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Metformin Attenuated the Autoimmune Disease of the Central Nervous System in Animal Models of Multiple Sclerosis¹

Narender Nath,* Musfiquidin Khan,* Manjeet K. Paintlia,* Md Nasrul Hoda,* and Shailendra Giri^{2†}

Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated autoimmune disease of the CNS. Metformin is the most widely used drug for diabetes and mediates its action via activating AMP-activated protein kinase (AMPK). We provide evidence that metformin attenuates the induction of EAE by restricting the infiltration of mononuclear cells into the CNS, down-regulating the expression of proinflammatory cytokines (IFN- γ , TNF- α , IL-6, IL-17, and inducible NO synthase (iNOS)), cell adhesion molecules, matrix metalloproteinase 9, and chemokine (RANTES). Furthermore, the AMPK activity and lipids alterations (total phospholipids and in free fatty acids) were restored by metformin treatment in the CNS of treated EAE animals, suggesting the possible involvement of AMPK. Metformin activated AMPK in macrophages and thereby inhibited biosynthesis of phospholipids as well as neutral lipids and also down-regulated the expression of endotoxin (LPS)-induced proinflammatory cytokines and their mediators (iNOS and cyclooxygenase 2). It also attenuated IFN- γ and IL-17-induced iNOS and cyclooxygenase 2 expression in RAW267.4 cells, further supporting its anti-inflammatory property. Metformin inhibited T cell-mediated immune responses including Ag-specific recall responses and production of Th1 or Th17 cytokines, while it induced the generation of IL-10 in spleen cells of treated EAE animals. Altogether these findings reveal that metformin may have a possible therapeutic value for the treatment of multiple sclerosis and other inflammatory diseases. *The Journal of Immunology*, 2009, 182: 8005–8014.

Multiple sclerosis (MS)³ is a chronic, relapsing-remitting inflammatory demyelinating disease of the CNS affecting more than one million people worldwide. The pathogenesis of the disease is characterized by activation and infiltration of mononuclear cells, predominantly Ag-specific CD4 and CD8 T cells in the CNS and their reactivation by resident Ag-presenting brain-spinal cord glial cells. These activated T cells and brain glial cells secrete proinflammatory cytokines/chemokines along with generation of inflammatory mediators including complement, highly reactive free radicals (reactive oxygen species, reactive nitrogen species) that leads to axonal loss (1, 2). Although genetic and environmental factors are implicated in the pathogenesis of MS (3), its precise etiology remains obscure.

Experimental autoimmune encephalomyelitis (EAE) is a well-established and widely studied animal model with many of the clinical,

immunological, and neuropathological features of MS (4). EAE is induced in susceptible mice by eliciting an immune response to injected myelin Ags, such as myelin oligodendrocyte glycoprotein (MOG) and peptide or proteolipid protein peptide (PLP) (5, 6). Direct evidence for the role of CD4 T cells in EAE induction has come from adoptive transfer studies in which myelin Ag-reactive CD4 Th1 clones induce encephalomyelitis and demyelination, leading to paralysis (7). Th1 cytokines are present in the inflammatory lesions in the CNS, whereas Th2 cytokines are absent, suggesting that Th1 cytokines play a role in the pathogenesis of disease (8, 9). Recovery from EAE in mice is associated with an increase in the presence of Th2 cytokines in the CNS (10). Recently, IL-17-producing Th cells (Th17 cells), a distinct subset from Th1 and Th2, have been characterized based on the secretion of proinflammatory cytokines IL-17, IL-6, and TNF- α (11, 12). Evidence has clarified that Th17 cells, rather than Th1 cells, are important in autoimmune inflammatory conditions such as EAE (13).

Metformin possesses several properties which are desirable for EAE/MS therapy. It has anti-inflammatory (14–16) and antioxidant (17–19) properties and the ability to restore endothelial dysfunction (20–22). Metformin mediates its mechanism of action via activation of AMPK. Studies have shown that activators of AMPK down-regulate inflammation in vitro and in vivo in different animal models (23–26). Moreover, metformin is already an approved drug for human consumption and is given orally for metabolic disorders (27).

In this study, we investigated the therapeutic potential of metformin on EAE disease progression in chronic EAE animal models (SJL; relapsing-remitting or C57BL/6; chronic progressive) and elucidated its possible mechanism via modulation of the proinflammatory environment of CNS and in macrophages and T cells.

Materials and Methods

Mice

Female 6- to 8-wk-old C57BL/6 and SJL mice were obtained from the National Cancer Institute (Bethesda, MD) and The Jackson Laboratory. All

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³ Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein peptide; AMPK, AMP-activated protein kinase; iNOS, inducible NO synthase; Cox2, cyclooxygenase 2; MMP9, matrix metalloproteinase 9; ACC, acetyl CoA carboxylase; CAM, cell adhesion molecule; FFA, fatty free acid; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; ROR γ t, retinoid-related orphan receptor-gamma.

