Caffeine Enhances Endothelial Repair by an AMPK-Dependent Mechanism

Ioakim Spyridopoulos, Stephan Fichtlscherer, Rüdiger Popp, Stefan W. Toennes, Beate Fisslthaler, Thomas Trepels, Alma Zernecke, Elisa A. Liehn, Christian Weber, Andreas M. Zeiher, Stefanie Dimmeler, Judith Haendeler

Objective—Migratory capacity of endothelial progenitor cells (EPCs) and mature endothelial cells (ECs) is a key prerequisite for endothelial repair after denuding injury or endothelial damage.

Methods and Results—We demonstrate that caffeine in physiologically relevant concentrations (50 to 100 μmol/L) induces migration of human EPCs as well as mature ECs. In patients with coronary artery disease (CAD), caffeinated coffee increased caffeine serum concentration from 2 μmol/L to 23 μmol/L, coinciding with a significant increase in migratory activity of patient-derived EPCs. Decaffeinated coffee neither affected caffeine serum levels nor migratory capacity of EPCs. Treatment with caffeine for 7 to 10 days in a mouse-model improved endothelial repair after denudation of the carotid artery. The enhancement of reendothelialization by caffeine was significantly reduced in AMPK knockout mice compared to wild-type animals. Transplantation of wild-type and AMPK$^{-/-}$ bone marrow into wild-type mice revealed no difference in caffeine challenged reendothelialization. ECs which were depleted of mitochondrial DNA did not migrate when challenged with caffeine, suggesting a potential role for mitochondria in caffeine-dependent migration.

Conclusion—These results provide evidence that caffeine enhances endothelial cell migration and reendothelialization in part through an AMPK-dependent mechanism, suggesting a beneficial role for caffeine in endothelial repair. (***Arterioscler Thromb Vasc Biol.* 2008;28:1967-1974**)

Key Words: caffeine ■ reendothelialization ■ endothelium ■ AMPK ■ mitochondria

Endothelial dysfunction plays a pivotal role for the development and progression of atherosclerosis. Atherogenic risk factors harbor the potential to injure the endothelial layer by promoting apoptosis, preceded by an inflammatory reaction in the vessel wall.[1] Therefore, the maintenance of the endothelial layer is a crucial process to ensure its integrity. Endothelial repair is also dependent on the capacity of mature ECs to proliferate and migrate.[2] More recent studies suggest an important role of circulating endothelial progenitor cells (EPCs) contributing to reendothelialization after vascular injury by homing to denuded parts of the artery after balloon injury.[3]

Caffeine (1,3,7-trimethylxanthine) exerts various functions including the modulation of glucose metabolism, alterations in apoptotic processes, immunomodulatory effects, and stimulation of muscle contraction.[4–6] Most of these effects are attributed to the psychostimulant properties of caffeine.[7] On a cellular level, caffeine acts as a competitive inhibitor of cyclic nucleotide phosphodiesterase isoenzymes in various tissues and, thereby, increases intracellular cAMP levels.[8] Caffeine has also been demonstrated to regulate glucose metabolism and increase energy production from mitochondria.[9] Furthermore, increasing levels of AMP lead to activation of AMP-activated protein kinases (AMPK, for review see[10]), which have been suggested to increase mitochondrial energy metabolism by activating the peroxisome proliferator-activated receptor-γ (PPAR-γ) coactivator 1α (PGC-1α).[11,12] Importantly, in endothelial cells mitochondria are not only required for ATP production, but also to act as signaling organelles.[13]

Therefore, we hypothesized the existence of a molecular link between caffeine, AMPK, mitochondrial energy metabolism, and endothelial repair. In this study, we show that (1) low concentrations of caffeine increase the migratory capacity of mature endothelial cells (ECs) in an AMPK-dependent manner; (2) In patients with coronary artery disease (CAD), coffee consumption increases migratory capacity and mitochondrial energy metabolism of EPCs; (3) Finally, pretreat-

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From Molecular Cardiology, Department of Internal Medicine III (I.M., I.S., S.F., T.T., A.M.Z., S.D., J.H.), University of Frankfurt, Germany; the Department of Physiology (R.P., B.F.), University of Frankfurt, Germany; the Department of Forensic Toxicology (S.W.T.), University of Frankfurt, Germany; the Institute for Molecular Cardiovascular Research (IMCAR) (A.Z., E.A.L., C.W.), RWTH Aachen University, Germany. Current affiliation for J.H.: Molecular Cell & Aging Research, Institut fuer umweltmedizinische Forschung (IUF), University of Duesseldorf GmbH, Germany.

