Biochemical and Biophysical Research Communications 447 (2014) 520–525

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

LPS inhibits caspase 3-dependent apoptosis in RAW264.7 macrophages induced by the AMPK activator AICAR

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ARTICLE INFO

Article history:
Received 25 March 2014
Available online 13 April 2014

Keywords:
AMPK
AICAR
LPS
Apoptosis
Macrophages
Caspase 3

ABSTRACT

AMP-activated kinase is a cellular energy sensor which is activated in stages of increased ATP consumption. Its activation has been associated with a number of beneficial effects such as decreasing inflammatory processes and the disease progress of diabetes and obesity, respectively. Furthermore, AMPK activation has been linked with induction of cell cycle arrest and apoptosis in cancer and vascular cells, indicating that it might have a therapeutic impact for the treatment of cancer and atherosclerosis. However, the impact of AMPK on the proliferation of macrophages, which also play a key role in the formation of atherosclerotic plaques and in inflammatory processes, has not been focused so far. We have assessed the influence of AICAR- and metformin-induced AMPK activation on cell viability of macrophages with and without inflammatory stimulation, respectively. In cells without inflammatory stimulation, we found a strong induction of caspase 3-dependent apoptosis associated with decreased mTOR levels and increased expression of p21. Interestingly, these effects could be inhibited by co-stimulation with bacterial lipopolysaccharide (LPS) but not by other proinflammatory cytokines suggesting that AICAR induces apoptosis via AMPK in a TLR4-pathway dependent manner.

In conclusion, our results revealed that AMPK activation is not only associated with positive effects but might also contribute to risk factors by disturbing important features of macrophages. The fact that LPS is able to restore AMPK-associated apoptosis might indicate an important role of TLR4 agonists in preventing unfavorable cell death of immune cells.

1. Introduction

The AMP-activated protein kinase (AMPK) is a highly conserved enzyme and constitutes an important regulator of cellular metabolic processes. It is a heterotrimeric protein which consists of 2 regulatory (β and γ) and one catalytic (α) subunits which all exist in at least 2 isoforms. AMPK is activated in states of increased ATP consumption occurring e.g., after heat stress, excessive training, hypoxia/ischemia and starvation [1]. Under these conditions, intracellular AMP rises and binds to the AMP-binding domain in the regulatory γ-subunit of the kinase resulting in increased enzyme activity. In addition, upstream kinases such as LKB1 or CaMKK2 are able to effectively increase AMPK activation by phosphorylating the catalytical α-subunit at Thr172. Chemical AMPK activators comprise 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR), as well as the well-established anti-diabetic drug metformin. Both drugs activate AMPK by increased AMP production; however by different mechanisms. While intracellular AICAR is phosphorylated by adenosine kinase to generate 5-aminoimidazole-4-carboxamide-1-D-ribofuranosyl-5′-monophosphate (ZMP) which is structurally related to AMP and can bind to the γ-subunit of AMPK [2], metformin interacts with the respiratory chain in mitochondria and thereby increases intracellular AMP [3,4]. AMPK activation influences glucose and lipid metabolism and decreases inflammatory processes. Therefore, it has been repeatedly suggested as a potential therapeutic target for the treatment of diabetes, obesity and inflammatory diseases [5]. In primary astrocytes,

Abbreviations: ACC, Acetyl-CoA-Carboxylase; FCS, fetal calf serum; LPS, lipopolysaccharide; MEF, mouse embryonic fibroblasts; ox-LDL, oxidized low density lipoprotein; PDGF, platelet derived growth factor; SRB, sulforhodamine B; TLR, Toll-like receptor; ZMP, 5-aminoimidazole-4-carboxamide-1-D-ribofuranosyl-5′-monophosphate.

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http://dx.doi.org/10.1016/j.bbrc.2014.04.008
0006-291X/

microglia and peritoneal macrophages and in the CNS of LPS-treated rats, AICAR-treatment reduced LPS-induced expression of pro-inflammatory cytokines *in vitro* and *in vivo* [6]. In RAW264.7 macrophages, AICAR attenuated LPS-induced TNFα, COX-2 and iNOS expression but these effects were shown to be mediated in an AMPK-independent manner [7,8].

