stress eg, by inhibition of vascular nicotinamide adenine dinucleotide phosphate oxidases10 or augmented superoxide dismutase (SOD) expression,11 increased number of endothelial progenitor cells (EPCs), and enhanced angiogenesis.12 Increased cardiac output and blood flow during exercise will enhance forces that act on the endothelium,13 and shear stress in particular is known to be 1 of the strongest stimuli for eNOS activation.14 However, so far no unifying

Received on: March 30, 2011; final version accepted on: April 13, 2012.
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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBHA.111.243980/-/DC1.
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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBHA.111.243980
signaling event has been characterized that is able to initiate all these beneficial effects in response to exercise. Because endothelial AMPK is activated in response to shear stress\textsuperscript{15} and exhibits similar vascular protective effects compared with exercise, we investigated whether exercise activates AMPK in the vasculature, and whether AMPK may be involved in the signaling processes leading to exercise-mediated vascular protection.

**Materials and Methods**

**Animals and Exercise Protocol**

All animal treatment was in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and was granted by the ethics committee of the University Hospital Mainz. To study the role of AMPK for the vascular effects of exercise, \( \alpha1 \)-AMPK knockout mice and corresponding littermate wild-type mice (C57BL/6J29Sv/FVB-N background) were used.\textsuperscript{16} Eight-week-old male mice were kept in individual cages for 8 weeks equipped with a running wheel and a mileage counter. Body weight and heart weight were assessed in all animals.

**Reagents**

Antibodies against serine 79p-acetyl-CoA carboxylase, total AMPK, and threonine 172p-AMPK were purchased from Cell Signaling (Boston, MA). The total eNOS, serine 1177p-eNOS, cell-cycle checkpoint kinase, p53, p16\textsuperscript{Nkp}, Sca-1-fluorescein isothiocyanate, and Flk-1 phycoerythrin antibodies were purchased from BD Biosciences (San Jose, CA). The Tie-2 phycoerythrin antibody was purchased from BD Biosciences (San Diego, CA). All other chemicals and reagents were of analytic grade purchased from Sigma-Aldrich. Colony forming units were assessed as described previously.\textsuperscript{23,24} Briefly, bone marrow-derived cells were cultured in endothelial cell–specific medium for 3 days, washed, and transferred in ColonyGEL 1202 methylcellulose (CellSystems, Troisdorf, Germany) containing Mouse VEGF (Miltenyi Biotec, Bergisch Gladbach, Germany). Colonies were counted after 2 weeks of culture.

**Serum Antioxidant Capacity**

Acetonitrile was added to freshly prepared serum in equal volumes, incubated for 4 minutes at room temperature, and centrifuged at 9500g at 4°C. The assay was started by addition of the deproteinized serum to a 50-μmol/L 2,2-diphenyl-1-picrylhydrazyl radical solution, and was measured at 517 nm during a period of 30 minutes. Antioxidants will reduce the 2,2-diphenyl-1-picrylhydrazyl radical resulting in the loss of its absorbance at 517 nm. Serum antioxidant capacity was calculated by the decrease in the absorbance of the 2,2-diphenyl-1-picrylhydrazyl radical as described previously.\textsuperscript{25}

**Vascular Reactive Oxygen Species Production**

The fluorescent dye dihydroethidine was used to detect vascular superoxide production in situ as described previously.\textsuperscript{26} In intact aortic rings, reactive oxygen species (ROS) levels including superoxide, hydrogen peroxide, and peroxynitrite were analyzed by L-012-enhanced chemiluminescence.

**Reverse Transcription Real-Time Polymerase Chain Reaction (quantitative Real-Time Polymerase Chain Reaction)**

mRNA expression was analyzed by quantitative real-time reverse transcription polymerase chain reaction as previously described.\textsuperscript{17} Briefly, total RNA from mouse aorta was isolated (RNeasy Fibrous Tissue Mini Kit; QIAGEN, Hilden, Germany), and 50 ng of total RNA was used for real-time reverse transcription polymerase chain reaction analysis with the QuantiTect Probe RT-PCR kit (QIAGEN). A TaqMan gene expression assay for TATA box binding protein, telomerase reverse transcriptase, telomere repeat binding factor 2, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), peroxisome proliferator-activated receptor \( \gamma \) coactivator 1\( \alpha \), and heme oxygenase-1 (HO-1) was purchased as probe and primer set (Applied Biosystems, Foster City, CA). The comparative delta-delta threshold cycle method was used for relative mRNA quantification.\textsuperscript{27} Gene expression was normalized to the endogenous control, TATA box binding protein mRNA, and the amount of target gene mRNA expression in each sample was expressed relative to that of wild-type.