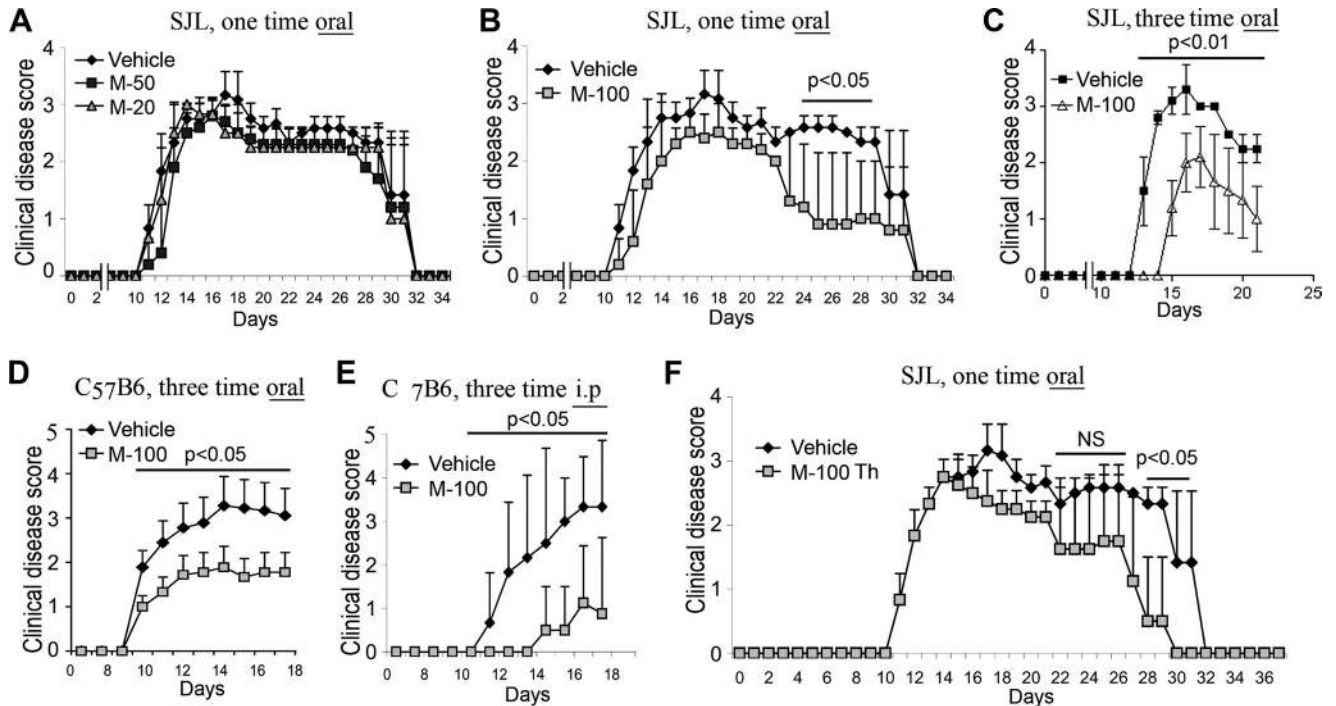


FIGURE 1. Metformin attenuates the EAE disease process in SJL and C57BL/6 mice. Active EAE was induced in SJL or C57BL/6 mice with immunization of 100 μ g of peptides PLP_{139–151} or MOG_{35–55} on days 0 and 7. **A** and **B**, Metformin was given at 20, 50, or 100 mg/kg by the oral route once daily starting day 0 of immunization. **C**, Metformin was given by the oral route three times a day starting on day 0 of immunization in a total of 100 mg/kg. **D** and **E**, Metformin in C57BL/6 mice was given by the oral (**D**) and i.p. (**E**) routes at 100 mg/kg daily divided in three doses. **F**, To examine the therapeutic efficacy, metformin was administered to ongoing active EAE in SJL mice, starting on day 14 (when all the mice had visible clinical symptoms of disease; 2.74 ± 0.27) at 100 mg/kg by the oral route and given once daily. The clinical symptoms were scored every day in a blinded manner. Data points are presented as the mean + SEM. Values of $p < 0.01$, $p < 0.05$, and NS refer to vehicle-treated EAE mice (two-way ANOVA). Numbers of mice used in the study are summarized in Table I.

animals were housed in the pathogen-free animal facility of Medical University of South Carolina (Charleston, SC). All animal protocols were approved by the Animal Care and Use Committee of the Medical University of South Carolina. Paralyzed mice were afforded facile access to food and water.

Reagents and cell line

Myelin proteolipid protein peptide (PLP_{139–151}) (HSLGKWLGHDPKF) and MOG_{35–55} (MEVGWYRSPFSRVVHLYRNGK) were purchased from Peptide International. Metformin was purchased from Sigma-Aldrich. The anti-phospho-ACC (Ser⁷⁹) and phospho-AMPK α (Thr¹⁷²) were purchased from Cell Signaling. Abs for iNOS, Cox2, and β -actin were purchased from Santa Cruz Biotechnology. Mouse IFN- γ and mouse IL-17 recombinant proteins were purchased from R&D Systems. The macrophage cell line (RAW267.4) was purchased from American Type Culture Collection and maintained in DMEM (4.5 g of glucose/L) containing 10% FBS and 10 μ g/ml gentamicin at 37°C in 5% CO₂/95% air. At 80% confluency, the cells were incubated and treated with metformin with serum-free DMEM for the indicated time period in the presence or absence of IFN- γ (50 U/ml) or IL-17 (50 ng/ml). After a 24-h incubation, cell lysate was processed for inducible NO synthase (iNOS) and cyclooxygenase 2 (Cox2) immunoblot analysis. Peritoneal macrophages were isolated and cultured in RPMI 1640 supplemented with heat-inactivated 1% FBS medium as described before (28).

Active EAE induction in SJL and C57BL/6 mice

Mice were immunized on day 0 by s.c. injections in the flank region with 100 μ l of an emulsion of PLP_{139–151} (SJL) and MOG_{35–55} (C57BL/6) peptide in CFA. The final concentrations of peptide and *Mycobacterium tuberculosis* H37Ra were 100 and 200 μ g/mouse, respectively. Each mouse additionally received 200 ng of pertussis toxin (Sigma-Aldrich) by i.v. injection in 300 μ l of PBS on day 0 and 72 h after the first immunization. Clinical disease was monitored daily in a blinded fashion by measuring paralysis according to the following conventional grading system: 0, no disease; 2, loss of tail tonicity; 2.5, partial hind limb paralysis (uneven gate of hind limb); 3, complete hind limb paralysis; 4, complete hind and

forelimb paralysis; and 5, moribund or dead. Clinical disease scores are presented as the average maximal scores over the treatment period (mean \pm SD) and were analyzed using the Kruskal-Wallis test.

Administration of metformin

The mice were administered metformin (20–100 mg/kg body weight in saline by oral gavage) every day from day 0 to until the end of the study (100 μ l/mouse, one or three times in a day with total 20, 50, or 100 mg/kg of drug) or i.p. as indicated following the induction of active EAE disease. Saline as a vehicle (200 μ l) was given to untreated EAE animals.

Histology

To assess the infiltration of immune cells in the CNS, the C57BL/6 mice in treated and untreated groups were killed at the peak of EAE disease (day 16) by CO₂ asphyxiation. The lumbar region of the spinal cords were taken out and fixed in 10% buffered formalin. Paraffin-embedded, 4- μ m-thick transverse sections of spinal cord (six sections per mouse) were stained with H&E to assess leukocyte infiltration and inflammation using an Olympus Compound Microscope (Olympus BX-60) with digital camera attached. The spinal cord sections from five representative animals (untreated and metformin treated) were processed and stained with Hoechst 33342 dye (for nuclei stain). Images of the various regions of the spinal cord were taken by fluorescence microscopy. Infiltrating cells labeled with Hoechst were analyzed using Image-Pro-Plus 4.0 (Media Cybernetics) software and plotted. Individual sections were analyzed and the mean and SD were calculated for each group ($n = 5$).

AMPK activity

AMPK α activity was assayed in total brain homogenate of metformin-treated and vehicle-treated EAE animals. In brief, brain was homogenized in lysis buffer (50 mM Tris-HCl (pH 7.4) containing 50 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol, and a protease inhibitor mixture). Approximately, 200 μ g of cell lysate was incubated with anti-AMPK α 1 Ab (Cell Signaling) for 2 h, then 30 μ l of protein A/G plus agarose was added and incubated for an additional 1 h at 4°C. The immune complexes were washed twice in lysis buffer and twice in kinase buffer (62.5 mM

Table I. Active EAE was induced in SJL or C57BL/6 mice with PLP_{139–151} or MOG_{35–55} peptide^a

Treatment	No. of Mice	Incidence	Clinical Disease Score	Peak Day	
SJL EAE mice EAE PLP _{139–151} peptide					
EAE + vehicle	One time orally	43	43/43	3.4 ± 0.21	17
EAE + 20 mg/kg metformin from day 0	One time orally	6	6/6	3.0 ± 0.0 ^b	14
EAE + 50 mg/kg metformin from day 0	One time orally	10	10/10	2.8 ± 0.27 ^b	17
EAE + 100 mg/kg metformin from day 0	One time orally	11	10/11	2.5 ± 0.35 ^b	17
EAE + vehicle	Three times orally	17	17/17	3.3 ± 0.35	17
EAE + 100 mg/kg metformin from day 0	Three times orally	17	15/17	2.1 ± 0.20 ^{**}	17
C57BL/6 EAE mice with MOG _{35–55} peptide					
EAE + vehicle	Three times orally	9	9/9	3.27 ± 0.48	15
EAE + 100 mg/kg metformin from day 0	Three times orally	9	7/9	1.88 ± 0.37 [*]	15
EAE + vehicle	Three times i.p.	4	4/4	3.47 ± 0.51	15
EAE + 100 mg/kg metformin from day 0	Three times i.p.	4	4/4	1.25 ± 1.35 [*]	17

^a Active EAE was induced in SJL and C57BL/6 mice and treated with different doses of metformin (20–100 mg/kg) by oral and i.p. route as indicated. Total number of mice used in study, treatment, incidence, clinical disease score, and peak day are reported in all experiments. Mean maximum scores are given at the peak of disease.