Furthermore, AMPK activation has also an impact on cell proliferation. Several studies showed proapoptotic effects of AICAR in a variety of primary tumor cells and tumor cell lines [9] as well as in non-malignant cells. In pancreatic beta cells, activation of AMPK or constitutively active AMPK resulted in induction of apoptosis [10]. In addition, AICAR blocked oxLDL-induced proliferation of macrophages and PDGF/FCS-induced proliferation of vascular smooth muscle cells which both have been associated to AMPK-dependent anti-atherosclerotic effects [11,12]. In general, mechanisms leading to AICAR-induced effects on cell proliferation could not be completely clarified so far and have been described as both AMPK-dependent and -independent, respectively [2,13,14].

Since inflammatory processes are very prominent in a number of macrophage-related diseases e.g., in atherosclerosis, we were interested in the effects of AMPK activation under inflammatory conditions in these cells. We treated RAW264.7 mouse macrophages with the AMPK activators AICAR or metformin, respectively, and assessed their effects on cell proliferation and apoptosis with and without additional proinflammatory stimulation with bacterial lipopolysaccharide.

Our results revealed a massive caspase 3-dependent induction of apoptosis after AMPK activation which, however, could be strongly inhibited by co-incubation with LPS but not by inflammatory cytokines. Since LPS constitutes an agonist of Toll-like receptor 4 (TLR) these results indicate that TLR4-dependent pathways might at least partially interfere with AMPK-mediated apoptosis.

2. Materials and methods

2.1. Drugs

AICAR and metformin, used as AMPK activators, were purchased from Merck Chemicals Ltd. (Darmstadt, Germany) and dissolved in phosphate buffered saline (PBS) at concentrations of 10 mg/ml and 1 mol/l, respectively. Cytokines were dissolved in sterile H_2O and cells were incubated with the following concentrations: TNFα [5 ng/ml], IL-1β [1 ng/ml] and IFN-γ [10 ng/ml]. Bacterial lipopolysaccharide was administered at 10 μg/ml cell culture medium.

2.2. Western blot analysis

Western blot analysis was performed as described earlier [15].

Blots were incubated with primary antibody against p-ACC, Caspase 3, pAkt1, Akt1, p21, mTOR, p-mTOR (all 1:250, Cell Signaling Technology, Heidelberg, Germany) Hsp90 (90 kDa) (Becton Dickinson GmbH, Heidelberg, Germany) was used as loading control.

2.3. Cell proliferation assays

The sulforhodamine B (SRB) assay is used to determine cell density, based on the analysis of the cellular protein content. For the sulforhodamine B assay, RAW264.7 macrophages were seeded in 6-well plates (10^6 cells/well) and cultured overnight prior to incubation. The cells were then incubated with 1 mM AICAR for 2, 20 and 24 h in quadruplicate. At the end of the incubation period the cells were fixed with 5% trichloroacetic acid (TCA) for 1 h at 4 °C. The supernatant was discarded; the plates were washed seven times with H_2O and then dried for 1 h at 60 °C. Staining of cell proteins was performed for 30 min at RT with sulforhodamine B (SRB) at a concentration of 0.4% in 1% acetic acid. The plates were washed five times with 1% acetic acid and then dried again 1 h at 60 °C. SRB was dissolved in 10 mM Tris pH 10.5 and the extinction of the stained supernatant was measured photometrically at 546 nm.