**Immunoblotting**

Isolated aortic tissue was frozen and homogenized in liquid nitrogen. Proteins were separated by SDS-PAGE, blotted
onto nitrocellulose membranes, and immunoblotting was performed as described previously.\(^2\)

### Statistical Analysis

Results are expressed as mean±SEM. One-way ANOVA (with Bonferroni or Dunn correction for comparison of multiple means) was used for comparisons of vasodilator potency and efficacy and vascular superoxide production. The EC\(_{50}\) value for vascular reactivity studies was obtained by log-transformation. \(P<0.05\) were considered significant.

### Results

#### Running Distance and Biometrical Data

The mean running distance in wild-type mice was 4336±842 m per 24 hours, and did not differ significantly in α1AMPK knockout mice (Figure 1A; 4002±1200 m). Body weight showed a significant decline in the exercise group, which was comparable in wild-type and α1AMPK knockout mice (Figure 1B and 1C).

#### Vascular AMPK Activity Is Increased During Chronic Exercise

Exercise resulted in a significant increase in vascular AMPK activity as monitored by AMPK phosphorylation at threonine 172 and phosphorylation of its downstream target acetyl-CoA carboxylase at serine 79 (Figure 1E–1G). Deletion of α1AMPK led to 80% reduction in total AMPK expression (Figure 1H) and a complete loss of vascular AMPK phosphorylation, confirming the prominent expression of the α1 containing AMPK in vascular cells.

#### Exercise Improves Endothelial Function in an α1AMPK-Dependent Manner

Exercise led to a marked improvement of endothelium-dependent relaxation, although it had no effect on endothelial function in α1AMPK knockout mice (Figure 2A). The degree of preconstriction after prostaglandin F2α was comparable among all groups (Figure IIA in the online-only Data Supplement).

Because AMPK and exercise are both known to activate eNOS,\(^2\,\,^8,\,\,^11\) we next investigated whether the improvement of endothelial function in wild-type mice was associated with increased vascular NO formation. As expected, plasma nitrite levels used as a surrogate for vascular NO production were increased during exercise in wild-type animals. In contrast, α1AMPK knockout animals had similar basal levels but showed no increase in response to exercise (Figure 2B). In accordance with previous studies, improved NO signaling in wild-type animals was associated with increased eNOS protein expression and its phosphorylation at serine 1177 (Figure 2C–2F). Because eNOS gene expression is mediated by Nrf2 in response to shear stress,\(^28\) this mechanism may also play an important role during exercise because hemodynamic changes will also increase the forces that act on the vascular endothelium. In fact we found increased aortic Nrf2 expression in response to exercise, an effect that was absent in the vasculature of α1AMPK knockout mice, suggesting that AMPK signaling must be upstream of Nrf2 (Figure 2G).

Although activation of forkhead box O3a was also shown to regulate eNOS expression in vivo\(^29\) and AMPK may directly phosphorylate forkhead box O3a at serine 413,\(^30\) we observed no significant change in forkhead box O3a phosphorylation among all treatment groups (Figure IA in the online-only Data Supplement). Taken together, our results strongly indicate that the blunted eNOS upregulation in α1AMPK knockout mice undergoing exercise is mediated by an attenuated Nrf2 expression, while forkhead box O3a did not modulate eNOS expression in our model. The decreased eNOS phosphorylation may be a direct consequence of diminished vascular AMPK activity, because eNOS is a known direct target of AMPK.\(^31\)

