Published in final edited form as:

Biochem Biophys Res Commun. 2009 August 14; 386(1): 16–20. doi:10.1016/j.bbrc.2009.05.106.

Loss of AMPK exacerbates experimental autoimmune encephalomyelitis disease severity

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Abstract

AMP-activated protein kinase (AMPK) is an energy sensing metabolic switch in mammalian cells. Here, we report our novel finding that AMPK is lost in all immune cells of experimental autoimmune encephalomyelitis (EAE), an inflammatory disease of Central Nervous System (CNS). AMPK α 1 is predominantly expressed in T cells and antigen presenting cells (APCs), which are primarily involved in EAE disease progression. AMPK is lost at protein level in spleen macrophages, total T cells and their subsets (CD4, CD8 and regulatory T cells) isolated from EAE afflicted animals compared to control, without affecting its mRNA levels suggesting that the loss of AMPK protein is the result of posttranscriptional modification. To examine its pathological relevance in inflammatory disease, EAE was induced in wild type (+/+) and AMPK α 1 null mice (−/−) using MOG_{35–55} peptide. AMPK α 1^{−/−} mice exhibited severe EAE disease with profound infiltration of mononuclear cells compared to wild type mice however, AMPK α 2 is not involved in enhancing the severity of the disease. Spleen cells isolated from AMPK α 1^{−/−} immunized mice exhibited a significant induction in the production of IFN γ . Our study identifies AMPK as a down regulated target during disease in all immune cells and possibly restoring AMPK may serve as a novel therapeutic target in autoimmune diseases like multiple sclerosis (MS).

Keywords

AMPK; EAE; Multiple sclerosis; T cells; Macrophage

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Introduction

AMP-activated protein kinase (AMPK) is a phylogenetically conserved intracellular energy sensor which plays a central role in the regulation of glucose and lipid metabolism [1]. It is a heterotrimeric complex enzyme comprising of a catalytic ($\alpha 1$ or $\alpha 2$), a regulatory ($\beta 1$ or $\beta 2$), and an AMP-binding regulatory ($\gamma 1$, $\gamma 2$, or $\gamma 3$) subunits [1]. AMPK gets activated by alterations in the AMP:ATP ratio in response to energetic stress and requires phosphorylation of Thr¹⁷² in the activation loop of the α catalytic subunit [2]. Once activated, AMPK induces ATP-generating catabolic pathways including glucose and fatty acid oxidation, while inhibiting ATP-consuming anabolic pathways including cholesterol, fatty acid, and triacylglycerol synthesis [1,3,2]. Three upstream kinases have been identified as activators of AMPK, the tumor suppressor LKB1, calcium/calmodulin-dependent protein kinase (CaMKK) and TGF-beta-activated kinase-1 (TAK1) [4–6].

MS is an inflammatory autoimmune demyelinating disease of CNS, in part, mediated by myelin-specific CD4 T cells [7]. Different classes of immunomodulatory drugs with distinct mechanisms of action have been approved for MS treatment [8–10]. However, current MS medications are either partially effective with significant side effects or less effective for long term treatment [11,12]. Therefore, identification of novel targets for developing a new class of drugs is essential, which can be used for MS therapy alone or in combination with existing drugs.

In the present work, we have investigated the status of AMPK in immune cells during EAE disease and its possible role in disease pathology.

Materials and methods

Animals

Female 6- to 8-wk-old C57BL/6 and SJL mice were obtained from National Cancer Institute (NCI), and were housed in pathogen free conditions. The generation of AMPK $\alpha 1$ ($-/-$) and $\alpha 2$ ($-/-$) mice has been described previously [13–15]. AMPK $\alpha 1$ ($-/-$)(background of C57B6/129), $\alpha 2$ ($-/-$) mice (background of C57B6) and their respective wild type (WT) mice were maintained on a 12:12-h light–dark cycle and received standard rodent chow and water *ad libitum*. Genotyping was performed by PCR using DNA from a tail-piece as described before [13–15]. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Medical University of South Carolina, Charleston. Paralyzed mice were afforded facile access to food and water.

Peptide, reagents and cell culture

Myelin proteolipid protein peptide (PLP_{139–151}) (HSLGKWLGHDPKF) and myelin oligodendrocytes protein peptide (MOG_{35–55}) (MEVGWYRSPFSRVVHLYRNGK) were purchased from Peptide International Inc. Louisville, KY USA. The anti-pAMPK α , pACC and AMPK α antibodies were purchased from Cell Signaling. The anti-AMPK $\alpha 1$, $\beta 1/2$ and $\gamma 1$ and $\gamma 2$ Abs were purchased from Epitomics (Burlingame, CA).