2.4. Caspase 3 assay

RAW264.7 macrophages were seeded in 24-well plates (50,000 cells/well) and cultured overnight prior to incubation. The cells were then incubated with the respective drugs at indicated concentrations and time periods. At the end of the incubation, cells were washed with PBS and then lysed by addition of 200 μl lysis buffer (10 mM HEPES, pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% CHAPS) supplemented with 1 mM DTT, 1 μg/ml Pepstatin A and 1 mM PMSF. 50 μl of the cell suspension were mixed with reaction buffer (25 mM HEPES, 1 mM EDTA, 0.1% Chaps, 10% Sucrose) supplemented with 1 mM DTT and 10 μM fluorogenic caspase 3-substrate (Enzo Life Science GmbH, Lörrach, Germany) in a 96-well fluorescence plate. The fluorescence read-out was determined 1 h after incubation at 37 °C in a Tecan plate reader at an excitation wavelength of 360 nm and an emission at 465 nm.

2.5. Detection of cell cycle distribution and apoptosis using flow cytometry

RAW264.7 were incubated with AMPK activators and LPS as indicated, respectively. At the end of the incubation period cells were harvested by scraping, washed twice with PBS, fixed with 80% (v/v) ethanol overnight at −20 °C. After two washing steps with PBS, cells were incubated for 5 min with 0.125% Triton X-100 on ice, washed again with PBS and then stained with propidium iodide (20 μg/ml) in PBS containing 0.2 mg/ml RNaseA. Stained cells were analyzed by flow cytometry (FACSCanto II, Becton Dickinson, Heidelberg, Germany). For each sample, cells were analyzed until 100,000 cells had been counted in a predefined G1-gate. The cell cycle distribution (percentage of cells in the G_0/G_1, S, and G_2/M phase) was assessed using FlowJo software. The percentage of apoptotic cells was obtained by calculating the percentage of cell fragments in subG1.

2.6. Data analysis

Statistical evaluation was done with SPSS 17.0 for Windows. Data are presented as mean ± SEM. Data were either compared by univariate analysis of variance (ANOVA) with subsequent *t*-tests employing a Bonferroni α-correction for multiple comparisons or by Student's *t*-test. For all tests, a probability value $p < 0.05$ was considered as statistically significant.

3. Results

3.1. AICAR inhibits cell proliferation and induces apoptosis in RAW264.7 macrophages

In the sulforhodamine B cell proliferation assay, RAW264.7 macrophages showed a significantly decreased cell number 24 h after treatment with 1 mM AICAR in comparison with untreated controls. Lipopolysaccharide also inhibited cell proliferation; however, when co-incubated with AICAR it could at least partially restore the AICAR-induced drop-down of cells (Fig. 1A). AMPK activation was confirmed by Western Blot analysis of

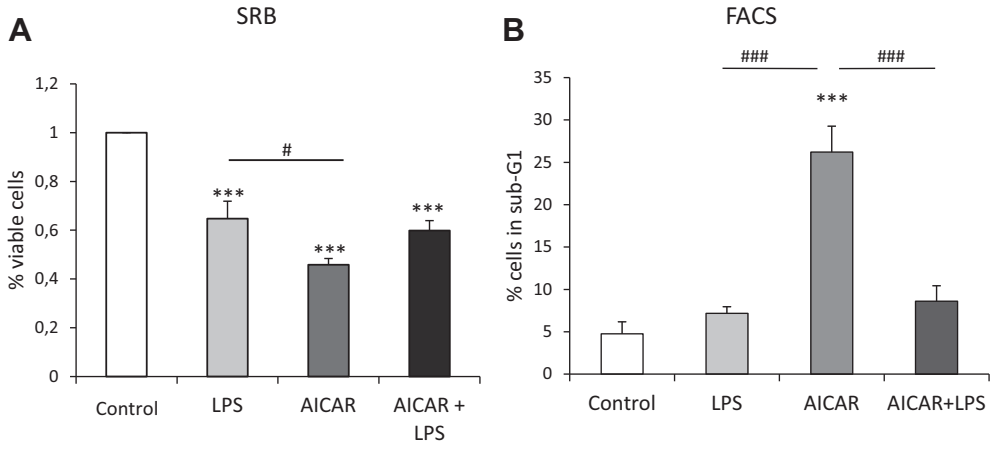

Fig. 1. AICAR attenuates cell proliferation in RAW264.7 macrophages. (A) Sulforhodamine B (SRB) cell proliferation assay. LPS [10 μg/ml] and AICAR reduce the proliferation of RAW264.7 macrophages ($n = 4$). (B) FACS analysis showing the percentage of cells in subG1 after an incubation period of 20 h. Effect of LPS, AICAR and a combination of both on induction of apoptosis ($n = 7$). $^{***}p < 0.001$, statistically significant difference compared to control, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, statistically significant difference between indicated samples.