#### Effects of α1AMPK Deletion on Antioxidant Capacity and Vascular Oxidative Stress During Exercise

Endothelial function largely depends on the critical balance between vascular NO production and its inactivation by ROS, in particular superoxide anions. Because exercise is known to decrease ROS levels by an upregulation of antioxidative enzyme systems such as HO-1,\(^32\) we next assessed serum antioxidant capacity by 2,2-diphenyl-1-pircylhydrazyl assay.\(^25\) Exercise resulted in a significant improvement of antioxidant capacity in wild-type mice whereas it failed to increase the antioxidant defense in α1AMPK knockout mice (Figure 3A). This was paralleled by an increased expression of peroxisome proliferator-activated receptor γ coactivator 1α (Figure 3B), an important regulator of the cellular antioxidative defense,\(^33\) and an upregulation of HO-1 (Figure 3C), which is known to be regulated in a Nrf2-dependent manner in endothelial cells.\(^34\) Because antioxidant serum capacity is a surrogate of systemic oxidative stress, we next examined vascular ROS levels. Our results show that exercise was able to reduce aortic ROS in wild-type mice but not in α1AMPK knockout mice as measured by L-012-enhanced chemiluminescence (Figure 3D) and dihydroethidine staining (Figure 3E). Due to the fast scavenging of superoxide anions by NO, increased NO production may contribute to the observed decline in vascular ROS levels. In order to evaluate the relative importance of reduced ROS levels for the improvement of vascular function by exercise, we performed additional isometric tension studies in aortic rings with addition of the superoxide scavenger polyethylene glycol-conjugated SOD in vitro. In wild-type mice, polyethylene glycol-conjugated SOD improved endothelial function only in aortic rings from untreated but not from running mice, suggesting that exercise already decreased ROS levels and therefore no further improvement after polyethylene glycol-conjugated SOD was observed. Aortic rings from AMPK knockout mice showed an improvement of endothelial function by polyethylene glycol-conjugated SOD preincubation in both the control and exercise group (Figure IIB in the online-only Data Supplement), compatible with increased basal oxidative stress in these animals. Endothelial-dependent relaxation was completely blunted after eNOS inhibition with L-NAME in all groups (Figure IIC in the online-only Data Supplement).

Because the modulation of antioxidative enzyme systems such as peroxisome proliferator-activated receptor γ coactivator 1α
and HO-1 goes in parallel with increased NO production (which may itself scavenge significant amounts of ROS), it seems that both of these \( \alpha \)1AMPK-mediated effects contribute to a reduction of vascular ROS levels and finally an improvement of endothelial function during exercise.

**Effects of \( \alpha \)1AMPK Deletion on Vascular Cell Senescence and Regeneration During Exercise**

Besides eNOS expression and activity, the balance between endothelial cell death and survival constitutes a distinctive factor of vascular function. In this respect, telomerase reverse transcriptase, the catalytic protein subunit of the enzyme telomerase and telomere repeat binding factors are important components of the telomere complex, which is a central regulator of cell senescence. In accordance with a previous study, physical exercise resulted in an increased expression of the telomere-regulating proteins, telomere repeat binding factor 2 and telomerase reverse transcriptase, (Figure 4A and 4B) in the vasculature of wild-type animals, which was absent in \( \alpha \)1AMPK knockout mice. In accordance with this observation, voluntary running wild-type mice showed a decreased protein expression of p53, cell-cycle checkpoint kinase 2, and p16 INK4 in comparison to sedentary mice, compatible with decreased vascular aging (Figure 4C–4E). Deletion of \( \alpha \)1AMPK resulted in a significant attenuation of these changes, indicating that the effects of exercise on cellular aging depend at least in part on the presence of \( \alpha \)1AMPK. Immunohistochemistry revealed that

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**Figure 1.** Effects of exercise on body weight, heart weight, and vascular AMP-activated protein kinase (AMPK) activity. Eight-week-old male mice were kept in individual cages for 8 weeks equipped with a running wheel and a mileage counter. Running distance is displayed as means±SEM of \( n=15 \) to 20 (A). Body weight (B) and heart weight/body weight ratio (C) were assessed in all animal groups at the end of the exercise period; data are mean±SEM of \( n=15 \) to 20. Aortic tissues were homogenized and the lysates corrected for protein content. Immunoblotting was performed using antibodies against serine 79p-acetyl-CoA carboxylase (ACC, E), threonine 172p-AMPK (F), ACC (G), total \( \alpha \)1AMPK (H), Alpha-actinin served as a loading control. Shown immunoblotting is representative of 8 independent experiments (D). *\( P<0.05 \) vs wild-type (WT); **\( P<0.05 \) vs \( \alpha \)1AMPK\(--\); #\( P<0.05 \) vs WT+exercise.
exercise led to a downregulation of p16INK4a and cell-cycle checkpoint kinase 2 in particular in the endothelium of wild-type but not α1AMPK knockout mice, suggesting that the prevention of endothelial cell senescence by α1AMPK may considerably contribute to the increased NO bioavailability in wild-type mice undergoing exercise (Figure IIIA and IIIB in the online-only Data Supplement).