EAE induction, Histology and recall response

AMPK null mice ($\alpha 1^{-/-}$ or $\alpha 2^{-/-}$) and respective WT mice (6–8 wk old) were immunized on day 0 and 7 by subcutaneous (s.c.) injections in the flank region with total 100 μ l of emulsion containing MOG_{35–55} peptide along with killed *Mycobacterium tuberculosis* H37Ra (400 μ g). In case of EAE induction in SJL mice, PLP_{139–151} (100 μ g/mouse) was used. Each mouse additionally received 200 ng of pertussis toxin (Sigma) by intravenous (i.v.) injection in 300 μ l of PBS on day 0 and 7 of immunization and clinical disease was monitored as described

earlier [16]. H&E staining of the lumbar region of the spinal cords were performed as described earlier [16]. Myelin MOG_{35–55}-immune spleen cells ($2 \times 10^5/100 \mu\text{l/well}$) isolated from WT and AMPK $\alpha 1^{-/-}$ mice were cultured in the presence of MOG_{35–55} (25 $\mu\text{g/ml}$). Cell proliferation and the production of cytokines (IFN γ and IL-17) were examined as before [16].

Separation of subpopulation of T cells

Mice were killed at the peak of EAE disease (day 20) and spleens were removed, the single cell suspension was prepared. Red blood cells (RBC) were lysed by $1 \times$ pharmalyse and then washed twice with RPMI-1640. Finally, the cells were resuspended in RPMI complete media and counted. Total CD3 T cells were enriched using T cells enrichment columns (R&D Systems) as per manufacturer's instructions. Different subpopulation of T cells (CD4, CD8 and CD4CD25) from control and EAE mice were enriched using Mag-Collect CD4, CD8 and CD4CD25 regulatory T cells isolation kits, respectively (R&D Systems).

Immunoblot analyses

Cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, and 0.5% Nonidet P-40] containing a protease inhibitor cocktail (Sigma) and 50 μg of proteins was used for immunoblot analysis of pAMPK $\alpha 1$, pACC, AMPK α , AMPK $\alpha 1$ and β actin using their specific antibodies as described before [16,17].

AMPK kinase assay

AMPK activity was assayed by immunoprecipitation followed by kinase assay using recombinant ACC protein (Upstate Biotech) as a substrate as described before [16,17].

Nucleotide assay

Adherent, non-adherent and T cells from control and EAE mice were lysed in perchloric acid as described [18]. These extracts were neutralized with 1.5 M KOH/KHCO₃ and then separated by high-performance liquid chromatography to measure ATP levels [18].

Statistical analyses

GraphPad Prism software (GraphPad Software Inc.) was utilized throughout for statistical analysis. Kruskal–Wallis test and Student's *t*-test were employed to analyze clinical disease score. Statistics for densitometric values comparison for proliferation and cytokine responses were analyzed with one-way multiple-range ANOVA and Student's *t*-test. A value of $p < 0.05$ and above was considered significant.

Results and discussion

Expression of AMPK subunits and their isoforms

We first examined the expression of different subunits of AMPK and their isoforms in T cells, antigen presenting cells (macrophages, dendritic and endothelial cells) and mouse brain (Suppl. Fig. S1A). The expression of $\alpha 1$, $\beta 1$ and $\gamma 2$ was found to be predominant in all the cells examined; however, expression of $\alpha 2$ was detected only in the brain (Suppl. Fig. S1A). Since, EAE disease is primarily mediated by T cells, therefore, we examined its expression in freshly isolated subsets of CD3 T cells (CD4, CD8 and CD4CD25) without any stimulation. The expression of $\alpha 1$, $\beta 1$, $\gamma 1$ and $\gamma 2$ was predominantly expressed in CD4, CD8 and in regulatory (CD4CD25) T cells (Suppl. Fig. S1B). These results were further supported by quantitative PCR using specific primers of AMPK subunits and their isoforms (Suppl. Fig. S1 C).

