The Effects of Novel AMPK Activator on Human Vascular Endothelial Cells

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Abstract—Adenosine 5'-monophosphate-activated protein kinase (AMPK) is a key regulator of cellular energy homeostasis and involved in modulating several important cellular mechanism including inflammation. Here we demonstrated that a novel AMPK activator, ENERGI-F704, dose-dependently activated AMPK in human umbilical vein endothelial cells (HUVECs). The pharmacological AMPK inhibitor, compound C, abolished the phosphorylation of threonine 172 residue on AMPK induced by ENERGI-F704. Importantly, ENERGI-F704 treatment did not affect HUVECs viability at the tested concentration. It was also observed that ENERGI-F704 significantly reduced the expression of inflammation cytokine, IL-6, in the high glucose cultured HUVECs during the long culture period. Furthermore, ENERGI-F704 suppressed the high glucose induced monocyte adhesion to HUVECs. Collectively, our data demonstrated that ENERGI-F704 is of use in the application of attenuating the high glucose induced chronic inflammation in endothelial cells.

Index Terms—AMPK, monocyte adhesion, AMPK activator

I. INTRODUCTION

Adenosine 5'-monophosphate-activated protein kinase (AMPK) is known as the enzyme response for energy homeostasis within cells [1]. AMPK is activated by phosphorylation on the conserved threonine 172 (thr172) residue of α subunit; catalyzed by kinases such as LKB1 and Ca²⁺/Calmodulin dependent kinase kinase [2] and [3]. High cellular AMP/ATP ratio may activate AMPK to suppress ATP consumption and promote ATP generation reactions [4]. Upon activation, AMPK activates catabolic pathways such as glycolysis, fatty acid oxidation, glucose uptake; inhibits anabolic pathways such as fatty acid/cholesterol synthesis, protein synthesis, gluconeogenesis [5]. In addition to the regulation of energy homeostasis, recent studies also revealed that AMPK might involve in modulating cellular stresses, such as inflammatory responses. Pharmacological AMPK activator, 5-aminomidazole-4-carboxamide ribose (AICAR) has been demonstrated to suppress inflammation of macrophages, glia cells, microglial cells and neutrophils [6]-[8]. The anti-inflammatory function of AMPK activators in LPS-induced lung injury or autoimmune disease was also observed in animals [7]-[9]. Herein, we demonstrated a novel AMPK activator which attenuated high glucose induced inflammatory response in vascular endothelial cells.

Vascular inflammation is a common pathophysiologic response in diseases including atherosclerosis, hypertension, diabetes and myocardial infarction [10] and [11]. One of the hallmarks of vascular inflammation is the monocytes and blood vessel interaction mediated by adhesion molecules. Stimuli such as reactive oxygen species (ROS) and cytokines induce expression of adhesion molecules including intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and endothelial selectin (E-selectin) in endothelial cells [12]-[14]. This up-regulation of adhesion molecules results in adhering of leukocytes to vascular endothelium and leads to endothelial dysfunction and tissue injury [15]. In the presence high glucose, endothelial cells also demonstrate similar inflammation phenotypes as aforementioned [16]. It has also been reported that high glucose condition induce the secretion of inflammatory cytokine, IL-6, in endothelia cells [17].

In the present study, we investigated anti-vascular inflammatory effects of a novel AMPK activator, ENERGI-F704, on human umbilical vein endothelial cells (HUVECs). We investigated the effect of ENERGI-F704 on AMPK activation and cell viability. It was found that in HUVECs the high glucose induced expression of IL-6 as well as the monocyte adhesion can be decreased by ENERGI-F704 upon AMPK activation.

II. MATERIALS AND METHOD

A. Reagents

All reagents were purchased from Sigma (St. Louis, MO, USA) except where otherwise specified. Dulbecco’s modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad,
ENERGI-F704 is a proprietary compound provided from Energenesis Biomedical Co. Ltd. Cell culture

Human umbilical vein endothelial cells (HUVEC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 5.56 mM glucose, 4 mM L-glutamine, 2 mM sodium pyruvate and 1% penicillin/streptomycin (Invitrogen GIBCO BRL, Carlsbad, CA, USA) at 37 °C under 5% CO₂ and 1% penicillin/streptomycin (Invitrogen GIBCO BRL, Carlsbad, CA, USA). ENERGI-F704 is a proprietary compound used in this experiment were between passage 3 and 8.

