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Metformin induces up-regulation of blood–brain barrier functions by activating AMP-activated protein kinase in rat brain microvascular endothelial cells

Fuyuko Takata [a,b], Shinya Dohgu [a], Junichi Matsumoto [a], Takashi Machida [a], Shuji Kaneshima [a], Mai Matsuo [a], Shinya Sakaguchi [a], Yuki Takeshige [a], Atsushi Yamauchi [a,c], Yasufumi Kataoka [a,b,*]

[a] Department of Pharmaceutical Care and Health Sciences, Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka, Japan
[b] BBB Laboratory, PharmaCo-Cell Co., Ltd., Nagasaki, Japan
[c] Academic, Industrial and Governmental Institute for Aging and Brain Sciences, Fukuoka University, Fukuoka, Japan

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ABSTRACT

Blood–brain barrier (BBB) disruption occurs frequently in CNS diseases and injuries. Few drugs have been developed as therapeutic candidates for facilitating BBB functions. Here, we examined whether metformin up-regulates BBB functions using rat brain microvascular endothelial cells (RBECs). Metformin, concentration- and time-dependently increased transendothelial electrical resistance of RBEC monolayers, and decreased RBEC permeability to sodium fluorescein and Evans blue albumin. These effects of metformin were blocked by compound C, an inhibitor of AMP-activated protein kinase (AMPK). AMPK stimulation with an AMPK activator, AICAR, enhanced BBB functions. These findings indicate that metformin induces up-regulation of BBB functions via AMPK activation.

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1. Introduction

The blood–brain barrier (BBB) is responsible for the proper functioning of the central nervous system (CNS) by regulating cellular and molecular trafficking between the blood and the nervous system. A continuous layer of cerebral endothelial cells attached to each other by tight junctions (TJs) constitutes the morphological basis of the BBB [1,2]. Disruption of the BBB has been observed in many CNS diseases and injurious events, such as traumatic injury, stroke, edema, and inflammatory responses [3]. Extended BBB disruption induces increased brain volume due to facilitation in the movement of water and plasma proteins into the CNS, resulting in vasogenic edema. These sequential events produce disruption of myelin sheaths, hyper-reactivity of astrocytes, and neuronal dysfunction [4]. Thus, protective manipulation of BBB disruption is required for treating a wide array of neurological disorders. However, only a few drugs, such as cilostazol and minocycline, have been shown to be therapeutic candidates for the facilitation of BBB functions.

Metformin is a drug widely used for the treatment of Type 2 diabetes mellitus. It has been well documented that metformin activates AMP-activated protein kinase (AMPK). AMPK activation is responsible for a number of actions of metformin related to its glucose-lowering effects, including a decrease in glucose production in hepatocytes and an increase in glucose uptake in skeletal muscle [5,6]. Chronic metformin treatment in clinical populations is associated with lowered risk of stroke and a reduction in cardiovascular mortality by 24% [7,8]. Metformin was associated with a greater protective effect against strokes compared with glibenclamide or insulin, although HbA1C values in patients treated with metformin were almost identical to those in patients treated with glibenclamide or insulin [8]. This evidence suggested that metformin has beneficial effects other than glucose-lowering effects. Moreover, metformin inhibited tumor necrosis factor-α-induced interleukin-6 production in human umbilical vein endothelial cells, suggesting that metformin has anti-inflammatory effects on endothelial cells in peripheral blood vessels [9]. However, effects of metformin on the functions of brain microvascular endothelial cells, including BBB functions, have not been investigated. Here,

Abbreviations: BBB, blood–brain barrier; RBEC, rat brain microvascular endothelial cell; AMPK, AMP-activated protein kinase; CNS, central nervous system; TJ, tight junction; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; DMEM/F12, DMEM/Ham's nutrient mixture F-12 medium; PDS, plasma-derived serum; TEER, transendothelial electrical resistance; Na-F, sodium fluorescein; EBA, Evans blue albumin.

* Corresponding author. Address: Department of Pharmaceutical Care and Health Sciences, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan. Fax: +81 92 862 2699.
E-mail address: ykataoka@fukuoka-u.ac.jp (Y. Kataoka).

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we show that metformin induces up-regulation of BBB functions via activation of AMPK.