AMPK activity was down regulated during EAE disease progression

To examine the status of AMPK under inflammatory disease condition, we measured AMPK activity and its phosphorylation in CNS at the peak of EAE disease (day 20). AMPK activity was determined by immunoprecipitation with AMPK α antibodies followed by kinase assay using recombinant Acetyl CoA Carboxylase (ACC) as a substrate. AMPK activity was significantly downregulated at the peak of EAE disease in brain compared to control mice (Fig. 1A). Similarly, phosphorylation of both AMPK and its bona fide substrate ACC was decreased in lymph nodes under EAE disease (Fig. 1B). AMPK activity examined by kinase assay in total spleen cells was also found significantly reduced (Suppl. Fig. S2). We further analyzed the expression of AMPK and observed that the protein levels of AMPK α 1, - β 1/2 and - γ 1 subunits were all significantly decreased in spleen cells isolated from EAE except - γ 2 (Fig. 1C). This data strongly suggests that AMPK activity gets downregulated during EAE disease in lymphoid and non lymphoid organs. Interestingly, the decreased AMPK activity was due to the loss of AMPK α 1 and other subunits (β 1/2 and γ 1) at protein levels without any changes in their mRNA expression (Data not shown) suggesting that AMPK is regulated at protein translation step or degradation. This also raises the question about the mechanism of regulation of AMPK protein levels during EAE disease. Whether inflammation is a causative effect or an upstream event in loss of AMPK or *vice versa*? Recent studies suggest that AMPK and AMPK-related kinases can be regulated by ubiquitin-dependent proteasome degradation [19,20], which may be one of the mechanisms of its regulation in immune cells during EAE.

To further investigate whether the inhibition of AMPK activity was confined to *in vivo* inflammatory condition or it could be mimicked *in vitro*, a macrophage cell line (Raw 267.4) was used and the inflammatory responses were stimulated with LPS/IFN γ (1 μ g/50 ng/ml) for 18 h followed by examination of phosphorylation of AMPK and ACC. The stimulus with LPS/IFN γ downregulated AMPK activity as documented by decreased phosphorylation of AMPK α and ACC (Suppl. Fig. S3Ai). To mimic EAE disease, we generated Th1 conditioned media from *in vitro* stimulated CD4 T cells and macrophage cells were treated with Th1 media (1:20 dilution) for 18 h. Interestingly, phosphorylation of AMPK α and ACC was observed very low compared to untreated cells (Suppl. Fig. S3Aii). Protein levels of AMPK- α 1, - β 1 and - γ 1 subunits were slightly decreased in Raw cells treated with LPS/IFN γ or Th1 media (Suppl. Fig. 3 Ai&ii). Since microglia are resident APC in the CNS and further activates infiltrated T cells during disease, therefore, we examined AMPK in these cells under inflammatory condition. For this, microglial cell line (BV2) was treated with Th1 conditioned media at different dilutions (from 5 to 100) for 24 h. The phosphorylation of AMPK and ACC was decreased with the increase of Th1 conditioned media (Suppl. Fig. S3B). Protein levels of AMPK- α 1, - β 1 and - γ 1 subunits were not changed in BV2 treated with Th1 conditioned media. These data strongly suggest that AMPK activity is down regulated under *in vitro* and *in vivo* inflammatory disease conditions.

AMPK was down regulated in sub-fractions of spleen cells during EAE disease

Spleen cells are comprised of mainly T cells, B cells, macrophages and dendritic cells. Since we observed the downregulation of AMPK in total spleen cells during EAE disease progression, we further examined its activity in subpopulations of spleen cells. For this, we separated spleen cells isolated from control and EAE mice in three different fractions: (1) adherent cells (mainly macrophages); (2) enriched CD3 positive T cells; and (3) non-adherent cells (total lymphocytes). We observed that phosphorylation of ACC and AMPK α was reduced in all fractionated cells isolated from total spleen cells including CD3 T cell, adherent and non-adherent cells as evident from immunoblot analysis of pACC and pAMPK α (Fig. 1D). We further examined the phosphorylation of ACC and AMPK α in total T cells (CD3) and its subsets (CD4, CD8 and CD4CD25) from control and EAE diseased mice. As depicted in Fig. 1E, we observed the inhibition of phosphorylation of ACC and AMPK α in total T cells (CD3) and

their subsets, which was found due to the loss of AMPK α 1 protein levels in EAE disease compared to control mice. These results indicate that reduced AMPK activity is a general phenomenon in immune cells during EAE disease process. Our findings are in contrast to others where they have shown the activation of AMPK by antigen receptor and Ca²⁺ in T lymphocytes mediated by CaMKK [21]. The difference in the outcome of their and our study may be due to the nature of stimulation as they have used PMA/ionomycin or TCR engagement by CD3 ligation (*in vitro* studies), whereas we have examined the AMPK in immune cells *in vivo* under disease condition without any *in vitro* stimulation. Our results have direct relevance to the inflammatory autoimmune disease like EAE/MS and may be implicated to other inflammatory diseases.