Because putative bone marrow–derived EPCs may replace injured endothelium, we also investigated the number of circulating Tie-2+Sca-1+Flk-1+ cells in α1AMPK knockout and corresponding wild-type mice. As described previously,12 the number of EPCs in wild-type mice was significantly increased during exercise, while these cells contained comparable amounts of the α1AMPK versus α2AMPK isoform (Figure V in the online-only Data Supplement). Compared with wild-type mice, α1AMPK knockout mice showed no significant increase of Tie-2+Sca-1+Flk-1+ cells in response to exercise (Figure 4F). Similarly, the colony forming capacity of EPCs, assessed by colony forming units assay, was markedly increased in wild-type mice during exercise whereas α1AMPK knockout mice showed no exercise-dependent increase (Figure 4G; Figure IV in the online-only Data Supplement). These results stress that AMPK is an important regulator of cell senescence and endothelial repair during chronic exercise training.

Discussion
In the present study, we provide evidence that AMPK activation is a key signaling event that mediates to a large part the protective effects of exercise in the vasculature. Deletion of the predominant vascular AMPK isoform α1AMPK prevented the manifestation of several protective effects of exercise including
Figure 3. Exercise decreases systemic and vascular oxidative stress in an α1AMP-activated protein kinase (α1AMPK)-dependent manner. Serum antioxidant capacity was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (A); data are mean±SEM of n=9 to 13. Aortic mRNA expression of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α, B) and heme oxygenase-1 (HO-1, C) was determined by reverse transcription real-time polymerase chain reaction (PCR); data are mean±SEM of n=8. Aortic reactive oxygen species (ROS) production was assessed by L-012-enhanced chemiluminescence (CL); data are mean±SEM of n=6 (D). Transverse aortic cryosections were labeled with dihydroethidine (DHE; 1 μmol/L), which produces red fluorescence when oxidized to 2-hydroxyethidium by superoxide. E indicates endothelium (points to the right); A, adventitia; lamina autofluorescence is green. (E). Summary of the densitometric analysis is shown on the right; data are representative of n=4. *P<0.05 vs wild-type (WT); #P<0.05 vs WT+exercise.
Figure 4. Effects of exercise on vascular cell senescence. Aortic mRNA expression of telomere repeat binding factor 2 (TRF2, A) and telomerase reverse transcriptase (TERT, B) was determined by reverse transcription real-time polymerase chain reaction (PCR). For vascular p33 (C), cell-cycle checkpoint kinase 2 (Chk2, D), and p16INK4 (E) protein expression, a representatives immunoblotting of n=6 independent experiments is shown; bar graphs were obtained by densitometry; data are means±SEM of n=6. *P<0.05 vs untreated wild-type (WT); #P<0.05 vs WT+exercise; **P<0.05 vs AMP-activated protein kinase (AMPK−/−). Endothelial progenitor cells (EPCs) were defined as Tie-2, Sca-1, and Flk-1–positive cells, and their number was assessed by flow cytometry (F) and by colony forming units (CFU) assay from bone marrow–derived cells (G); data are means±SEM of n=10 to 14. *P<0.05 vs WT; #P<0.05 vs WT+exercise.
increased eNOS expression/serine 1177-phosphorylation, decreased oxidative stress, an attenuation of cell senescence, and an increase of circulating Tie-2\textsuperscript{+}Sca-1\textsuperscript{+}Flk-1\textsuperscript{+} EPCs.

AMPK is usually known to be activated by stimuli that lead to increased ATP consumption and a concomitant rise in cellular AMP. In this respect, it is conceivable that AMPK is activated in skeletal muscle during exercise, because muscle work largely depends on rapid available energy stores in the form of ATP and creatine phosphate. Because AMPK can also improve cellular glucose uptake, it was postulated that the positive effects of exercise on glycemic control in diabetics are AMPK-dependent. A recent report confirmed this hypothesis, and showed that intact AMPK signaling is required for the metabolic response to exercise in vivo. In the present study, we can extend these observations by showing that AMPK activation in response to exercise occurs also in the vasculature. It is tempting to speculate that an increasing AMP/ATP ratio is the initiating event to stimulate exercise-mediated AMPK activation in the vessel wall similar to the findings in skeletal muscle. Despite this appealing hypothesis, it remains to be established whether energy-dependent mechanisms are the primary means of AMPK regulation in the vasculature, in particular because upstream activators of AMPK such as liver kinase B1 have been described. Shear stress may be another important factor to drive AMPK activation in the vasculature, while NO itself may help to maintain AMPK activation in a positive feedback loop. Independent of its mode of activation, AMPK appears to be a proximal signal in the adaptation to exercise, because our results demonstrate that AMPK is clearly upstream of Nrf2 and eNOS signaling, and therefore establish AMPK as an early signaling event that mediates the protective effects of exercise in the vasculature (proposed scheme in Figure 5).