Correspondence to Judith Haendeler, PhD, Molecular Cell & Aging Research, Institut fuer Umweltmedizinische Forschung, University of Duesseldorf GmbH, Auf'm Hennekamp 50, 40225 Duesseldorf, Germany. E-mail juhae001@uni-duesseldorf.de and Ioakim Spyridopoulos, Molecular Cardiology, Department of Internal Medicine III, University of Frankfurt, 60590 Frankfurt, Germany. E-mail: spyridopoulos@uni-frankfurt.de.

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ment of mice with caffeine profoundly enhances reendothelialization after denudation of the carotid artery, in part in an AMPK-dependent manner.

Methods

For detailed Methods, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Cell Culture and Isolation of EPCs

EPCs were isolated as previously described.[14] Three days after isolation cells were incubated with caffeine. ECs were cultured as previously described.[15]

Assessment of Migratory Capacity of EPCs

After 3 days of culture, progenitor cells were resuspended in EBM medium without FCS, containing 1% bovine serum albumin. 5×10^4 EPCs were placed in the upper chamber of a modified Boyden chamber. For quantification, cells were stained with Dil-acetylated LDL and were counted manually in 3 random microscopic fields per well.

Healthy Volunteers and Patients With CAD

To assess migratory capacity and mitochondrial function of EPCs, venous blood samples before and after coffee consumption of a total of 4 standardized cups of espresso (Lavazza; Saeco espresso machine; Saeco GmbH Eigeltingen) were obtained in 9 healthy, nonsmoking volunteers (age 34.1±6.6 years; range 26.5 to 44.5) without any evidence of CAD by history and physical examination. 22 patients with stable CAD, documented by coronary angiography, were also included into the study.

Analysis of Plasma Samples for Caffeine

Plasma samples (50 μL) were analyzed for caffeine after acetonitrile precipitation using LC-TOF MS (Agilent) operated in positive electrospray ionization mode with a Polaris C18-Ether column (Varian) at 50°C using a 6 minutes gradient consisting of 0.1% formic acid and acetonitrile at 0.4 mL/min.

Scratch Wound Assay of ECs

For detection of cell migration, in vitro "scratch" wounds were created by scraping cell monolayers with a sterile disposable rubber policeman.[16]

ATP Measurements

ATP levels were determined with a bioluminescence assay in total cell lysates and mitochondrial fractions (BioThema AB).

Mitochondrial Membrane Potential

The membrane potential of the inner mitochondrial membrane was measured using JCI dye added to the cell culture medium at a final concentration of 0.5 μmol/L for 30 minutes.

Western Blot Analysis

Immunoblotting was performed as previously described.[14] The following primary antibodies were used: AMPK-α (Cell Signaling #2532 rabbit polyclonal antibody, 1:200) and phospho AMPK-α (Thr 172, Cell Signaling #25359 rabbit polyclonal antibody, 1:100).

Mouse Carotid-Artery Injury Model

Female 8-week-old C57BL/6 mice (n=6 per group) or AMPK α1–deficient mice were untreated (control) or received caffeine (0.05% in 200 mL drinking water daily) for 3 days before and after injury (pre-/post-), or only after injury (post-). Arterial wire-injury was performed as described previously.[17] Endothelial recovery was evaluated 7 days after wire-injury by staining denuded areas with Evans blue dye (Sigma).[17] The blue-stained and total luminal areas were determined (Diskus software, Hilgers), and the non-reendothelialized area was expressed as percentage of the total surface (% area).[18]

Bone Marrow Reconstitution

Bone marrow reconstitution was performed essentially as previously described.[19] Briefly, donor bone marrow was prepared from femurs and tibias from wild-type or $AMPK^{-/-}$ mice, and cells in phosphate-buffered saline (2×10^6) were administered by intravenous injection to $AMPK^{+/+}$ wild-type mice 24 hours after an ablative dose of whole-body irradiation (2×5.5 Gy). After 4 weeks of recovery, mice were placed on caffeine treatment 3 days before and for 7 days after wire-induced denudation injury of the left carotid artery.

Creation of Rho^0-ECs

The mtDNA-depleted (rho^0) ECs were generated as previously described[20] with some modifications. In brief, ECs were treated with 0.05 μg/mL ethidium bromide and 50 μg/mL uridine for 17 days. The control ECs were maintained for the same time period in normal culture medium. Depletion of mitochondrial DNA was measured by polymerase chain reaction (PCR) as previously described.[21]

Statistics

All values are expressed as mean±SEM. Differences between groups were assessed by using either the Mann–Whitney test, unpaired *t* test (2 groups), or 1-way ANOVA (>2 groups). Subsequent multiple comparisons for ≥3 groups were performed only, if 1-way ANOVA reached statistical significance $P<0.05$, using Dunnett's post test to compare each group against its control, and Bonferronis multiple comparison test. Statistical calculations were carried out with GraphPad Prism version 5.00 for Windows (GraphPad Software, www.graphpad.com).