^b Not significant.

^{**}, $p < 0.01$ and ^{*}, $p < 0.05$ refer to control saline-treated EAE respective group (Student's *t* test).

HEPES (pH 7.0), 62.5 mM NaCl, 62.5 mM NaF, 6.25 mM sodium pyrophosphate, 1.25 mM EDTA, 1.25 mM EGTA, and 1 mM DTT) and incubated at 30°C in 30 μ l of kinase assay buffer containing 200 μ M AMP and ATP mixture (200 μ M ATP and 1.5 μ Ci of [γ -³²P]ATP), with acetyl CoA carboxylase (ACC) recombinant protein (Upstate Biotechnology) for 20 min. The reaction was terminated by adding sample buffer followed by boiling for 3 min. Samples were loaded onto a SDS-PAGE 4–20% Tris-glycine gel and transferred onto nitrocellulose membranes followed by exposure on x-ray film (ECL Hyper film; Amersham Life Science).

Recall responses

Myelin MOG_{35–55}-immune spleen cells ($2 \times 10^5/100 \mu$ l/well) isolated from treated and untreated groups with metformin were cultured in 96-well, round-bottom microculture plates (Falcon Labware) in 0.1 ml of RPMI complete medium in the presence of 10 μ g/ml MOG_{35–55} peptide. For proliferation, [³H]thymidine (1 μ Ci/well) was added at 48 h and mean incorporation of thymidine in DNA was measured after 72 h by a 1450 Microbeta Wallac Trilux Liquid Scintillation Counter (PerkinElmer Life Sciences). Supernatant was used for various cytokine analysis (IFN- γ , IL-17, IL-6, and IL-10) by the ELISA OptEIA system (BD Pharmingen and R&D Systems) as per the manufacturer's instructions.

Isolation of splenic macrophages

The 8- to 10-wk-old female C57BL/6 mice were killed and spleens were taken out. The spleens were lysed and prepared as single-cell suspensions followed by removal of all of the RBC with 1 \times pharma lyses (BD Biosciences). The cells were washed twice and resuspended in RPMI 1640 and the concentration adjusted to 5×10^6 cells/ml total spleen cells was plated in 24- and 6-well tissue culture plates and incubated at 37°C for 2 h. After 2 h, the nonadherent (mostly lymphocytes) cells were removed and adherent cells (96% macrophages) were washed twice with RPMI 1640 and finally resuspended in RPMI 1640. The macrophages were pretreated with different concentrations of metformin (5–10 mM) for 2 h followed by stimulation with LPS (1 μ g/ml) for 48 h. The production of NO and proinflammatory cytokines (IFN- γ , TNF- α , and IL-6) and expression of iNOS and Cox2 was determined by ELISA and immunoblot, respectively (28–30).

RNA analysis and real-time PCR (quantitative PCR)

Total cellular RNA from brains of control, untreated EAE, or metformin-treated EAE or CD3-positive total T cells, as indicated, were isolated using TRIzol reagent (Invitrogen) per the manufacturer's protocol. cDNA synthesis was performed using an iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. RT-PCR was performed with SYBR Green PCR master mix (Bio-Rad) and Bio-Rad iCycler iQ PCR using the following primers: mouse IFN- γ sense, GCA TTC ATG AGT ATT GCC AAG and antisense, GGT GGA CCA CTC GGA TGA; TNF- α sense, CAT CTT CTC AAA ATT CGA GTG ACA A and antisense, TGG GAG TAG ACA AGG TAC AAC CC; IL-17 sense, CTC CAG AAG GCC CTC AGA CTA C and antisense, AGC TTT CCC TCC GCA TTG ACA CAG; IL-1 β sense, TGG AAA AGC GGT TTG TCT TC and antisense,

TAC CAG TTG GGG AAC TCT GC; IL-6 sense, GAG GAT ACC ACT CCC AAC AGA CC and antisense, AAG TGC ATC ATC GTT GTT CAT ACA; iNOS sense, GGA AGA GGA ACA ACT ACT GCT GGT and antisense, GAA CTG AGG GTA CAT GCT GGA GC; matrix metalloproteinase 9 (MMP9) sense, TGT ACA CAG GCA AGA CCG TGC TG and antisense, CTC ATG GTC CAC CTT GTT CAC CTC; RANTES sense, AGC TGC CCT CAC CAT CAT C and antisense, CTC TGG GTT GGC ACA CAC TT; ICAM sense, GCA GAG TGT ACA GCC TCT TT and antisense, CTG GTA TCC CAT CAC TTG; VCAM sense, GCA GAG TGT ACA GCC TCT TT and antisense, CTG GTA TCC CAT CAC TCG AG; E-selectin sense, ACT TCA GTG TGG TCC AAG AG and antisense,

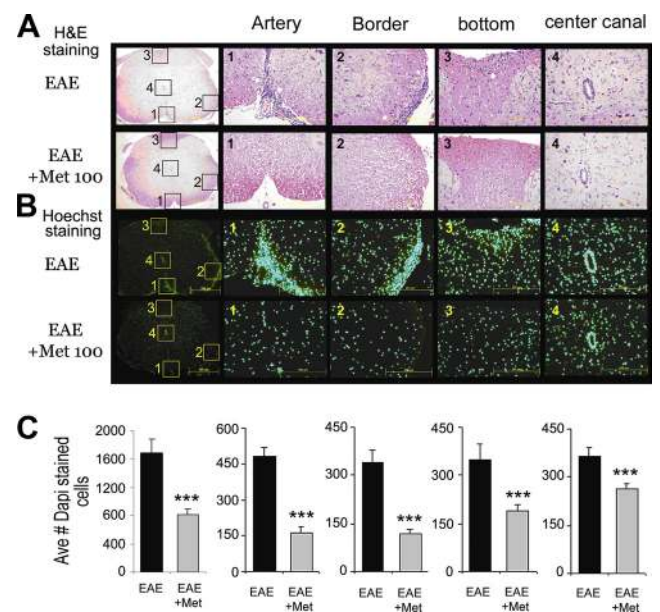


FIGURE 2. Metformin (Met) restricts infiltration of mononuclear cells into the CNS of treated EAE animals. Active EAE was induced in the C57BL/6 mice with MOG_{35–55} and one group was treated with metformin orally three times a day (100 mg/kg daily) as described in *Materials and Methods*. At the peak of disease (day 16), animals were killed and spinal cords were harvested for infiltration studies. Infiltration of mononuclear cells in spinal cords sections were examined by H&E stain (A) and by Hoechst 33342 dye (for nuclei stain) (B) in various regions of the spinal cord like artery, border, and bottom and central canal. C, Representative bar graphs of the nuclei count in different regions of spinal cords for comparison. ^{***}, $p < 0.001$ compared with metformin-treated and EAE-untreated mice ($n = 5$).