2. Materials and methods

2.1. Reagents

Metformin, the AMPK activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), DMEM/Ham's nutrient mixture F-12 medium (DMEM/F12), and an antibody against β-actin were purchased from Sigma (St. Louis, MO, USA). Compound C was purchased from TOCRIS bioscience (Ellisville, MO, USA). Plasma-derived serum (PDS) was purchased from Animal Technologies Inc. (Tyler, TX, USA). Antibodies against phospho-AMPKα (Thr-172) and AMPKα were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against ZO-1 and claudin-5 were obtained from Invitrogen (Carlsbad, MA, USA). Antibodies against occludin were obtained from BD Transduction Laboratories (Franklin Lakes, NJ, USA).

2.2. Cell cultures

Primary cultures of rat brain capillary endothelial cells (RBECs) were prepared from 3 week-old Wistar rats, as previously described [10]. All procedures involving experimental animals adhered to the law (No. 105) and notification (No. 6) of the Japanese Government, and were approved by the Laboratory Animal Care and Use Committee of Fukuoka University.

2.3. In vitro evaluation of barrier functions

Confluent RBECs were seeded on the insides of inserts (Transwell™ inserts, diameter 6.5 mm, 0.4 μm pore size; Corning, Midland, MI, USA) coated with collagen type 4 and fibronectin, and then placed into 24-well plates. Metformin was dissolved in RBEC medium I [DMEM/F12 supplemented with 10% PDS, basic fibroblast growth factor (1.5 ng/mL, Roche), heparin (100 μg/mL, Sigma), insulin (5 μg/mL), transferrin (5 μg/mL), sodium selenite (5 ng/mL; insulin-transferrin-sodium selenite media supplement, Sigma) and gentamicin (50 μg/mL)] without PDS. AICAR and compound C were dissolved in sterile water. Each original solution was then diluted in RBEC medium I with 550 nM hydrocortisone and without PDS (treatment medium). RBECs were then exposed for 24 h to each compound into the inside and outside of the insert (luminal and abluminal side). In parallel, RBECs were exposed to the treatment medium containing the corresponding amount of sterile water as vehicle.

Transendothelial electrical resistance (TEER), reflecting the flux of ions through cell layers in culture conditions, was measured by an epithelial-volt-ohm meter and Endohm-6 chamber electrodes (World Precision Instruments, Sarasota, FL, USA). The TEER values of the cell-free Transwell inserts were subtracted from the measured TEER values of the RBECs, shown as $\Omega \times cm^2$.

The flux of sodium fluorescein (Na-F) and Evans blue albumin (EBA) across the endothelial cell layers of the *in vitro* BBB models was determined as previously described [10].

2.4. Western blot analysis

Cells were scraped and lysed in lysis buffer (10 mM Tris–HCl, pH 6.8, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM Na_3VO_4, 50 mM NaF, 20 mM sodium pyrophosphate decahydrate and 50 μg/mL phenylmethylsulfonyl fluoride) containing 1% phosphatase inhibitor cocktail 1 (Sigma), 1% phosphatase inhibitor cocktail 2 (Sigma), and 1% protease inhibitor cocktail (Sigma). The total protein concentration in the cell lysates was determined using a BCA Protein assay kit (Pierce, Rockford, IL, USA). Proteins from each sample were electrophoretically separated on 5–20% SDS-polyacrylamide gels (Bio Craft Co., Ltd., Tokyo, Japan), and then immunoblotted using rabbit polyclonal anti-AMPKα, anti-phospho-AMPKα (Thr172), anti-ZO-1, and mouse monoclonal anti-occludin, anti-claudin-5, and β-actin. The band images were digitally captured with a FluorChem SP imaging system (Alpha Innotech, San Leandro, CA, USA) and the band intensities were quantified using a public domain software Image J (NIH Image, Bethesda, MD, USA).

2.5. Measurement of intracellular cAMP levels

cAMP measurements for cell lysates in each well were performed using the cAMP Biotrak enzyme immunoassay system (GE Healthcare, Milwaukee, WI, USA) according to the manufacturer's instructions, as previously described [10].