Results

Caffeine Increases Migratory Capacity of EPCs and ECs

We first examined whether low concentrations of caffeine would affect migration of EPCs. When incubating EPCs in a dose-dependent manner, caffeine concentrations of 50 to 100 μmol/L increased migratory capacity of EPCs as measured in a modified Boyden-chamber assay, whereas higher concentrations (200 to 300 μmol/L) had no effect (Figure 1a). The effect of caffeine on EPC migration was equipotent to the prototypical endothelial cell migration stimulus VEGF (Figure 1a). In accordance with the dose-dependent effects of caffeine in EPCs, caffeine also enhanced the migratory capacity of mature ECs in a scratch wound assay in low concentrations (Figure 1b and 1c).

Physiological Concentrations of Caffeine Enhance Migratory Capacity of EPCs in Patients With CAD

Next, we determined the influence of coffee consumption on the dysfunctional migratory capacity of EPCs derived from patients with CAD. Four cups of caffeinated coffee increased blood caffeine concentrations up to 20 μmol/L in 9 healthy volunteers (Figure 2a), whereas consumption of 4 cups of decaffeinated coffee did not increase blood caffeine concentrations (data not shown). The subsequent experiments in CAD patients were performed in a randomized and double-blinded fashion. Caffeinated coffee increased caffeine serum concentration from 2 μmol/L to 23 μmol/L (n=14, $P=0.023$; Figure 2b), coinciding with a significant increase in migratory activity of patient-derived EPCs (46±8 versus 20±4 cells/high power field; $P=0.006$, Figure 2c). Decaffeinated coffee did not cause a significant increase in caffeine serum levels of patients with CAD (n=8; Figure 2

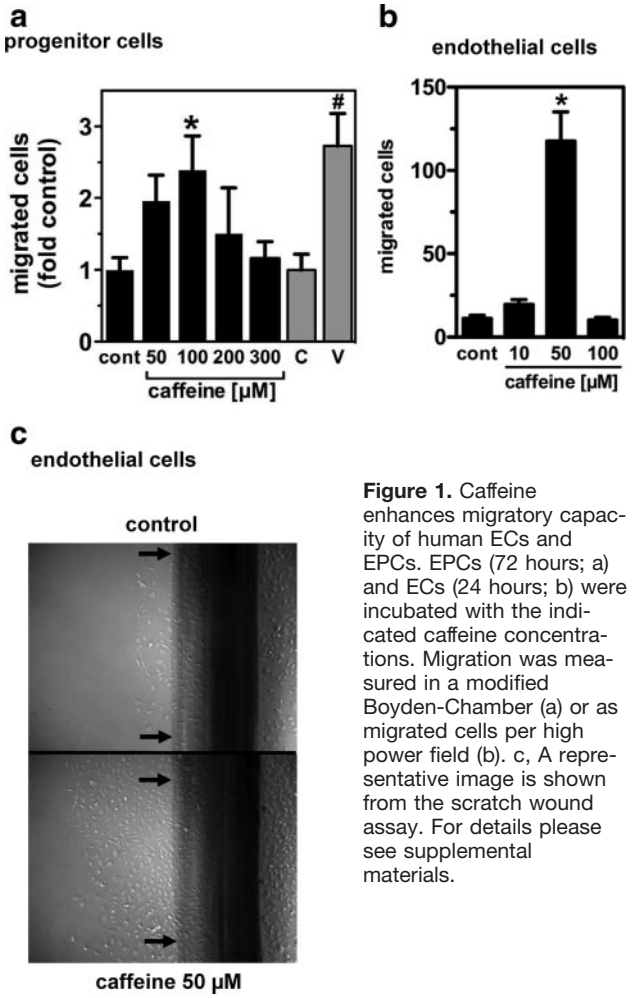

Figure 1. Caffeine enhances migratory capacity of human ECs and EPCs. EPCs (72 hours; a) and ECs (24 hours; b) were incubated with the indicated caffeine concentrations. Migration was measured in a modified Boyden-Chamber (a) or as migrated cells per high power field (b). c, A representative image is shown from the scratch wound assay. For details please see supplemental materials.