phospho-Acetyl-CoA-Carboxylase (pACC), a well known AMPK substrate (Suppl. Fig. 1). To clarify the mechanism leading to the reduced cell number after addition of AICAR, a FACS analysis of cell cycle progression was performed. The results revealed a strong increase in the subG1 fraction after AICAR treatment (Fig. 1B) which was dose- and time-dependent (Suppl. Fig. 2) and indicates induction of apoptosis. The shift to subG1 was compensated by decreases of cells in the G_1-, S- and G_2-phases of the cell cycle; however, these changes were not significant (not shown). Next, we applied the adenosine kinase inhibitor iodotubericidin to evaluate if AICAR-processing to ZMP contributes to the increase of cells in subG1. 0.1 μM iodotubericidin completely abolished the AICAR-mediated apoptotic response thus showing that the effect was dependent on ZMP-formation. To further confirm the role of AMPK activation we repeated the experiment with the anti-diabetic drug metformin, which is another well-known AMPK activator. Similar to AICAR, metformin also induced apoptosis in RAW264.7 macrophages at concentrations of 5 and 10 mM (Suppl. Fig. 2). These concentrations are frequently used in cell culture, however they are high (~650 and 1300 mg/l, respectively) compared to therapeutically reached plasma concentrations (~0.5–8 mg/l) [16].

Interestingly, co-stimulation with the TLR4 agonist lipopolysaccharide (LPS), which did not induce apoptosis by itself, reversed AICAR-mediated apoptosis (Fig. 1B). Control experiments with other pro-inflammatory stimuli such as TNFα, IL-1β and IFNγ failed to reverse AICAR-induced cell death indicating that the effects might be specific for TLR4 activation (Suppl. Fig. 3).

3.2. AICAR induced apoptosis is mediated by caspase 3 activation

Caspase 3 is a major effector caspase which can be activated by the extrinsic and intrinsic apoptosis pathways. We examined the activation of caspase 3 by Western Blot analysis detecting cleaved and thereby activated caspase 3, as well as by a caspase 3-specific activity assay to elucidate the mechanism of AICAR-induced apoptosis. The results showed a time-dependent increase in caspase 3 activity after treatment of RAW 264.7 cells with AICAR indicating that a caspase 3-dependent pathway is contributing to AICAR-induced apoptosis in macrophages. In accordance with the effects of the FACS analysis, we observed that stimulation with lipopolysaccharide, which alone did not affect caspase 3 activity, almost completely reversed the AICAR-induced caspase 3 activation (Fig. 2).

3.3. AICAR decreases mTOR expression and activity

It has been shown for many times that mammalian target of rapamycin (mTOR) is a major target of AMPK which is inhibited after AMPK activation. Furthermore, mTOR inhibition has been linked to antiproliferative effects. In our experiments we could show that mTOR was strongly downregulated on the total protein level as well as in its phosphorylated state 20 h after AICAR exposure. Since other proteins were well detectable at that time point we would like to suggest that the effect is due to AMPK activation rather than to decreased global protein expression caused by induction of cell death. This assumption was supported by the fact that the decrease in mTOR could be completely blocked by iodotubericidin. Similarly, as already shown in caspase activity assays and the FACS analyses, LPS was able to counteract the AICAR-induced downregulation of mTOR (Fig. 3).

3.4. AICAR induces Akt activation and p21 expression

AICAR-induced apoptosis has been linked to a prolonged increase in the expression of the cell cycle protein p53 and its target protein p21. In accordance with such data we could show that p21 is strongly augmented after AICAR treatment. In contrast, the anti-apoptotic protein kinase B (Akt 1) was activated after AMPK activation which might constitute a cellular counterregulation to the apoptotic response. Both effects were also alleviated by iodotubericidin and LPS (Fig. 4, Suppl. Fig. 4).