2.6. Statistical analysis

Results are shown as means ± SEM. Statistical analysis was performed using Student's *t* test to compare two groups. The statistical significance of differences between groups was assessed using one-way analysis of variance for factorial comparisons, and by the Tukey–Kramer's test for multiple comparisons. Differences were considered significant at $P < 0.05$, and all statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA).

3. Results

3.1. Effects of metformin on BBB integrity

Metformin at a concentration of 1 mM significantly increased TEER at 12 and 24 h after addition (110.1 ± 3.5 and 126.7 ± 5.1% of vehicle, respectively), but not at 1–6 h after (Fig. 1A). Thus, an exposure period of 24 h was used in the following experiments. RBECs exposed to metformin (0.1, 0.5, and 1 mM) for 24 h showed a dose-dependent increase in TEER (Fig. 1B). The permeability coefficients of Na-F and EBA for RBECs were dose-dependently decreased by 37% and 47%, respectively, after exposure to metformin (Fig. 1C and D). Metformin increased, although not significantly, the expression levels of ZO-1 and occludin in RBECs by 31% and 34%, respectively (Fig. 1E, F, G, and H).

3.2. Involvement of AMPK in metformin-enhanced BBB functions

To determine whether AMPK is involved in metformin-induced up-regulation of BBB functions, we pretreated RBECs with an AMPK inhibitor (compound C) for 15 min prior to 24 h exposure to metformin. Compound C dose-dependently blocked metformin-induced increases in TEER and decreases in Na-F and EBA permeability; however, these changes were reversed by administration of 1 μM of compound C by 77.2%, 50.3%, and 76.7%, respectively (Fig. 2A–C). To determine whether AMPK activation in RBECs induced up-regulation of barrier functions, we tested the effects of AICAR on TEER and permeability to Na-F and EBA in RBECs after a 24 h exposure to AICAR (0.1–1 mM). AICAR dose-dependently and significantly increased TEER of RBECs (0.5 and 1 mM: 121.2 ± 4.1 and 124.7 ± 5.6% of vehicle, respectively) (Fig. 2D). The permeability coefficients of Na-F and EBA for RBECs were dose-dependently reduced by AICAR (0.1–1 mM) to 101.4–74.2% and 100.6–82.0%, respectively (Fig. 2E and F). Metformin (1 mM) time-dependently increased the phosphorylation levels of AMPK in RBECs (Fig. 2G).