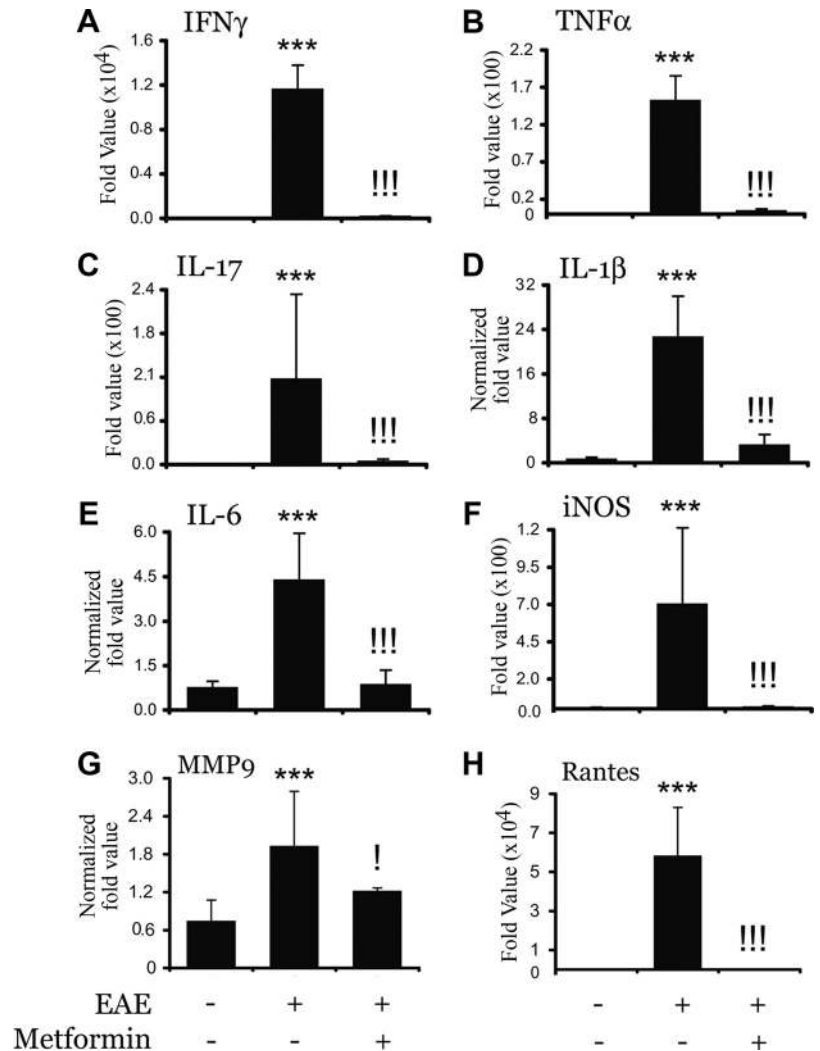


FIGURE 3. Metformin treatment inhibits the expression of inflammatory cytokines and their mediators in the CNS of EAE mice. Active EAE was induced in the C57BL/6 mice with MOG_{35–55} as described in *Materials and Methods*. The mice were euthanized in treated and untreated groups at the peak of disease (day 16). Brains were taken out and analyzed for inflammatory cytokines IFN- γ (A), TNF- α (B), IL-17 (C), IL-1 β (D), IL-6 (E), iNOS (F), MMP9 (G) and chemokine RANTES (H) by RT-PCR. Data are expressed fold change normalized with housekeeping gene *HPRT*. ***, $p < 0.001$ compared with unimmunized control mice; !!!, $p < 0.001$ and !, $p < 0.05$ treated animals compared with untreated EAE mice.

GCA CAT GAG GAC TTG TAG GT; T-bet sense, CAA CAA CCC CTT TGC CAA AG and antisense, TCC CCC AAG CAG TTG ACA GT; retinoid-related orphan receptor gamma (ROR γ t) sense, CCG CTG AGA GGG CTT CAC and antisense, TGC AGG AGT AGG CCA CAT TAC A; and *HPRT* (hypoxanthine phosphoribosyltransferase) sense, GCT TTC CCT GGT TAA GCA GTA CA and antisense, CAA ACT TGT CTG GAA TTT CAA ATC. Thermal cycling conditions were as follows: activation of DNA polymerase at 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 30 s and at 63.0°C for 30 s.

Immunoblot analysis

Primary macrophages and RAW267.4 cells were incubated in the presence or absence of various concentrations of metformin as indicated in the figure legends followed by lysis 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 mixture (Sigma-Aldrich). Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The membrane was then blocked for 1 h in 5% nonfat dry milk and TTBS (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20, pH 7.5) and incubated overnight in primary antisera (phospho-ACC, iNOS, Cox2, and β -actin) containing 5% nonfat dry milk or 5% BSA in the case of phospho-Abs. Blots were washed with TTBS (four times, 5 min each) and incubated for 45 min at room temperature with HRP-conjugated anti-rabbit or anti-mouse secondary Ab at a dilution of 1/5000. The blots were washed three times in TTBS and developed with an ECL-Plus Detection System (28–31).

Fatty acid and cholesterol biosynthesis

Treated macrophage cells were preincubated in complete medium with [2-¹⁴C]acetate (5 μ Ci/well). After 4 h or the indicated time, the cells were processed for lipid extraction and incorporation of labeled acetate in fatty acid and cholesterol was analyzed as mentioned previously (28, 31, 32).

Brain extract from various groups were processed for lipid extraction as previously described (28, 31, 32).

Statistical analysis

GraphPad Prism software was used throughout for statistical analysis. The Kruskal-Wallis test and Student *t* test were used to analyze clinical disease score. Statistics for Hoechst-stained nuclei, cell proliferation, and cytokine responses were analyzed with one-way ANOVA and Student's *t* test. Significances (*p*) between groups were determined using the Newman-Keuls test. A value of $p < 0.05$ and above was considered significant.

Results

Metformin attenuates EAE disease progression in chronic EAE mice models

To evaluate the efficacy of metformin, EAE was induced in SJL mice with myelin PLP_{139–151} peptide (100 μ g/mouse). The mice developed clinical symptoms of EAE disease from day 10 \pm 0.8 (Fig. 1A) and reached a maximum mean clinical score of 3.4 \pm 0.21 on day 17. To test the prophylactic efficacy in EAE, metformin was given orally as 20, 50, or 100 mg/kg starting from day 0 of immunization. Compared with the untreated EAE group, metformin treatment with 20 or 50 mg/kg did not provide any significant protection in disease induction (Fig. 1A). However, at the dose of 100 mg/kg, it provided promising results, especially at the chronic phase of disease ($p < 0.05$; Fig. 1B). The reason for lower efficacy of metformin at 100 mg/kg could be due to the fast clearance from the body when given once a day; therefore, we decided

to give metformin three times a day in a 100- μ l volume each time with a total dose of 100 mg/kg. Following this regimen, we observed a significant delay of onset and disease attenuation ($p < 0.01$; Fig. 1C and Table I).

Similarly, active EAE disease was induced with immunization of MOG_{35–55} peptide (100 μ g/mouse) in C57BL/6 mice and metformin was given orally or by the i.p. route three times daily. Oral administration of metformin significantly ($p < 0.05$) reduced the clinical disease progression; however, we did not observe any delay in the disease onset (Fig. 1D). Metformin-treated animals did not have 100% EAE incidence. We calculated clinical score without nonresponsive mice and observed a significant difference (3.27 ± 0.48 vehicle-treated vs 2.35 ± 0.47 metformin-treated excluded nonresponsive mice; $p < 0.05$). The animals treated by the i.p. route (100 mg/kg) exhibited both delayed onset of disease and lower clinical disease scores ($p < 0.05$) with 100% disease incidence (Fig. 1E and Table I).