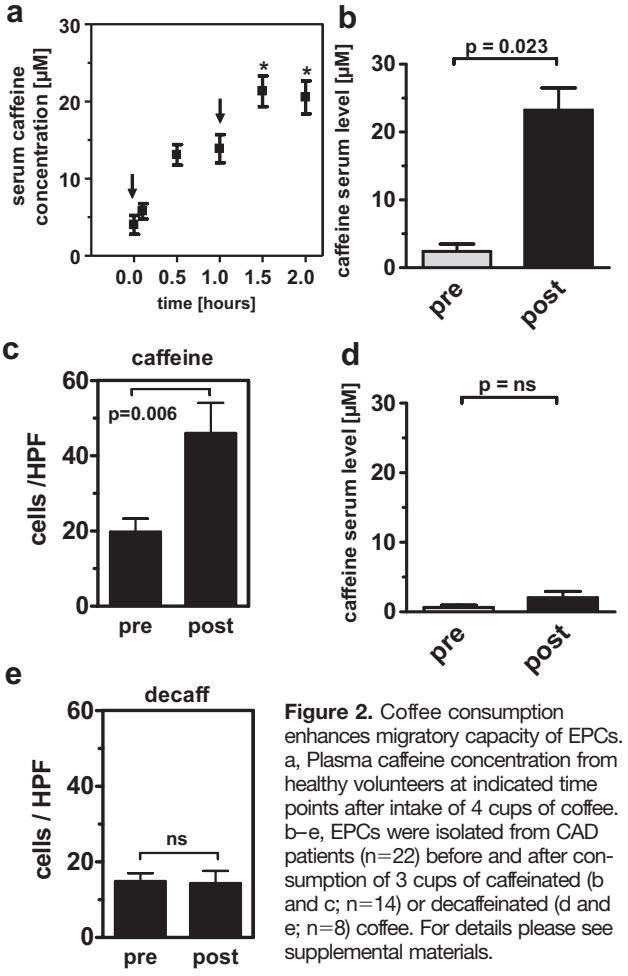

Figure 2. Coffee consumption enhances migratory capacity of EPCs. a, Plasma caffeine concentration from healthy volunteers at indicated time points after intake of 4 cups of coffee. b–e, EPCs were isolated from CAD patients (n=22) before and after consumption of 3 cups of caffeinated (b and c; n=14) or decaffeinated (d and e; n=8) coffee. For details please see supplemental materials.

days, d). Accordingly, migratory capacity of patient-derived EPCs was not altered by decaffeinated coffee (Figure 2e). Of note, the caffeine-induced increase in migratory capacity did not differ between CAD patients and healthy volunteers (data not shown).

Caffeine Enhances Reendothelialization in a Carotid-Artery Injury Mouse Model

Having demonstrated that caffeine increases migratory capacity of mature ECs and EPCs, we next assessed the effect of caffeine in a mouse model of reendothelialization. Caffeine (0.05%) was added every day to the drinking water, resulting in serum levels of approx. 30 μmol/L (Figure 3a). Evans blue exclusion revealed that caffeine significantly enhanced reendothelialization after denuding injury, when added for 7 days after wire injury (5.7% versus 13% nonreendothelialized area, $P<0.05$; Figure 3b). Interestingly, pretreatment of mice for 3 days with caffeine improved the protective effect of caffeine even further (1.7% nonreendothelialized area, $P<0.001$ versus control), suggesting that caffeine enhances endothelial repair in vivo (supplemental Figure III). Similar results were obtained when mice were harvested 3 days after wire injury (data not shown).

Caffeine Increases Migratory Capacity and Reendothelialization by an AMPK-Dependent Mechanism

AMPK, and more precisely AMPK α1, plays an important role for endothelial cell biology.[22] Indeed, as recently reported,[23] caffeine led to enhanced phosphorylation of AMPK at Thr-172 of the α subunit in mature ECs (Figure 4a). Stimu-

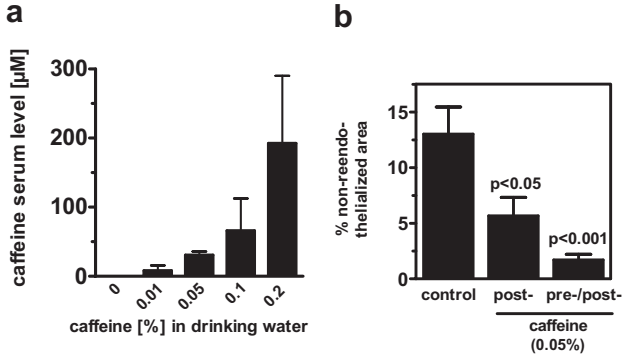

Figure 3. Caffeine enhances reendothelialization in a mouse carotid-artery model. a, Caffeine was freshly added to the drinking water once a day at the indicated concentrations and caffeine serum levels measured. b, Drinking water was supplied with caffeine (0.05%) either for 7 days after carotid injury (post-) or 3 days preinjury and 7 days postinjury (pre-/post-). For details please see supplemental materials.