B. Cell Viability Assay

Cell viability was analyzed using XTT assay. Briefly, HUVECs were seeded in 96-well plates at 4x 10⁵ cells/ml for 18 h and treated with various concentration of ENERGI-F704 or vehicle. After 24 h incubation, 50 μl of XTT (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide) reagent was added. The plates were then incubated for 4 h at 37°C. The absorbance at 490 nm and 690 nm were measure using microplate reader.

C. Western Blot Assays

HUVECs were collected at indicated time point and were lysed using cell lysis buffer [10 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton-X 100, 1×Protease inhibitor cocktail (Roche, Basel, Switzerland), 1× PhosSTOP phosphatase inhibitor cocktail (Roche, Basel, Switzerland)] for 30 min at 4°C, and then centrifuged at 15,000×g for 1 min. The equal amount of cell lysates were resolved by 10% SDS-PAGE and then transfer to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% bovine serum albumin (BSA) in PBS and incubated with an anti-phospho-AMPK (Thr172) antibody (1:2000, Cell signaling) or an anti-actin antibody (1:5,000; Cell signaling) at 4° C overnight followed by the corresponding secondary antibody for 1 h at room temperature (RT). Immunoreactive bands were detected by chemiluminescence (VisGlow™, Visual Protein, Taipei, TW) and recorded using Kodak XAR-5 film (Rochester, NY, USA). The detected signals were scanned and then quantified using TotalLab Quant software (TotalLab).

D. Enzyme-linked Immunosorbent assay (ELISA) Examination

The level of secreted IL-6 in culture medium was determined by ELISA following manufacturer’s instruction (R&D Systems, Minneapolis, MN, USA). Briefly, polyclonal rat anti-mouse cytokine antibodies were used as capturing antibodies, and biotinylated polyclonal rat anti-mouse cytokine antibodies were used for detection. Streptavidin-horseradish peroxidase and tetrathymethylenebenzidine sulphonate were added as color indicators, and plates were read at 405 nm.

E. Monocyte-endothelial Cell Adhesion Assay

The monocyte adhesion assay was modified as describe [18]. Briefly, HUVECs were seeded in 6-well plates at 4x 10⁵ cells/ml for 18 h and were stimulated with 10 ng/ml of TNF-α for another 6 h in the present of ENERGI-F704 or compound C. Regular passaged THP-1 cells were incubated with 10 μM of Calcein AM (Sigma-Aldrich) at 37°C for 30 min. Calcein AM labeled cells were harvest by centrifugation (1,000xg, 5 min) and washed three times with PBS. The fluorescent THP-1 cells were re-suspended in medium and were added into stimulated HUVECs. After 30 min, nonadhering THP-1 cells were washed twice with PBS and THP-1 cells bound to HUVEC were analyzed with fluorescence microscopy.

III. RESULTS

A. ENERGI-F704 Treatment Has No Effect on HUVEC Viability

We first examined the cytotoxic effect of ENERGI-F704 on HUVECs using XTT assay. As shown in fig. 1, ENERGI-F704 did not affect the HUVECs viability at 20 μM to 1200 μM when compared with vehicle treated cells (fig. 1).

B. ENERGI-F704 Activates AMPK in HUVECs

In the experiment to determine whether ENERGI-F704 activates AMPK, various concentration of ENERGI-F704 or AICAR were added to HUVECs for 6 h and then assessed western blot analysis. As shown in fig. 2, ENERGI-F704 activated AMPK in a dose-dependent manner as monitored by phosphorylation of AMPK. Co-treatment of compound C suppressed the AMPK phosphorylation induced by ENERGI-F704.

**Figure 1.** ENERGI-F704 has no effect on HUVECs viability in high glucose-containing medium. HUVECs were treated with variously concentration of ENERGI-F704 in DMEM containing 25 mM of glucose for 24 h. The cell viability of each condition was analyzed using XTT assay. Data are presented as the mean ± SEM of three experiments.