The anti-inflammatory effects of metformin on the induction of EAE led us to investigate whether initiation of therapy after onset of disease can induce any therapeutic effects. To address this hypothesis, metformin was administered to ongoing active EAE in SJL mice, starting on day 14 (when all of the mice had visible clinical symptoms of disease; 2.74 ± 0.27). Metformin at 100 mg/kg given once daily significantly decreased the clinical disease severity of EAE (Fig. 1F).

Metformin limits the infiltration of immune cells to the CNS

The hallmark of EAE disease is the infiltration of inflammatory cells into the CNS, leading to tissue damage (33). Therefore, we aimed to investigate the effect of metformin on the infiltration of inflammatory cells into the CNS of untreated and treated EAE animals. As shown in Fig. 2A, EAE, *top panel*, the untreated EAE mice showed profound infiltration of immune cells in the CNS. Treatment with metformin (100 mg/kg) significantly reduced the infiltration of these inflammatory cells into the CNS (Fig. 2A, EAE plus Met 100, *lower panel*). Nuclei are shown by Hoechst staining in Fig. 2B corresponding to the infiltration of immune cells in the CNS and the total counts of these nuclei are presented as bar graphs in Fig. 2C.

Metformin treatment inhibits inflammatory cytokines in the CNS

MS lesions are associated with expression of a wide range of proinflammatory cytokines (IFN- γ , TNF- α , IL-17, IL-1 β , and IL-6) and their mediators (iNOS) (34, 35). To ascertain the status of these inflammatory cytokines in metformin-treated EAE mice, we examined the levels of mRNA in brain tissues from the metformin-treated and untreated EAE animals in chronically ill C57BL/6 mice at the peak of disease. We observed significantly higher levels of IFN- γ , TNF- α , IL-17, IL-1 β , and IL-6 in the EAE animals in the CNS, whereas metformin-treated animals showed a marked decrease in the expression of these cytokines (Fig. 3, A–E). Furthermore, the expression of the inflammatory mediator iNOS in the CNS of EAE animals was down-regulated by the metformin treatment (Fig. 3F). Also, the CNS from metformin-treated EAE animals exhibited significantly less expression of MMP9 and chemokines (RANTES or CCL5) which play a critical role in the infiltration of immune cells during EAE disease progression (Fig. 3, G and H).

Metformin inhibits cell adhesion molecule (CAM) expression in the CNS of EAE-diseased animals

The selective entry of activated leukocytes into the CNS is regulated by specific interactions at the endothelial boundary (36, 37). Under inflammatory conditions encountered in the CNS in EAE/MS, activated endothelial cells up-regulate the expression of

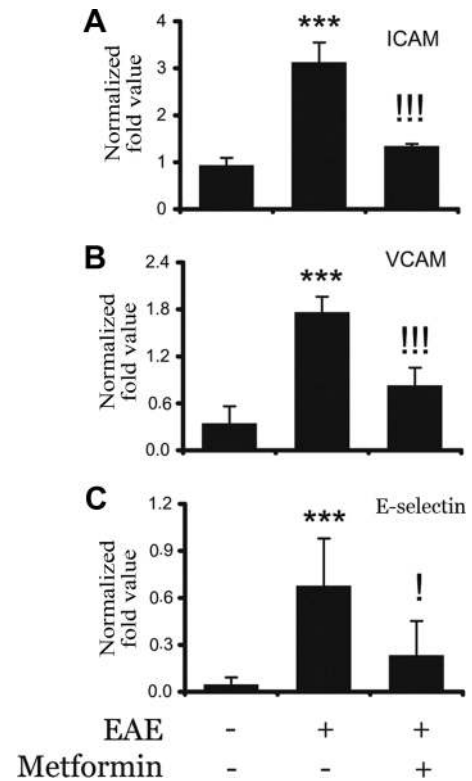


FIGURE 4. Metformin treatment inhibits the expression of CAMs in the CNS of EAE mice. Active EAE was induced in the C57BL/6 mice with MOG_{35–55} as described in *Materials and Methods*. The mice in treated and untreated groups were killed at the peak of disease and brains were taken out and analyzed for ICAM (A), VCAM (B), and E-selectin (C) by RT-PCR. ***, $p < 0.001$ compared with unimmunized control mice; !!!, $p < 0.001$ and !, $p < 0.05$ metformin-treated vs untreated EAE mice.

CAMs, including ICAM-1, VCAM-1, and E-selectin (38, 39). These CAMs interact with their ligands present on the leukocytes and aid in their infiltration. There are studies demonstrating that adhesion molecule expression precedes EAE clinical symptoms, suggesting a critical role of adhesion molecules in the initiation of CNS inflammation (38, 40). Thus, we investigated the effect of metformin on expression of CAMs (ICAM, VCAM, and E-selectin) in the CNS of EAE-treated and untreated animals. Quantitative RT-PCR analysis shows that CNS tissues from untreated EAE animals have significantly induced expression of CAMs compared with control mice (Fig. 4). Treatment with metformin markedly down-regulated the expression of these CAMs in the CNS of EAE-treated animals (Fig. 4, A–C).

Metformin restored lipid alterations in the CNS of EAE mice

One of the mechanisms of action of metformin has been shown to be via activation of AMPK (24, 26). Recently, we have investigated the status of AMPK during EAE disease progression and observed that the activity of AMPK is significantly down-regulated in the brain (41). To examine the effect of metformin on AMPK activity in brain, mice were killed at the peak of disease (day 16) and brains were processed for AMPK activity by immunoprecipitating AMPK α followed by a kinase assay using recombinant ACC as a substrate. As depicted in Fig. 5A, metformin treatment restored AMPK activity in total brain homogenates in treated EAE compared with vehicle-treated EAE mice. Decreased AMPK activity may lead to increased ACC and HMG-CoA reductase activities, which are the rate-limiting enzymes for fatty acid and cholesterol synthesis, respectively. Phosphorylation of