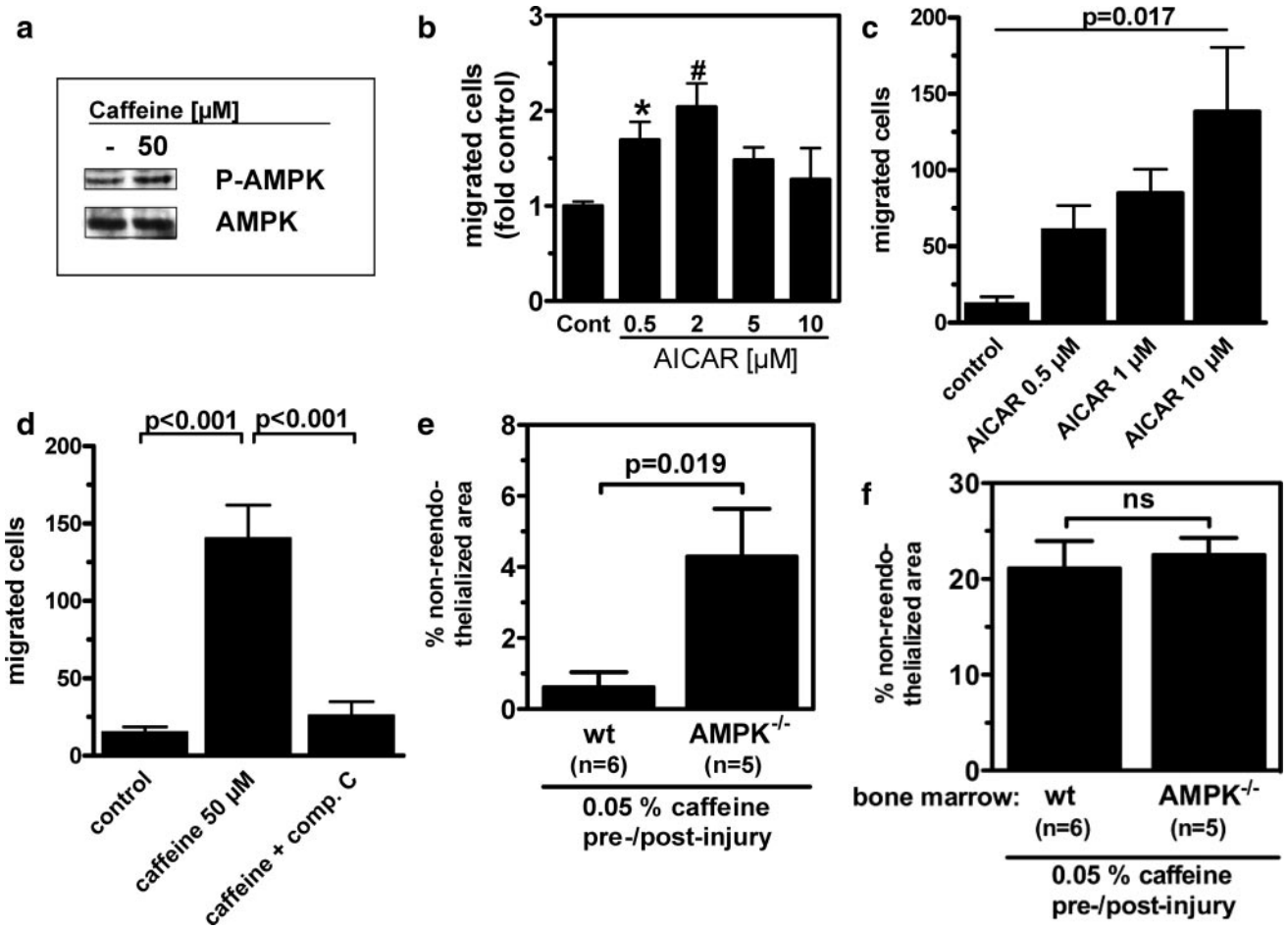

Figure 4. Caffeine enhances migratory capacity and reendothelialization by an AMPK-dependent mechanism. a, Western blot analysis of AMPK and phospho-AMPK levels in either control (-) or caffeine treated (50 μmol/L) ECs. Migration of EPCs (b) or ECs (c and d) incubated with normal media (control) or indicated concentrations of the AMPK-inducer AICAR or 1 μmol/L compound C, respectively. Nonreendothelialized area of (e and f) wild-type, AMPK$^{-/-}$, as well as transplanted mice 7 days after wire-induced denudation injury of the left carotid artery. For details please see supplemental materials.