A well characterized mechanism of AMPK activity modulation is the alteration of cellular energy levels reflected by an altered AMP: ATP ratio [1]. Therefore, we investigated the energy status in immune cells under disease by measuring the intracellular ATP and AMP levels in total spleen cells, spleen macrophage and CD3 T cells isolated from control and EAE mice. Higher levels of ATP was detected in total spleen cells (10.3 vs. 6.9 nmol/10⁶ cells, $P < 0.001$) and in spleen macrophage cells (10.8 vs. 5.46 nmol/10⁶ cells, $P < 0.001$), however, no change was found in ATP levels in T cells (7.7 vs. 8.6 nmol/10⁶ cells, $P < 0.089$) isolated from EAE mice compared to the control (Suppl. Fig. S4). We were unable to detect AMP levels in total spleen cells, CD3 and spleen macrophage cells suggesting these cells exhibit a normal energy status in EAE mice.

AMPK α 1 null mice (–/–) exhibited exacerbated EAE disease severity compared with WT littermates

Since we observed loss of AMPK α 1 at protein level in all immune cells, next we examined the effect of AMPK α 1^{–/–} gene disruption on the development of EAE disease using AMPK α 1 null mice. EAE was induced in AMPK α 1^{–/–} and WT mice following active immunization with MOG_{35–55}. As shown in Fig. 2 and in Table 1, the severity of disease was enhanced in AMPK α 1^{–/–} mice (3.37 ± 0.1 ; $p < 0.001$) compared with their WT littermates (2.59 ± 0.35) without any change in the onset of the disease (Table 1). The development of severe paralytic symptoms was apparent in AMPK α 1^{–/–} by day 16 with the maximal disease severity score of 3.37 being observed till end of the study (day 26). Although all mice in both groups succumbed to the disease, there was no mortality associated with the acute attack. The hallmark of EAE disease is the infiltration of inflammatory cells into the CNS, leading to tissue damage [22]. Therefore, we examined the status of infiltration of inflammatory cells into the CNS of WT and AMPK α 1^{–/–} mice. As shown in figure 2B, AMPK α 1^{–/–} EAE mice showed profound infiltration of immune cells in the CNS compare to WT mice. We also examined EAE disease induction in AMPK α 2^{–/–} mice and did not observe any significant change in EAE clinical score in AMPK α 2^{–/–} mice compare with WT mice (Supp. Fig. S5), suggesting that α 2 isoform does not play role in EAE pathology. Induction of EAE disease predominantly involved the participation of APCs and T cells to induce inflammatory cascade, which express mainly α 1. Our novel observations, the loss of AMPK α 1 during EAE disease and exacerbation of EAE clinical symptom in AMPK α 1^{–/–} null mice suggesting a critical role of AMPK in the regulation of inflammatory disease progression.

To confirm that AMPK α 1^{–/–} mice were sensitized to MOG_{35–55} peptide, we measured the Ag-induced T cell proliferation *ex vivo*. As shown in Fig. 2C, *in vitro* culture of spleen cells from WT and AMPK α 1^{–/–} mice showed an antigen-dependent proliferation with no significant change suggesting that the severity of disease in AMPK α 1^{–/–} mice is not due to the higher proliferation of MOG-specific T cell responses. Further, in response to Ag stimulation, spleen cells from WT and AMPK α 1^{–/–} mice induced production of IFN γ and IL17 (Fig. 2D&E), however, AMPK α 1^{–/–} cells produced higher amount of IFN γ with lesser IL17 compare with

WT cells. Spleen cells isolated from AMPK α 1^{-/-} mice displayed normal cell proliferation and pro-inflammatory cytokines production suggesting that loss of AMPK did not affect the normal cellular functions which is consistent with a recent report [23]. However, loss of AMPK α 1 enhances the production of IFN γ with lesser production of IL17 compare with WT mice required further detail study to establish the role of AMPK α 1 in modulation of pro-inflammatory cytokines under inflammatory environment. Loss of AMPK α 1 expression in all immune cells and the higher production of proinflammatory cytokines during EAE disease indicate an inverse correlation. But, to establish this relationship, enzymatic active AMPK α 1 has to be restored in immune cells and examine if levels of cytokines and EAE disease course can be modulated.