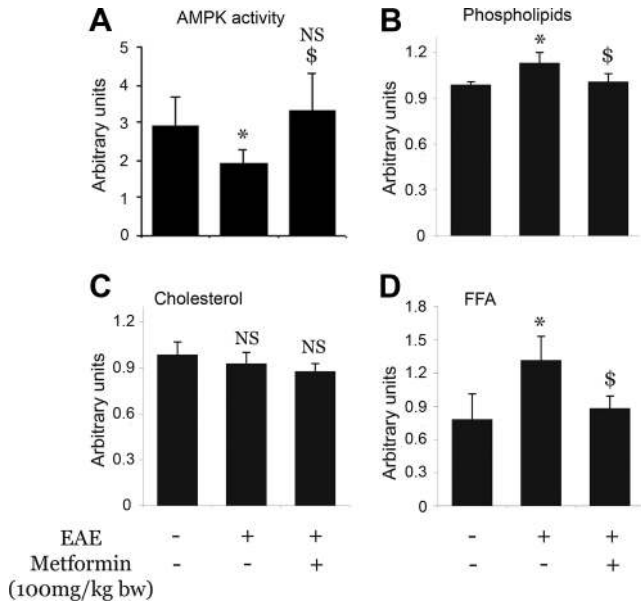


FIGURE 5. Metformin corrects AMPK activity and lipid alteration in brains of treated EAE animals. Active EAE was induced in the C57BL/6 mice with MOG₃₅₋₅₅ and treated with metformin (100 mg/kg). At day 16, total brain homogenate was processed for the AMPK assay (A) and for total lipid isolation (phospholipids (B), cholesterol (C), and FFA (D)). For the AMPK assay, 200 μ g of protein was immunoprecipitated followed by kinase assay using recombinant ACC as a substrate (A). Densitometric values were plotted as bar graphs ($n = 4$). Data are means \pm SD of the individual four values. *, $p < 0.05$ and NS compared with control mice. \$, $p < 0.05$ compared with EAE-untreated mice. Lipids were separated by TLC. Densitometry analysis of total phospholipids (PE, PI, PS, PC), cholesterol, and FFA was performed and plotted as a bar graph ($n = 4$; B–D). Data are means \pm SD of the individual four values. *, $p < 0.05$ and NS compared with control mice. \$, $p < 0.05$ compared with EAE-untreated mice.

ACC and HMG-CoA reductase by AMPK leads to inhibition of fatty acid and cholesterol biosynthesis. Therefore, we examined the effect of metformin on neutral and phospholipids in the brain during EAE from treated and untreated animals. We observed that total phospholipids and free fatty acids (FFA) were significantly increased in total brain homogenates during EAE disease without modulation of cholesterol levels which were restored by metformin treatment in treated animals (Fig. 5, B–D). These data suggest that metformin restores the activity of AMPK in brain, which gets down-regulated during disease condition, and thereby corrects lipids alteration in the CNS.

Metformin mediated its anti-inflammatory effects via activation of AMPK in macrophages

To examine the effect of metformin on AMPK activity, we treated the macrophage cell line RAW276.4 with various concentrations of metformin (1–10 mM). After 4 h of incubation, cells were processed for detection of the phospho-status of ACC using its specific Ab by immunoblot analysis. We observed that metformin induced the phosphorylation of ACC, which is a substrate of AMPK α , and indicates its intracellular activation (Fig. 6A, *i* and *ii*). Treatment with metformin also down-regulates the biosynthesis of phospholipids and neutral (cholesterol and FFA) lipids in RAW276.4 cells (Fig. 6B).

The expression of inflammatory mediators (iNOS and Cox2) has been implicated in various disease conditions. To determine the anti-inflammatory effect of metformin, we treated RAW276.4 cells with metformin (1–10 mM) for 2 h followed by stimulation with LPS (1 μ g/ml) and the protein expression of iNOS and Cox2 was

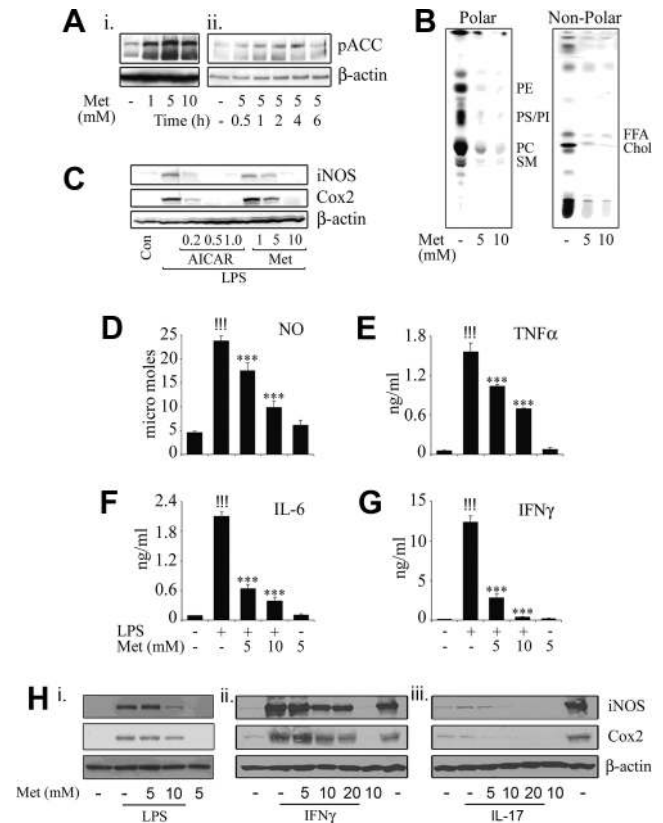
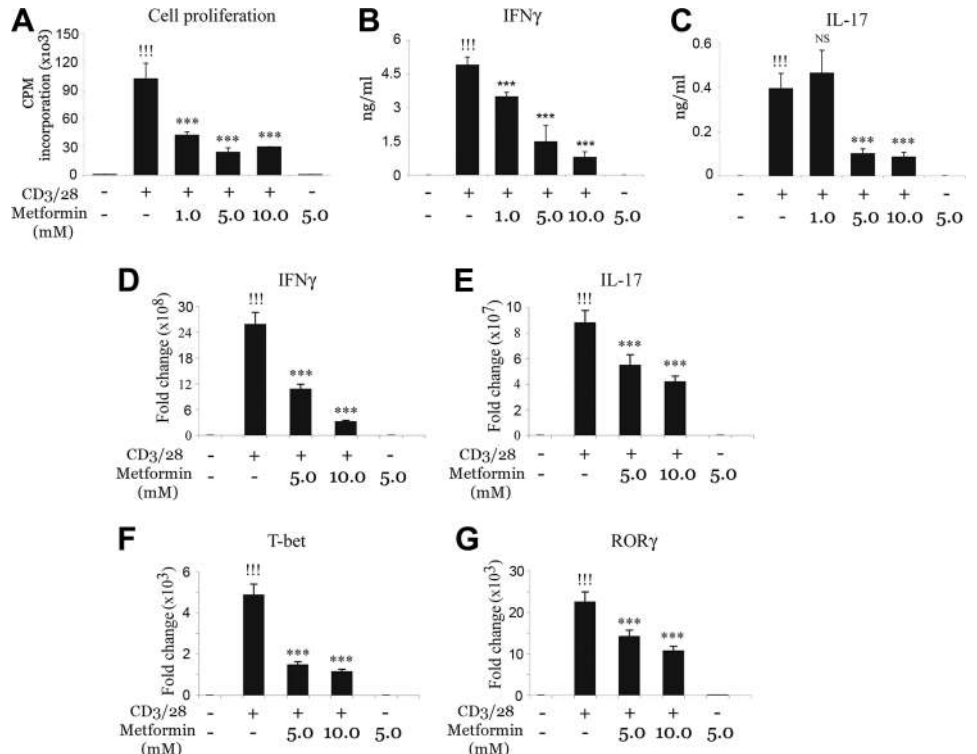