Physical exercise increases cardiac output and vascular perfusion, which will automatically increase shear stress that acts on the vascular wall. As a consequence, shear stress leads to eNOS activation and vasodilation, and it is attractive to speculate that endothelium-dependent processes are crucial for the manifestation of protective vascular effects in response to exercise. Our data implicate that AMPK and may be Nrf2, which both are activated in response to shear stress, are part of a signaling cascade that leads to eNOS upregulation and vascular protection during exercise.

Previous studies regarding the effects of exercise on oxidative stress have yielded equivocal results. High endurance training was even shown to increase systemic oxidative stress whereas moderate physical activity had no such effect. These findings might also relate to the importance of antioxidant defense strategies in this setting, because exercise is able to increase HO-1\textsuperscript{12} or SOD activity.\textsuperscript{13} Our observations are in line with these findings because serum antioxidant capacity and the expression of HO-1, Nrf2, and peroxisome proliferator-activated receptor γ coactivator 1α were increased by exercise in an α1AMPK-dependent manner, leading to a significant decline in aortic ROS levels. In addition, the experimental setting with voluntary exercise in mice may have prevented prooxidative effects associated with forced exercise or high endurance training.

Many of the vascular effects in response to exercise have been described in a similar fashion for AMPK activation, including eNOS activation, protection against oxidative stress and angiogenesis, which prompted us to investigate the role of AMPK for the protective effects of exercise. In addition to the modulation of eNOS activity, endothelial function may also depend on the critical balance between endothelial cell senescence and regeneration, in particular by putative vascular progenitor cells. A previous study by Werner et al\textsuperscript{36} demonstrated that exercise is able to prevent vascular cell senescence in a telomerase reverse transcriptase- and eNOS-dependent manner. Here we demonstrate that AMPK is required to prevent cellular senescence by exercise, because AMPK deletion prevented exercise-induced increases of telomere-regulating proteins and downregulation of cell-cycle checkpoint kinase 2, p53, and p16\textsuperscript{INK4}. Regarding endothelial repair, a potential mechanism is the replacement of injured endothelial cells by bone marrow–derived EPCs.\textsuperscript{42} A previous study demonstrated that AMPK plays a pivotal role in the differentiation of putative vascular progenitor cells.\textsuperscript{43} Here we demonstrate for the first time that α1AMPK regulates the number of circulating Tie-2\textsuperscript{+}Sca-1\textsuperscript{+}Flk-1\textsuperscript{+} myeloid progenitor cells in vivo. Mechanistically, α1AMPK may prevent oxidative damage

**Figure 5. Proposed scheme to illustrate the AMP-activated protein kinase (AMPK)-dependent vascular effects during exercise.** Chronic exercise will temporarily increase cardiac output and thereby augment shear stress that acts on the endothelial cell layer. Shear stress in turn activates AMPK, which mediates endothelial NO synthase (eNOS) protein upregulation and its phosphorylation at serine 1177. These events are paralleled by increased nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression, which will lead to heme oxygenase-1 (HO-1) upregulation. Together with an increased NO production, these events will lead to a reduction of vascular reactive oxygen species (ROS) levels. The effects of exercise on endothelial progenitor cell (EPC) number and endothelial function are AMPK-dependent, but also require intact eNOS signaling.\textsuperscript{42}
of these cells in a similar fashion as previously described for endothelial cells, even though vascular progenitor cells have been reported to exhibit a higher resistance to oxidative stress. Because our study uses a global (and not tissue-specific) knockout model, we cannot rule out that α1AMPK deletion in other cell types besides endothelial cells or circulating EPCs contribute to the observed vascular phenotype. A landmark study by Narkar et al demonstrated that AMPK activation has endurance enhancing properties, making it an attractive target for pharmacological interventions in order to simulate the beneficial effects of exercise. Current available strategies to activate AMPK pharmacologically include biguanides and glitazones. However, their AMPK activating properties are secondary to the inhibition of the mitochondrial respiratory chain, and therefore, the development of more potent and specific compounds remains a future goal. Our current results support the concept that the vascular protection through exercise is AMPK-dependent, and that AMPK may be an attractive future pharmacological target to simulate the protective effects of exercise.