lation of EPCs (72 hours, Figure 4b) or mature ECs (24 hours, Figure 4c) with the AMPK-inducer 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) led to a dose-dependent increase in migratory function comparable to caffeine. AMPK inhibition by compound C resulted in an abrogation of caffeine-induced migration in mature ECs (Figure 4d). Most importantly, deletion of the catalytic α1 subunit of AMPK in mice significantly reduced the enhanced reendothelialization by caffeine compared to the wild-type animals (0.6±1.0% versus 4.3±3.0% nonreendothelialized area, P=0.019; Figure 4e, see also supplemental Figure IV). In contrast, blockade of Akt 1 and Akt 2 by the specific Akt inhibitor IV did not inhibit caffeine-induced migration (data not shown). To elucidate the respective role of the action of caffeine on EPCs and on local ECs, we grafted bone marrow from AMPK$^{-/-}$ mice or their wild-type littermates to irradiated wild-type mice and performed arterial denudation injury and treatment with caffeine before and after the injury. As shown in figure 4f (see also supplemental Figure V), we did not detect a significant difference in the area of reendothelialization between these two groups after 7 days, suggesting that the deficiency of AMPK predominantly affects the potential of mature endothelial cells for reendothelialization and a lack of AMPK in bone marrow–derived EPCs is not sufficient to induce a phenotype.

Functional Mitochondria Are Required for Caffeine-Induced Migration

AMPK has recently been implicated in mitochondrial biogenesis.[24] Because mitochondrial function has also been associated with migratory capacity,[25] we investigated whether caffeine influences mitochondrial function. AICAR and caffeine dose-dependently increased total cellular ATP concentration both in EPCs (Figure 5a) as well as in ECs (Figure 5b). Moreover, mitochondria isolated from ECs treated with caffeine showed an increase in ATP concentration (Figure 5c). In addition, we determined the effect of low concentrations of caffeine on the mitochondrial membrane potential. Caffeine significantly increased the mitochondrial membrane potential, as measured with JC1 and fluorescence-activated-cell sorter (FACS) analysis (Figure 5d and 5e). To test whether mitochondria are also affected by coffee consumption in vivo, we measured mitochondrial membrane potential of EPCs derived from patients after consumption of coffee. Indeed, those patients who received caffeinated coffee dis-