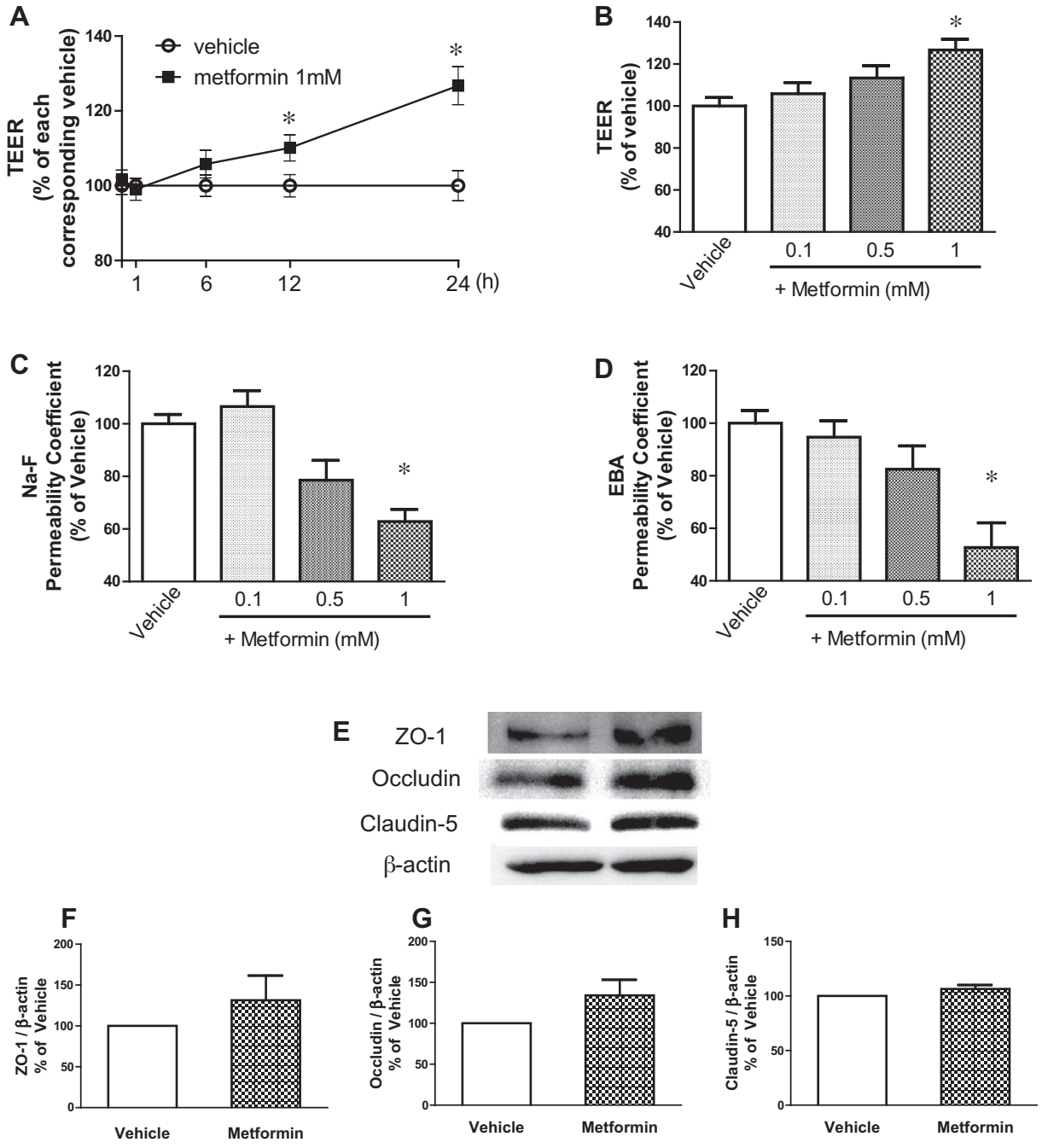

Fig. 1. Metformin induced up-regulation of barrier function in an RBEC monolayer. Time-course of changes in TEER of an RBEC monolayer treated with metformin 1 mM (A). Results are expressed as % of vehicle. Values are means ± SEM ($n = 12$). $^{*}P < 0.05$, significantly different from each corresponding vehicle-treated group. Effects of metformin on the TEER (B), RBEC permeability to Na-F (C), and EBA (D) in an RBEC monolayer. RBECs were treated with metformin (0.1–1 mM) for 24 h. Results are expressed as % of vehicle ($76.0 \pm 8.05\ \Omega\,cm^2$ (B); $8.29 \pm 0.63 \times 10^{-5}$ cm/min (C); $3.23 \pm 0.84 \times 10^{-5}$ cm/min (D)). Values are means ± SEM ($n = 10$–12). $^{*}P < 0.05$, significantly different from vehicle-treated group. Representative western blots show the expression of ZO-1, occludin, and claudin-5 in RBECs treated with 1 mM of metformin for 24 h (E). β-Actin was used as a loading control. Quantitative analysis of the expression levels of ZO-1 (F), occludin (G), and claudin-5 (H) in RBECs. Values are means ± SEM ($n = 5$–6).

3.3. Effect of metformin on intracellular cAMP levels

Next, we tested the effect of metformin on intracellular cAMP levels. Exposure of RBECs to metformin (1 mM) for 24 h produced no effect on intracellular cAMP levels (89.5 ± 4.6% of vehicle) (Fig. 2H). The effect of forskolin, an adenylyl cyclase activator, on intracellular cAMP levels was used as a positive control for metformin. Forskolin (10 μM) increased intracellular cAMP levels in RBECs to 167.7 ± 14.9% of vehicle (Fig. 2H).