Altogether, our study identified for the first time that energy sensor is lost during disease in all immune cells and its genetically deficient mice exhibits an increased severity of EAE disease suggesting its critical role in inflammatory disease progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Ms. Joyce Bryan and Carrie Barnes for their technical assistance. Authors also acknowledge generous support of Prof. Inderjit Singh for providing infrastructure for conducting this study. This investigation (S.G.) was supported by Grants (RG 3810-A-1, PP1283) from the National Multiple Sclerosis Society. This work was also supported by Extramural Research Facilities Program of the National Center for Research Resources (Grants C06 RR018823 and No C06 RR015455). C.d.M. is supported by the Fund for Scientific Research in Industry and Agriculture (F.R.I.A.), Belgium. L.B. is Research Associate of the Fonds National de la Recherche Scientifique, Belgium.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.bbrc.2009.05.106.

Fig. 1.

AMPK was down regulated at the peak of EAE disease. (A) EAE was induced in C57BL/6 mice using MOG_{35–55} peptide and at the peak of disease, mice were killed and AMPK activity was examined in brain homogenate using AMPK α antibody followed by kinase assay as described in methods. $*P < 0.05$ compared to control mice ($n = 3$). (B) Lymph nodes were isolated from control and EAE (SJL) mice at the peak of disease (Day 20) and phosphorylation of ACC and AMPK α was examined by immunoblot and densitometry analysis of pACC and pAMPK α were normalized with β actin. $***P < 0.001$ compared to control mice ($n = 3$). (C) Immunoblot of various proteins including pAMPK α , $\alpha 1$, $\beta 1/2$, $\gamma 1$, $\gamma 2$ and β actin were performed in the lysate isolated from spleen cells from control and EAE mice (C57B6) as described above (A). Blots are representation of three independent experiments. (D) Spleen cells were isolated from the control and EAE diseased mice (B6) at peak of disease (day 20) and T cells, adherent and non-adherent cells were fractionated as described in method. Phosphorylation of ACC and AMPK α was examined using immunoblot analysis. (E) Subpopulations of CD3 T cells (CD4, CD8 and CD4CD25) were isolated from control and EAE mice (B6) and processed for analysis of pACC, pAMPK α , $\alpha 1$ and β actin by immunoblot using their specific antibodies as described in methods.

Fig. 2.

AMPKα1^{-/-} mice exhibited severe EAE disease compared to WT. (A) Active EAE was induced in AMPKα1^{-/-} mice and WT littermate (C57B6/129) with immunization of 100 μg of peptides MOG₃₅₋₅₅ on day 0 and 7. The clinical symptoms were scored every day in a blinded manner. Data points are presented as the mean ± SEM. $P < 0.001$ refers to wild type EAE mice (two-way ANOVA). Numbers of mice used in the study are summarized in Table 1. (B) Spinal cords (lumbar regions) were harvested at the peak day of the disease from AMPKα1^{-/-} and WT mice. Tissues were fixed (10% buffered formalin) and embedded in paraffin, and the paraffin sections were stained with H & E and visualized at 10× to determine infiltration. (C) At the peak of EAE disease, spleen cells from AMPKα1^{-/-}, AMPKα1^{+/-} and WT mice were isolated and stimulated *ex vivo* with MOG₃₅₋₅₅ (25 μg/ml) peptide for 48 h and then [³H]-thymidine was added to the cells and incubated for additional 18 h and counted for cell proliferation. (D and E) The culture supernatants of spleen cells were collected at 72 h and analyzed for inflammatory IFNγ and IL17 by OptEIA system. *** $p < 0.001$ compared with MOG₃₅₋₅₅ untreated spleen cells; \$\$\$ $p < 0.001$, NS, not significant from control MOG₃₅₋₅₅ treated spleen cells.

Table 1

EAE in AMPKα1 WT, heterozygote and in homozygous littermates.

	Number of mice	Incidence	% Incidence	Mean maximum score at peak	Peak day
WT	14	14/14	100	2.59 ± 0.35	20
AMPKα1 (+/-)	13	13/13	100	3.05 ± 0.37**	21
AMPKα1 (-/-)	7	7/7	100	3.37 ± 0.10***	20

Active EAE was induced in AMPKα1 WT, heterozygote and in homozygous mice with MOG35–55 peptide. Total number of mice used in study, incidence, clinical disease score and peak day are reported in this table. Mean maximum score are given at the peak of disease.

*** $P < 0.001$,

** $P < 0.01$ as compare with WT EAE (Student *t*-test).