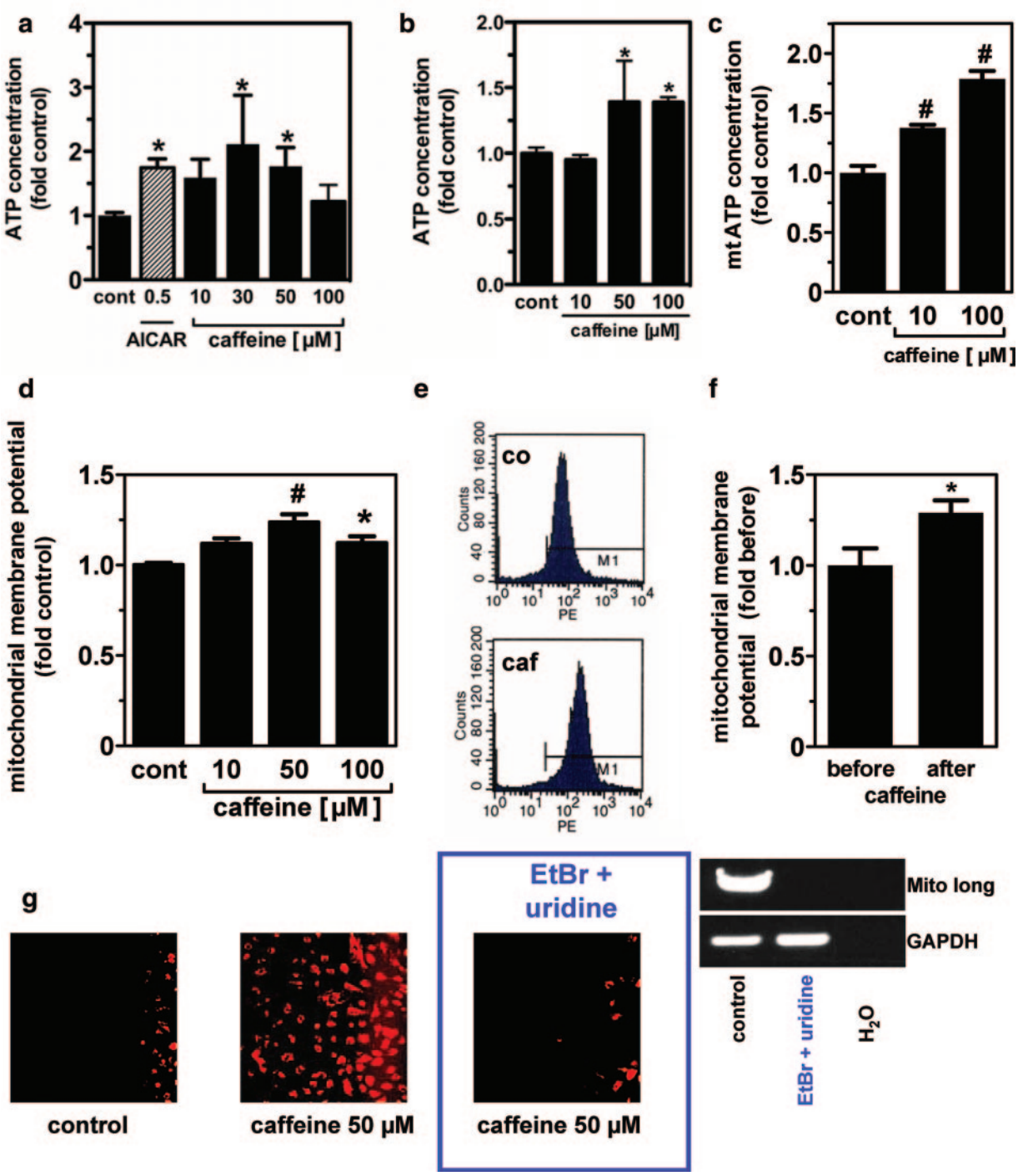

Figure 5. Caffeine increases ATP concentration and mitochondrial membrane potential. Total ATP (a, EPCs; b, ECs) and mitochondrial ATP production (c). Mitochondrial membrane potential of EPCs (d), ECs (e), and isolated EPCs from CAD patients (f). Migration of ECs after depletion of mitochondrial DNA (g). For details please see supplemental materials.

played increased mitochondrial membrane potential (Figure 5f). To demonstrate that migration of ECs depends on mitochondrial ATP production, we generated rho^0 ECs by ethidium bromide treatment.[20] Rho^0 ECs were completely depleted of mitochondrial DNA (inset Figure 5g) and were unable to migrate under basal conditions (control: 10±2 migrated cells; rho^0 ECs: 1±1 migrated cells). Treatment with caffeine did not enhance migration in these rho^0 ECs, further demonstrating an important role for functional mitochondria in the migratory response of ECs (Figure 5g).

Discussion

Our study finds that coffee consumption or ex vivo treatment of cells with caffeine significantly improves migration of ECs and EPCs by an AMPK-dependent mechanism. Importantly, AMPK also contributed to the enhanced reendothelialization

induced by caffeine in vivo. The beneficial influence of caffeine on reendothelialization seen in the mouse model could be explained by both migration of mature ECs as well as attachment of circulating EPCs, accelerating recovery of the endothelial monolayer. Several studies have documented the fundamental role of EPCs in the healing process of vascular endothelium alone,[26] after mobilization with GM-colony stimulating factor (CSF),[27] erythropoietin,[28] or pretreatment with statins,[3] estrogen[29,30] or the peroxisome proliferator-activated receptor-gamma agonist rosiglitazone.[31] So far, two studies have investigated a potential role of AMPK for migration at least in mature ECs. Nagata et al have shown that overexpression of a dominant-negative mutant of the AMPK α2-subunit inhibited endothelial cell migration toward VEGF in hypoxic cultures.[11] Ouchi et al have found that adiponectin can induce endothelial cell migration through stimulation of AMPK under normoxic conditions.[12] Again, overexpression of a dominant-negative mutant of AMPK abrogated adiponectin-induced migration of endothelial cells.

AMPK functions as an intracellular energy stress sensor regulating metabolism and cell proliferation.[10] Caffeine-induced AMP kinase activation may be mediated by different ways: (1) increasing intracellular AMP levels thus acting like a fuel gauge[32]; (2) by mitochondrial reactive oxygen species[13]; or (3) by the Peutz-Jeghers syndrome gene product LKB1.[33,34] Several lines of evidence argue against the first and second possibilities: Human endothelial cells mainly express the α1 catalytic isoform of AMPK,[13] which is far less dependent on AMP levels than the α2 isoform.[35] In addition, we could find an increase rather than a decrease in total or mitochondrial ATP production. Quintero et al have shown that eNOS activation facilitated production of mitochondrial reactive oxygen species leading to AMPK phosphorylation.[13] However, Quintero et al performed these studies under low oxygen concentrations (0.5% to 3%), whereas in the present study we used culture conditions with 20% oxygen for our experiments. As caffeine is known to increase intracellular cAMP levels, the activation of protein kinase A (PKA) and subsequently LKB1 could be a potential signaling pathway.