4. Discussion

In the present study, we provided evidence that metformin induces up-regulation of BBB functions via activation of AMPK *in-vitro*. Two evaluated parameters, TEER and the permeability of Na-F were used to detect the barrier integrity of TJs [2]. Metformin produced a significant increase in TEER and a decrease in Na-F permeability (Fig. 1). These findings suggest that metformin tightens TJ barriers. The expression of TJ-associated proteins, ZO-1 and

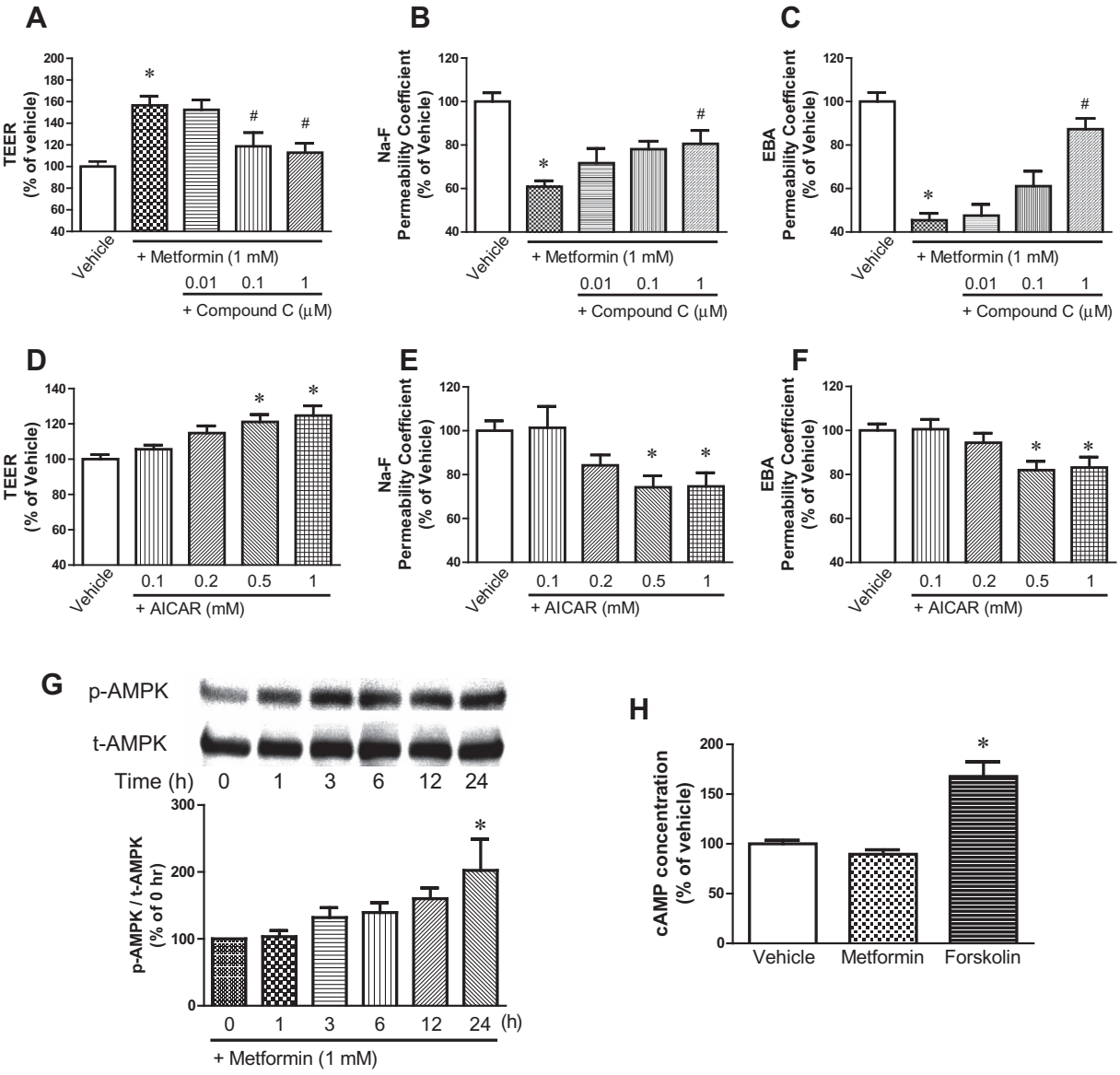

Fig. 2. Metformin up-regulated barrier function of an RBEC monolayer by activating AMPK. Effects of compound C on metformin-induced increases in TEER (A), and decreases in permeability to Na-F (B) and EBA (C) in an RBEC monolayer. Results are expressed as % of vehicle ($43.2 \pm 3.53\ \Omega\ cm^2$ (A); $1.20 \pm 0.13 \times 10^{-4}$ cm/min (B); $6.04 \pm 0.27 \times 10^{-5}$ cm/min (C)). Values are means ± SEM ($n = 7–8$). $^{*}P < 0.05$, significantly different from vehicle-treated group. $^{\#}P < 0.05$, significant difference between metformin-treated groups and metformin plus compound C-treated groups. Effects of AICAR on TEER (D), and permeability to Na-F (E) and EBA (F) in an RBEC monolayer. Results are expressed as % of vehicle ($84.8 \pm 5.06\ \Omega\ cm^2$ (D); $5.64 \pm 0.32 \times 10^{-5}$ cm/min (E); $7.75 \pm 1.08 \times 10^{-5}$ cm/min (F)). Values are means ± SEM ($n = 6–12$). $^{*}P < 0.05$, significantly different from vehicle-treated group. Time-course of metformin-induced phosphorylation of AMPK in RBECs (G). Representative western blots (top panel) and densitometric analysis (bottom panel) of phosphorylation levels of AMPK in RBECs. RBECs were treated with metformin (1 mM) for the indicated time periods. Results are expressed as the % of 0 h. Values are means ± SEM ($n = 3$). $^{*}P < 0.05$, significant difference from 0 h group. Changes in intracellular cAMP levels in RBECs exposed to metformin (1 mM) or forskolin (1 μM) for 24 h (H). Results are expressed as % of vehicle (109.7 ± 6.62 fmol/well). Values are means ± SEM ($n = 8–12$). $^{*}P < 0.05$ significantly different from control.

occludin, in RBECs tended to be increased by metformin. It is, therefore, conceivable that metformin can enhance the barrier function of brain endothelial cells with a slight or moderate, but not marked increase in the expression of TJ proteins.