Acknowledgments

We thank Angelica Karpi, Nicole Papaioannou, and Jörg Schreiner for excellent technical assistance. This article contains results that are part of the doctoral thesis of Felix Hauptmann.

Sources of Funding

The present work was supported by a grant from the German Heart Foundation (grant No. F39/10) and by a grant from the Stiftung Bildung und Forschung (grant No. F/39/10) and by a grant from the Stiftung Krebsforschung (grant No. F/39/10) and by a grant from the Stiftung Bildung und Forschung. We thank Angelica Karpi, Nicole Papaioannou, and Jörg Schreiner for excellent technical assistance. This article contains results that are part of the doctoral thesis of Felix Hauptmann.

Disclosures

None.

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AMP-Activated Kinase and Exercise


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Arterioscler Thromb Vasc Biol. published online April 26, 2012;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2012/04/26/ATVBAHA.111.243980

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Methods

Reagents
Antibodies against Foxo3a and ser413p-Foxo3a were from Cell Signaling (Boston, MA, USA). The Chk-2 and p16\textsuperscript{INK4} antibodies were purchased from BD biosciences (San Jose, CA, USA).

Isometric Tension Studies
Isometric tension studies in aortic rings (3mm in length) were performed to assess vasodilator responses to acetylcholine (ACh). To investigate the role of superoxide anions regarding changes in endothelial function, selected rings were preincubated with polyethylene glycol-conjugated superoxide dismutase (PEG-SOD, 100 U/ml) for 30 minutes and a dose response curve for ACh was performed. Similarly, inhibition of NO production was achieved in separate experiments by preincubation of aortic rings with L-NAME 200\textmu M for 30 minutes.

Immunohistochemistry
Immunostaining of mouse aortic sections (5\mu m) were performed using the Vector M.O.M. immunodetection kit (Vector Laboratories, Burlingham, CA). The mouse anti human p16\textsuperscript{INK4} antibody and mouse anti Chk-2 antibody from BD biosciences (San Jose, CA, USA), were used a dilution of 1:100. A negative control was included in every experiment to rule out unspecific antibody binding. The pictures were adjusted for contrast and brightness (the same way in all treatment groups) in order to allow easier identification of the positive staining (brown). Background staining was performed with hematoxilin in order to visualize the vascular structure.
Reverse transcription real-time PCR (qRT-PCR)
mRNA expression in EPC’s was analyzed by quantitative real-time RT-PCR as described in
detail in the main Methods section. A TaqMan® Gene Expression assay to analyze Foxo3a,
\( \alpha_1 \)AMPK and \( \alpha_2 \)AMPK expression was purchased as probe-and-primer set (Applied
Biosystems, Foster City, CA). Relative expression of \( \alpha_1 \)AMPK and \( \alpha_2 \)AMPK in wildtype mice
was normalized to the endogenous control TATA box binding protein (TBP) mRNA. Foxo3a
mRNA expression was determined by the comparative \( \Delta \Delta \)Ct method. Foxo3a gene
expression was normalized to the endogenous control, TATA box binding protein (TBP)
mRNA, and the amount of target gene mRNA expression in each sample was expressed
relative to that of wildtype.
Figure Legends

Suppl. Figure I: Effects of α1AMPK deletion or exercise on Foxo3a expression and phosphorylation. (A) Aortic mRNA Expression of Foxo3a was determined by reverse transcription real-time PCR. (B&C) Aortic tissues were homogenized and the lysates corrected for protein content. Immunoblotting was performed using antibodies against total Foxo3a and serine413p-Foxo3a. β-actin served as a loading control. (D) Ratio between Foxo3a phosphorylated at serine413 and total Foxo3a protein. The shown PCR and immunoblots are representative of 6-8 independent experiments. * indicates p<0.05 vs. WT, ** indicates p<0.05 vs. α1AMPK -/-, # indicates p<0.05 vs. WT + exercise.