Data from other studies showing that caffeine increases AMP levels[8] are consistent with our finding that caffeine increases phosphorylation of AMPK in mature ECs and EPCs. Moreover, inhibition of AMPK by its specific inhibitor compound C abrogated caffeine-induced migration, demonstrating that activation of AMPK is necessary for caffeine-induced migratory capacity of cells. It has also been demonstrated that migration of endothelial cells depends on the activation of the PI3 kinase/Akt pathway in several cell types using different stimuli. Therefore, we also investigated whether caffeine influenced the PI3 kinase/Akt pathway. Our data revealed that caffeine specifically activates the AMPK pathway rather than Akt 1 and 2. Genetic ablation of AMPK in AMPK α1–deficient mice significantly reduced the enhanced reendothelialization by caffeine compared to wild-type animals. Our results from bone marrow–transplanted mice suggest that the deficiency of AMPK rather affects the potential of mature endothelial cells for reendothelialization than the bone marrow–derived EPCs, which of course does not rule out their participation in these processes. The lesser degree of reendothelialization as evident by a higher percentage of Evans blue staining in these 2 groups compared to nonirradiated controls is likely attributable to the direct effects of irradiation.

Recent studies suggested that cellular ATP levels may contribute to the regulation of cell migration. Wu et al demonstrated that inhibition of mitochondrial membrane potential and ATP concentration are paralleled by reduced migration of macrophages.[25] Moreover, cell division has been shown to be sensitive to alterations in the cellular energy pool.[36] Our data now demonstrate that AMPK stimulation by AICAR increases cellular ATP levels. Furthermore, depletion of mitochondrial DNA inhibited the migration of ECs, suggesting that intact mitochondrial function is required for the migratory capacity of these cells. Thus, caffeine was not able to induce migration in rho^0-endothelial cells devoid of functional mitochondria. We show that caffeine improves mitochondrial function, which by itself is required for migratory capacity. Mitochondria, thereby, may not only be required for ATP production, but also to act as signaling organelles in endothelial cells as recently postulated.[13]

Considerable evidence suggested that caffeine antagonizes adenosine receptors and raises the intracellular calcium levels both in the central nervous and in the cardiovascular system.[37] However, analysis of the IC_{50} and EC_{50} for antagonism of the adenosine receptors and the intracellular calcium increase, respectively, revealed that caffeine concentrations in the millimolar range are needed to elicit such a response.[7] This is in accordance with our own findings, that intracellular calcium levels are not increased with caffeine concentrations of up to 100 μmol/L (data not shown). The lower concentrations of caffeine used in our experiments, however, promoted cell migration and are likely to be physiologically relevant. Ingestion of 3 cups of coffee did not elevate caffeine serum concentrations above 50 μmol/L, which are far lower serum concentrations then those of 500 μmol/L caffeine, causing lethal intoxication in vivo.[7] To perform dose-response experiments with EPCs, which would require large amounts of mononuclear cells (approx. 250 mL peripheral blood), we were constrained to use buffy-coats from healthy blood donors up to 24 hours after blood donation. Therefore, ATP measurements had to be performed from different isolates for functional assays. We believe that the heterogeneity in cell quality had led to slightly different concentrations of caffeine necessary to achieve the maximum in vitro effect on cell migration or ATP content. In fact, our in vivo experiments in healthy volunteers and CAD patients strongly suggest, that caffeine concentrations of 20 to 30 μmol/L are sufficient to achieve significant improvement in migratory capacity and increase in ATP content.

Several studies have documented improvement of mitochondrial function associated with an increase in AMP kinase activity, mostly AMP kinase α1. In a recent article by

Kukidome et al, the authors demonstrate that metformin and AICAR normalize hyperglycemia-induced production of mitochondrial reactive oxygen species (mtROS) and promoted mitochondrial biogenesis.[24] This effect was, at least in part, mediated through AMPK, leading to induction of PGC-1α and MnSOD.

Mitochondrial biogenesis and function has been suggested as a relevant pathophysiological process underlying metabolic syndrome, diabetes, and cardiovascular disease. Caffeine-mediated improvement of mitochondrial function, therefore, may not only augment endothelial repair, but also contribute to the prevention of metabolic syndrome. Indeed, a meta-analysis of clinical studies supports the hypothesis that habitual coffee consumption is associated with a substantial lower risk of type 2 diabetes.[6,38] Further studies are required to elucidate a potential impact of caffeine-induced AMPK and mitochondrial function on metabolic syndrome or diabetes.

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Disclosures

None.

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