4. Discussion

Activation of the AMP-activated kinase by AICAR has been repeatedly associated with cell cycle arrest and apoptosis of tumor cells (e.g., childhood acute lymphoblastic leukemia cells (ALL) [17], multiple myeloma cells [18], and colon cancer cells [19]) suggesting its function as a tumor suppressor. Moreover, inhibition of cell proliferation has also been shown in mouse embryonic fibroblasts and aortic smooth muscle cells [12,20]. On the other hand AMPK was linked to cell survival, mainly in non-malignant cells [21]. In neurons, a dual function for AMPK has been described which provides protective survival effects during transient energy depletion, while prolonged AMPK activation leads to neuronal apoptosis. From these results it might be suggested that AMPK-mediated effects on cell proliferation are dependent on the cell type, the

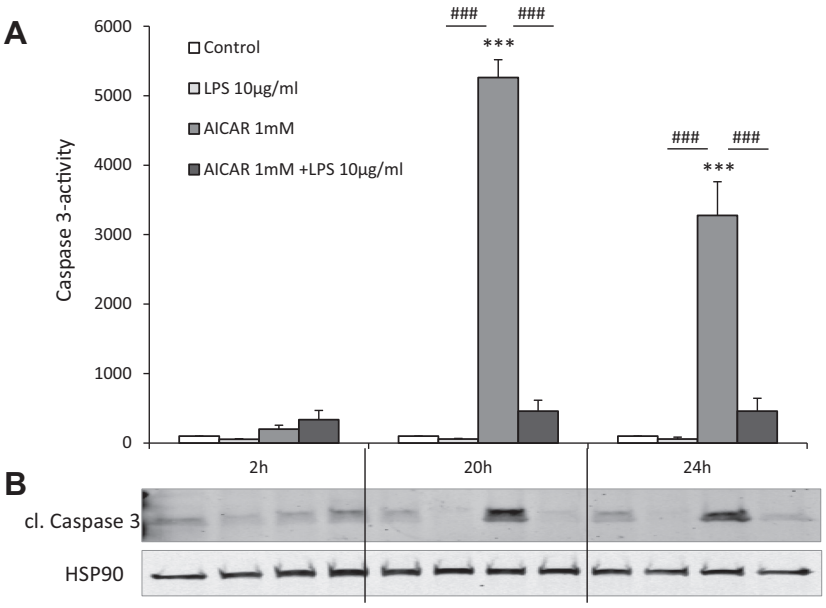

Fig. 2. AICAR-induced apoptosis is mediated by caspase 3. Time course of caspase 3 activity in RAW264.7 cells treated with LPS, AICAR or a combination of both, respectively. (A) Caspase activity assay, (B) cleaved caspase 3 as assessed by Western Blot analysis. The figure shows one representative Blot of 3 independent experiments. ($n = 3$), $^{***}p < 0.001$, statistically significant difference compared to control, $^{\#\#\#}p < 0.001$, statistically significant difference between indicated samples.