Next, we determined the mechanisms by which metformin induced up-regulation of the barrier functions of RBECs. AMPK, a serine/threonine kinase, is a target for metformin in hepatocytes and skeletal muscle. Hence, we examined whether AMPK mediates metformin-enhanced barrier functions in RBECs. Our results showed that metformin activated AMPK in RBECs and that pharmacological inhibition of AMPK with compound C inhibited metformin-induced up-regulation of BBB functions (Fig. 2). These results provide evidence that metformin enhances barrier functions of RBECs by activating AMPK. In addition, AMPK stimulation with AICAR in RBECs induced intensified BBB functions, similar to metformin (Fig. 2). AICAR increased the expression levels of TJ proteins (ZO-1, occludin, and claudin-5) [11]. This difference between metformin and AICAR in the expression levels of TJ proteins should be addressed in future studies. Our present results suggest that AMPK activation is involved in the fundamental mechanisms underlying the maintenance of brain endothelial barrier functions.

AMPK activation promotes ATP synthesis, leading to increased intracellular cAMP levels [12]. Conversely, elevated levels of cAMP produced facilitation of the AMPK activity as well as BBB functions [13]. Metformin produced no effect on intracellular cAMP levels in

RBECs (Fig. 2). Thus, it is unlikely that cAMP production in RBECs is involved in mediating metformin-induced BBB up-regulation and AMPK activation.

BBB breakdown in stroke allows the entry of plasma proteins and immune molecules, leading to edema, neural dysfunction, and ultimately neural degeneration [3]. Our EBA data demonstrated that metformin reduces albumin permeability in RBECs through AMPK activation (Fig. 1). This finding suggests that metformin prevents plasma albumin from penetrating into the brain parenchyma. In fact, metformin reduced infarct volume in experimental stroke [14,15]. Thus, metformin-enhanced BBB functions may contribute, in part, to the protective effect of metformin on stroke.

In conclusion, we demonstrated that metformin facilitates barrier properties of the BBB by activating AMPK. These results suggest that metformin may have considerable therapeutic benefits to prevent the formation and/or development of BBB breakdown. This pharmacological intervention may be useful to block the pathological path between BBB dysfunction and various neurodegenerative diseases.

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