FIGURE 6. Metformin (MET) activates AMPK, thereby inhibiting lipid biosynthesis and inflammation in RAW267.4 cells and splenic macrophages. The macrophage cell line RAW267.4 was treated with various concentrations of metformin (1–10 mM) for 4 h (Ai) or 5 mM (Aii) at different time points (0.5–6 h) followed by immunoblot analysis with phospho-ACC Ab. The same blots were reprobbed with β -actin for equal loading of protein (A, *i* and *ii*). RAW267.4 cells were treated with metformin (5–10 mM) for 4 h followed by [¹⁴C]acetate pulse for an additional 4 h and lipids were isolated to measure the incorporation of labeled acetate in total phospholipids, FFA, and cholesterol by high-performance TLC as described in *Materials and Methods* (B). RAW267.4 cells were treated with metformin (1–10 mM) and AICAR (0.2–1.0 mM) for 2 h followed by LPS stimulation (1 μ g/ml) and then analyzed for iNOS, Cox2, and β -actin (C). Splenic macrophages were isolated as described in *Materials and Methods* and treated with metformin (5–10 mM) for 2 h followed by LPS stimulation (1.0 μ g/ml). After 48 h, the cell supernatant was analyzed for secretion of NO and proinflammatory cytokines (TNF- α , IL-6, and IFN- γ) (D–G). ***, $p < 0.001$ compared between cells with or without metformin treatment. Splenic macrophage cell lysates were processed for iNOS, Cox2, and β -actin by immunoblot analysis using their specific Abs as described before (Hi). RAW267.4 cells were treated with various concentrations of metformin (5–20 mM) in the presence or absence of IFN- γ (50 U/ml) or IL-17 (50 ng/ml) as indicated. After a 24-h incubation, cell lysate was processed for iNOS, Cox2, and β -actin by immunoblot analysis (H, *ii* and *iii*).

examined in cell lysates after 18 h of incubation with LPS using immunoblot analysis. Metformin treatment inhibited the LPS-induced expression of iNOS and Cox2 at 5 and 10 mM concentrations (Fig. 6C). To strengthen our hypothesis that the anti-inflammatory effects of metformin were mediated by activation of AMPK, we used another activator of AMPK, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), which has been shown previously to be anti-inflammatory (28). As shown in Fig. 6C, AICAR also inhibited the induction of iNOS and Cox2 in these cells. To further establish the anti-inflammatory activity of metformin, we used primary macrophages isolated from spleens of C57Bl/6 mice and treated them with metformin followed by LPS

FIGURE 7. Metformin inhibits naive T cell proliferation and Th1 and Th17 immune responses. The naive CD3-positive T cells were isolated from B6 mice and stimulated with anti-CD3 ϵ and anti-CD28 (1 μ g/ml) for 24–72 h in the presence or absence of different concentrations of metformin (1–10 mM; A–G). For proliferation, the [3 H]thymidine (1 μ Ci/well) was added at 48 h followed by an 18-h incubation and incorporation of thymidine was examined (A). The T cell supernatant was collected at 72 h and the level of Th1 (IFN- γ) and Th17 (IL-17) were detected by an OptEIA system (B and C). For detection of Th1 or Th17 cell-specific transcription factors T-bet or ROR γ , naive T cells were stimulated with CD3/28 as above and after 24 h of incubation, total RNA was isolated and processed for quantitative PCR to analyze the expression of IFN- γ , IL17, T-bet, and ROR γ (D–G). Data are representative of two independent experiments. !!!, $p < 0.001$ as compared between unstimulated and CD3/28 stimulated and ***, $p < 0.001$ compared between CD3/28 stimulated and metformin treated.



(1 μ g/ml) stimulation as described above. LPS induced production of NO and proinflammatory cytokines (TNF- α , IL6, and IFN- γ) from macrophages that was significantly attenuated by metformin treatment in a dose-dependent manner (Fig. 6, D–G). Metformin also inhibited the expression of LPS (1 μ g/ml)-, IFN- γ (50 U/ml)-, or IL-17 (50 ng/ml)-induced iNOS and Cox2 in macrophages (Fig. 6H, *i-iii*), suggesting that the anti-inflammatory action of metformin is not just confined to LPS signaling but also inhibits Th1- and Th17-mediated responses.

Metformin inhibits proliferation and production of proinflammatory cytokines in naive CD3-positive T cells

Next, we examined the effect of metformin on naive CD3-positive T cells. For this, naive T cells were isolated from normal

B6 mice and treated with various concentrations of metformin (1–10 mM) for 2 h followed by stimulation with plate-bound CD3/28 Abs. As depicted in Fig. 7A, metformin inhibits the T cell proliferation and production of proinflammatory cytokines IFN- γ (Th1) and IL-17 (Th17) (Fig. 7, B and C). These observations are further supported by the quantitative analysis of mRNA for Th1 or Th17 cytokines by RT-PCR. As shown in Fig. 7, D–G, metformin treatment inhibited expression of CD3/28-induced expression of IFN- γ and IL-17 along with their specific transcription factors T-bet (Th1) and ROR γ t (Th17). These data suggest that metformin inhibits T cell proliferation, proinflammatory cytokines, and their specific transcription factors which may be one of the mechanisms through which metformin mediates its protection in EAE animals.

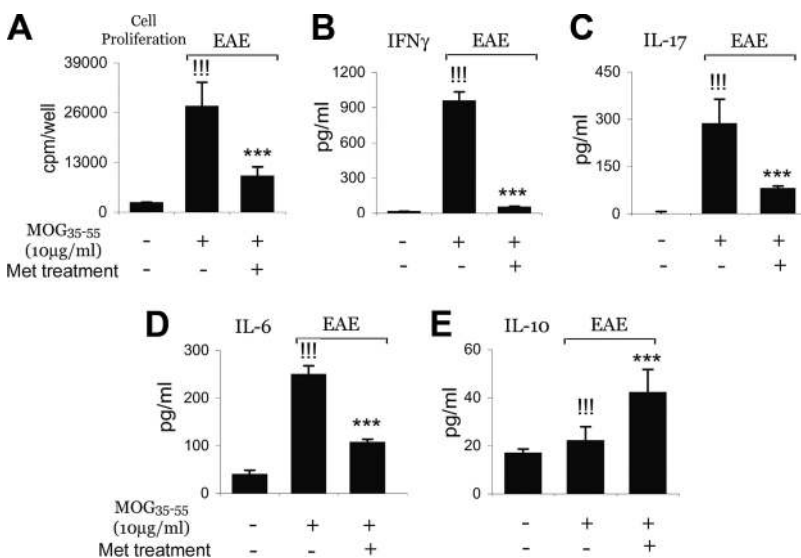


FIGURE 8. Metformin (Met) inhibits Ag-specific T cell immune responses. Active EAE was induced in C57BL/6 mice with MOG₃₅₋₅₅ as described in *Materials and Methods*. The mice in treated and untreated groups were killed at the peak of EAE disease and spleen cells were isolated and stimulated ex vivo with MOG₃₅₋₅₅ (10 μ g/ml) peptide for 48 h and then [3 H]thymidine was added to the cells and incubated for an additional 18 h and incorporation of thymidine was measured (A). The culture supernatants of spleen cells were collected at 72 h and analyzed for inflammatory IFN- γ (Th1), IL-17 (Th17), IL-6, and anti-inflammatory IL-10 cytokines by an OptEIA system (B–E). Data are representative of two different experiments. ***, $p < 0.001$ compared between spleen cells from untreated EAE and treated mice.