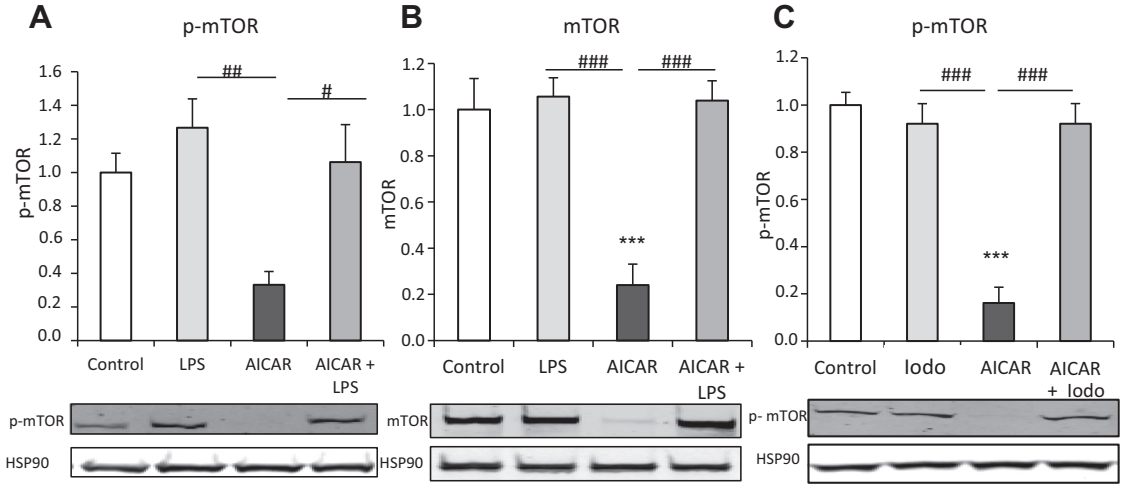

Fig. 3. AICAR suppresses mTOR expression and activation. Expression and activation of mTOR in RAW264.7 cells. (A) Expression of phosphorylated mTOR ($n = 4$) and (B) mTOR total protein in macrophages with and without treatment with LPS and/or AICAR ($n = 4$), respectively. (C) Iodotubericidin (Iodo) inhibits AICAR-induced downregulation of p-mTOR ($n = 3$). $^{***}p < 0.001$, statistically significant difference compared to control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, statistically significant difference between indicated samples.

culture conditions, the duration of AMPK stimulation and the downstream targets which are involved. These targets comprise upregulated p53 [17], increased expression of p27 and p21 [17], alleviated NF-κB activity [2,19], and modulation of protein kinase B/Akt and mTOR [18,22].

The effects of AMPK on cell proliferation and viability in macrophages have not been extensively studied so far although a number of studies described a decrease of inflammatory mediators in these cells after AICAR treatment at high concentrations (1–3 mM) [7,8]. In this study we have assessed the impact of AMPK activation on proliferation and survival of unstimulated or LPS-treated RAW264.7 macrophages, respectively. We found that two AMPK activators with different activation mechanisms, AICAR and metformin, were both potent inducers of apoptosis in these cells at relatively high concentrations. AICAR-induced apoptosis in RAW264.7 macrophages was mediated in a ZMP-dependent manner via a strong inhibition of the growth-supporting protein mTOR and activation of caspase 3. In addition, AICAR significantly increased p21 expression. These results were in good accordance with AMPK-mediated mechanisms of apoptosis induction mentioned above and with studies showing caspase 3 dependent apoptosis after AICAR-mediated AMPK activation in liver cells [23]. Unexpectedly, p-Akt (Ser473) increased after AMPK activation, which is in contrast to its role as anti-apoptotic protein, but has nevertheless been described earlier [24] and might be regarded as a compensatory survival mechanism of the cells.

Interestingly, AICAR-induced apoptosis could be suppressed by addition of lipopolysaccharide whereas other proinflammatory stimuli such as the cytokines IL1β and TNFα as well as IFNγ were not able to inhibit AICAR-induced effects indicating that reduction