Suppl. Figure II: Effects of superoxide scavenging and eNOS inhibition on endothelial function.
Endothelial dependent relaxation in response to acetylcholine was performed in intact aortic rings after preconstriction with 1µM prostaglandin F$_2$α (PGF$_2$α), the degree of preconstriction obtained by PGF$_2$α is summarized in (A). To assess the relative importance of superoxide anions or NO production for the observed changes in endothelial function, selected aortic rings were preincubated with PEG-SOD 100 U/ml (B) or L-NAME 200 µM (C) for 30 min. Data are means ± SEM of n=8 independent experiments. # indicates p<0.05 vs. α1AMPK -/-, ++ indicates p<0.05 vs. α1AMPK -/- + exercise.

Suppl. Figure III: Immunohistochemistry for aortic expression of cell senescence markers.
Immunohistochemistry by using p16$^{INK4}$ (A) and Chk-2 (B) was performed in paraformaldehyde fixed aortic sections. Brown color identifies a positive staining. “E” indicates the endothelium, the shown sections are representative of 5 independent experiments.
Suppl. Figure IV: Phenotypical characterisation of expanded EPCs.

Uptake of acetylated LDL on EPC’s was visualised with fluorescence microscopy using Dil-labelled acetylated LDL. Representative cell stainings after 3 days in culture are shown in (A), phase contrast images are summarized in (B). The shown cell stainings are representative of 4 independent experiments.

Suppl. Figure V: Expression of AMPK α-isoforms in endothelial progenitor cells.

mRNA was isolated from bone marrow derived EPC’s and the relative expression of α1AMPK vs. α2AMPK was determined by reverse transcription real-time PCR. Gene expression was normalized to the endogenous control TATA box binding protein (TBP) mRNA. Data are mean ± SEM of n=6 independent experiments. n.d. = not detectable.
References


Suppl. Figure I

A

![Bar chart showing % of WT for different groups: WT, WT + exercise, α1AMPK -/-, α1AMPK -/- + exercise.](chart)

B

![Bar chart showing % of WT for different groups: WT, WT + exercise, α1AMPK -/-, α1AMPK -/- + exercise.](chart)

C

![Bar chart showing % of WT for different groups: WT, WT + exercise, α1AMPK -/-, α1AMPK -/- + exercise.](chart)

D

![Bar chart showing % of WT for different groups: WT, WT + exercise, α1AMPK -/-, α1AMPK -/- + exercise.](chart)

* p < 0.05 vs. WT; ** p < 0.05 vs. AMPK -/-; # p < 0.05 vs. WT + exercise
Suppl. Figure II

A

WT
WT
α1AMPK−/− α1AMPK−/−

0.0
0.2
0.4
0.6
0.8
1.0

0
0.2
0.4
0.6
0.8
1.0

WT
WT
α1AMPK−/− α1AMPK−/−

B

Endothelium-dependent Relaxation

WT
WT + PEG-SOD
WT + exercise
WT + exercise + PEG-SOD

% Relaxation

Log (Ach) M

Endothelium-dependent Relaxation

α1AMPK−/− + exercise
α1AMPK−/− + exercise + PEG-SOD
α1AMPK−/−
α1AMPK−/− + PEG-SOD

% Relaxation

Log (Ach) M

C

Endothelium-dependent Relaxation
L-NAME

α1AMPK−/− + L-NAME
α1AMPK−/− + exercise + L-NAME
WT + L-NAME
WT + exercise + L-NAME

% Relaxation

Log (Ach) M

# p < 0.05 vs. α1AMPK−/−, ++ p < 0.05 vs. α1AMPK−/− + exercise
Suppl. Figure III

A  \( p16^{\text{INK4}} \) immunohistochemistry

B  Chk-2 immunohistochemistry
Suppl. Figure IV

A

WT

WT + exercise

α1AMPK -/-

α1AMPK -/- + exercise

B

WT

WT + exercise

α1AMPK -/-

α1AMPK -/- + exercise
Suppl. Figure V

The figure shows the relative expression of α1 and α2 subunits in different genotypes and conditions:

- WT
- WT + exercise
- AMPK -/-
- AMPK -/- + exercise

The expression levels are indicated as follows:

- α1 and α2 for WT and WT + exercise are shown.
- α1 and α2 for AMPK -/- are not detectable (n.d.).
- α1 and α2 for AMPK -/- + exercise are also not detectable (n.d.).