Metformin inhibits recall responses and production of Th1 and Th17 cytokines in MOG-primed T cells

To examine the effect of metformin on Ag-specific immune responses in T cells, the mice were killed at the peak of EAE disease in untreated and treated groups. The spleen cells were stimulated *ex vivo* with MOG_{35–55} peptide (10 μ g/ml) and recall responses were measured by tritiated [³H]thymidine uptake and proinflammatory cytokines profile as described in *Materials and Methods*. The spleen cells from metformin-treated mice showed significantly reduced recall responses compared with untreated EAE animals when stimulated *ex vivo* with MOG peptide (Fig. 8A). To define whether the anti-inflammatory effects of metformin on EAE disease are associated with modulation of inflammatory cytokines, we analyzed the production of pro- and anti-inflammatory cytokines (IFN- γ , IL-17, IL-6, or IL-10) in the supernatant of spleen cells of metformin-treated and untreated EAE mice after *ex vivo* stimulation with MOG_{35–55} peptide. As shown in Fig. 8, B–D, metformin reduced the production of inflammatory cytokines (IFN- γ , IL-17, and IL-6); however, the production of IL-10 (anti-inflammatory) was significantly up-regulated in spleen cells of treated mice (Fig. 8E). These data suggest that metformin possibly suppressed the encephalitogenic nature of T cells inhibiting the recall responses and production of inflammatory cytokines.

Discussion

Metformin was discovered in the 1920s in a search for guanidine-containing compounds with anti-diabetic activities and was introduced clinically in Europe in the 1950s. Since then it has a long history of human consumption; however, it was approved for diabetes in the United States in 1995 (42). Metformin is often the first drug used for newly diagnosed type 2 diabetic patients and in 2006 it accounted for 37% of the noninsulin diabetes prescriptions in the United States. A major action of metformin is suppression of hepatic glucose production (26). A minimal risk of hypoglycemia has been reported; however, it has some gastrointestinal side effects and very rarely causes lactic acidosis (43).

The present study is designed to investigate the prophylactic efficacy of metformin on the progression of an autoimmune inflammatory disease mediated by the infiltration of activated macrophages and T cells into the CNS. In this study, we used two different rodent models which partially mimic the relapsing-remitting (SJL) and chronic progressive (C57BL/6) form of MS. We observed that metformin was very effective in both models of EAE disease and exhibited anti-inflammatory effects on the CNS, macrophages, and T cells. Metformin exerted its immunosuppressive effect by inhibiting the expression of proinflammatory mediators (IFN- γ , TNF- α , IL-1 β , IL-6, IL-17, iNOS, MMP9, and RANTES) and infiltration of immune cells, which was blocked by reducing the expression of CAMs (ICAM, VCAM, and E-selectin) on vascular cells. The observed inhibition of inflammatory cytokines in the CNS suggests that cells responsible for the production of these cytokines may be the direct target of metformin. Therefore, to examine this possibility, we used macrophages *in vitro* with inflammation induced by endotoxin (LPS). Metformin treatment blocked the production of NO and proinflammatory cytokines and expression of iNOS and Cox2 in macrophages, further supporting its anti-inflammatory action. These observations are in agreement with other reports where the anti-inflammatory effects of metformin have been documented (15, 44).

Metformin mediates its action by activating AMPK, which is a master sensor and regulator of energy homeostasis in mammalian cells and also a therapeutic target for metabolic disorders (45). AMPK activation affects many pathways, generally causing con-

servation and generation of ATP (46). Our data support this notion because metformin induced activation of AMPK in the brain of treated EAE mice and in macrophages and inhibited the expression of inflammatory cytokines and their mediators. It has been shown that another AMPK activator (AICAR) also inhibited the production of proinflammatory mediators (TNF- α , IL-1 β , IL-6, and NO) in brain glial cells and in macrophages via inhibition of NF- κ B and the C/EBP pathways (28) and attenuated LPS-induced inflammation in rats (28). Under recall response, we observed that metformin-treated mice exhibited inhibition in the production of proinflammatory cytokines and induction of IL-10 compared with vehicle-treated mice. A recent study suggested that AMPK acts as a potent counterregulator of inflammatory signaling pathways in macrophages since transfection of constitutively active AMPK in macrophages inhibited proinflammatory cytokines (TNF- α and IL-6) and, on the other hand, increased the production of an anti-inflammatory cytokine (IL-10) (47). The ability of AMPK to be rapidly activated by anti-inflammatory stimuli, or rapidly inactivated by proinflammatory stimuli, combined with the downstream consequences of AMPK activity that we observed herein, implicates AMPK as a “master regulator” of macrophage functional polarization. This study supports our finding published in 2004 (28), which indicates AMPK as an anti-inflammatory molecule.

Earlier it has been shown that AICAR attenuated EAE disease progression in mice by switching Th1 to Th2-type immune responses, suggesting that these molecules exert their anti-inflammatory effects on T cells (29). Metformin showed similar properties in T cells as it inhibited the recall responses and the production of inflammatory cytokines (IFN- γ , IL-17, and IL-6) in encephalitogenic T cells from treated EAE mice with increased production of IL-10. The role of IFN- γ (Th1)- or IL-17 (Th17)-producing cells is controversial in EAE disease since both of the cells have been shown to induce the disease (13, 48). Recently, it has been reported that Th1 cells induce EAE disease with the help of CD11b-positive macrophages; however, Th17 cells need Ly6G-positive neutrophils to exert similar effects (13, 48–51). Metformin inhibits the production of both Th1 and IL-17 cytokines in naive T cells simulated with CD3/28 along with their associated transcription factors T-bet and ROR γ , which primarily regulate the generation of these cytokines in Th1 or Th17 cells. Similarly, we observed inhibition of Th1 (IFN- γ) or Th17 (IL-17) in the CNS of metformin-treated EAE animals, suggesting the possible mechanism of its protective action.

Metformin inhibited the expression of adhesion molecules like ICAM, VCAM, and E-selectin and thereby reduced the infiltration of mononuclear cells into the CNS, as CAMs are directly involved in the transmigration of activated macrophages across the blood-brain barrier. These observations are in agreement with data published from two different clinical trials conducted in patients with impaired glucose tolerance and diabetes where metformin administration reduced the levels of soluble ICAM, VCAM, and E-selectin (52, 53). Inhibition of RANTES or CCL5 with metformin suggests that it limits the migration of T cells as these chemokines attract the activated T cells to the site of inflammation (54). Moreover, the mechanism of metformin is proposed to be via down-regulation of the NF- κ B pathway in endothelial cells (44), which is a prerequisite transcription factor for expression of CAMs (ICAM, VCAM, and E-selectin) (55–57). The mechanism of action of metformin seems to be similar to AICAR (58) since metformin also inhibits degradation of I κ B α by inhibiting I κ B kinase activity that results in suppression of cytokine-induced NF- κ B activation in HUVECs (44).

There is no cure available for MS; however, a number of medications can be used to treat the disease symptomatically such as

corticosteroids, IFN- β -1B (Betaseron), IFN- β (Avonex), and copolymer 1. Our study provides evidence that metformin has anti-inflammatory properties in vivo and in vitro and, most interestingly, it has good bioavailability and can be given orally which may reduce the cost of therapy, frequent doctor visits, and painful administration of drugs. Our work shows great implications for clinical research and offers the possibility of developing effective therapy alone or in combination with existing therapies for MS.

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Disclosures

The authors have no financial conflict of interest.

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Corrections

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The fourth author's name was inadvertently excluded from the article. The corrected author and affiliation lines are shown below.

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The seventh author's name was omitted from the article. The corrected author and affiliation lines are shown below.

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