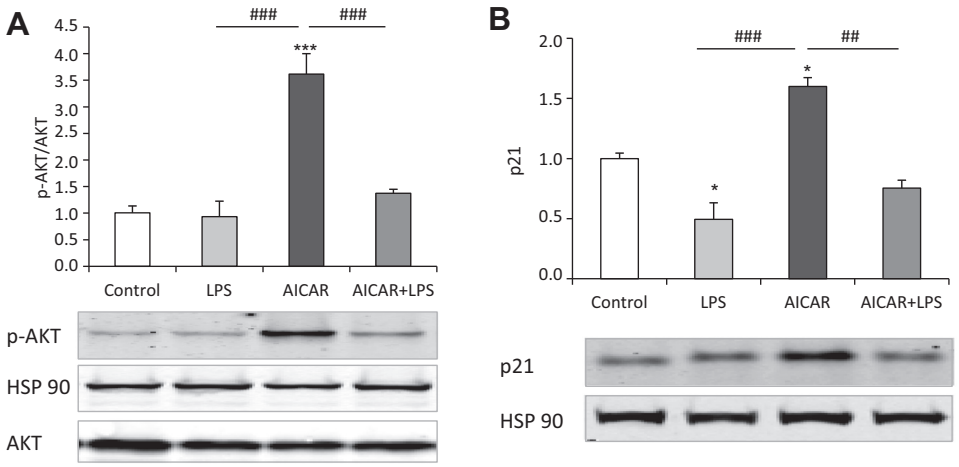

Fig. 4. AICAR increases expression of p-Akt and p21. (A) Activation of protein kinase B/Akt after AICAR treatment as assessed by Western Blot of p-Akt vs. Akt total protein ($n = 3$). (B) Expression of p21 after AICAR treatment. The diagram shows the densitometric analysis of 3 independent experiments, the Blot one representative example. Hsp 90 served as a loading control ($n = 3$). $^{*}p < 0.05$, $^{***}p < 0.001$, statistically significant difference compared to control, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ statistically significant difference between indicated samples.

of apoptosis is mediated in a TLR4-specific manner. This observation fits well with reports showing that stimulation of macrophages with anti-inflammatory cytokines (e.g., IL-10 and TGF-β) provoked a rapid AMPK phosphorylation and thereby activation [25] while pro-inflammatory TLR4 activation by LPS inhibits AMPK activation in immune cells [25,26] by disturbing its interaction with LKB1 [27]. Since LPS has also been associated with inhibitory effects on cell cycle progression, which was confirmed by our results, another potential explanation for inhibition of AICAR-induced apoptosis might be that the LPS-induced cell cycle arrest prevents cells from a shift to subG1.

AICAR-mediated effects on cell viability and cell cycle progression have already been associated with AMPK-dependent as well as -independent mechanisms. A recent report suggested AMPK-independent apoptosis induction by AICAR since MEFs from AMPKα1 and α2 knock-out mice underwent apoptosis after AICAR treatment. The study showed that BAX and Bad are essential players in AICAR-induced apoptosis which was mediated by ZMP [28]. However, other studies which used cells with dominant negative or constitutively activated AMPK revealed that AICAR-induced effects on cell proliferation are clearly mediated by AMPK [2]. A number of results in our study support an AMPK dependent mechanism of AICAR-induced apoptosis comprising upregulation of pACC, ZMP-dependency and mTOR inhibition. Furthermore, the second AMPK activator, metformin, also induced apoptosis. On the other hand we found that a direct AMPK activator, A769662, failed to induce apoptosis and also another cell line, NIH3T3 murine fibroblasts, did not show induction of apoptosis (not shown). In comparison to a number of macrophages which do not express the α2 isoform of AMPK [25], NIH3T3 cells express AMPKα1 as well as α2 indicating that AMPKα might prevent cells from apoptosis which was also suggested in studies with AMPKα knock-out MEFs. In these cells, AICAR-induced apoptosis was increased after single knock-out of α1 or α2 and further augmented by double knock-out of both subunits which further supports a protective role of AMPKα subunits in the induction of apoptosis [28].

Several studies already revealed anti-inflammatory effects of AMPK activation in macrophages, however, mainly by using relatively high concentrations of AMPK activators. We could now show that these high concentrations are associated with the induction of cell death in macrophages. Since macrophage apoptosis is an important feature of chronic inflammatory diseases including atherosclerosis [29] and might impair resolution of inflammation due to dysfunction of host phagocyte-mediated innate immunity, these results have to be taken into account as a potential risk factor of AMPK activation. However; taking into account that plasma concentrations of metformin which result from severe overdose do not exceed 200 mg/l [30] these might only occur in rare cases.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

The authors would like to thank Julia Häusler and Julian Sommer for excellent assistance. This study was supported by the Deutsche Forschungsgemeinschaft (DFG, NI-705/3-1, GRK 1172).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.008.

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