**Figure 2.** ENERGI-F704 increases phosphorylation of AMPK in high glucose-treated HUVECs. HUVECs were treated with variously concentration of ENERGI-F704 or AICAR in the present of 4 M of compound C in DMEM containing 25 mM of glucose for 6 h. Cell lysates collected from each condition were used to determine the phosphorylation of AMPK using Western blot analysis.
C. ENERGI-F704 Inhibits the Induction of IL-6

It has been demonstrated that IL-6 increased adhesion molecules expression in endothelial cells and led to activate endothelial cells. To further evaluate the effect of ENERGI-F704 on high glucose induced IL-6 secretion, the level of IL-6 in medium was analyzed using ELISA assay. The same with previous reports, high glucose time-dependently increased IL-6 secretion (Fig. 3). ENERGI-F704 suppressed high glucose induced IL-6 secretion in dose-dependent manner. The IL-6 secretion in 600 μM ENERGI-F704 treated cells was inhibited to 50% relative to that in vehicle treated cells in high glucose DMEM.

D. ENERGI-F704 Inhibits High Glucose Induced Monocyte Adhesion

To further explore the functional significance of ENERGI-F704 to endothelial cells-monocytes interaction, we examined the adhesion of THP1 to high glucose-induced HUVECs. HUVECs were treated with 25 mM glucose in the present of ENERGI-F704 or vehicle for 72 h. ENERGI-F704 significantly inhibited the monocytes adhesion (Fig.4). The monocyte adhesion in 600 μM ENERGI-F704 treated cells was inhibited to 32% relative to that in vehicle treated cells in high glucose DMEM. More importantly, the inhibition of monocytes adhesion caused by ENERGI-F704 was fully blocked by AMPK inhibitor, compound C.

![Figure 3](image1.png)

Figure 3. Effect of ENERGI-F704 on high glucose-induced IL-6 secretion. HUVECs were treated with variously concentration of ENERGI-F704 in DMEM containing 25 mM of glucose. After 24 h (A), 48h (B) or 72 h incubation, the level of IL-6 in culture medium of each condition was accessed using ELISA analysis. Data are presented as the mean ± SEM of three experiments.

![Figure 4](image2.png)

Figure 4. Effect of ENERGI-F704 on high glucose-induced monocyte adhesion. HUVECs were treated with indicated compound in DMEM containing high-glucose for 72 h. The adhesion of Calcein AM labeled THP-1 to HUVECs was analyzed with fluorescence microscopy. (A) Control (5.5 mM of glucose). (B) High glucose (25 mM). (C) Co-treated ENERGI-F704. (D) Co-treated with high glucose, 600 M of ENERGI-F704 and 4 M of compound C. Representative images of three independent experiments are shown. Data are presented as the mean ± SEM of three experiments. ** p < 0.01, one-way ANOVA.

IV. DISCUSSION

In the present study, we reported that ENERGI-F704 has significant anti-inflammatory activity in high glucose stimulated HUVECs. The western blot analysis showed that ENERGI-F704 dose-dependently increased AMPK phosphorylation in HUVECs suggesting that ENERGI-
F704 is an AMPK activator (Fig. 2). This result also supported by the increased phosphorylation of AMPK downstream targets such as ACC, TSC2, Raptor, (data not shown). Extensive studies demonstrate AMPK is a repressor of inflammation but there are limited studies related to the effect of AMPK activators on high glucose-induced vascular inflammation. We demonstrated herein that ENERGI-F704 suppressed high glucose induced IL-6 secretion and monocyte adhesion to HUVECs (Fig. 3 and Fig. 4). ENERGI-F704 had no effect on HUVECs viability at 20 μM to 1200 μM (Fig. 1), suggesting that the inhibitory effect on ENERGI-F704 in high glucose induced IL-6 secretion and monocyte adhesion is not caused by cytotoxic effect.

Hyperglycemia is considered the major factors for chronic inflammation in diabetes mellitus. The hyperglycemia enhances generation of reactive oxygen intermediates, inflammatory activation, and conducts endothelial dysfunction [19] and [20]. In diabetes patients, the level of IL-6 is much higher than that in normal glucose tolerance individuals. As the same with phenomenon in vivo, high glucose also increased IL-6 secretion in HUVECs (Fig. 3). The increased IL-6 secretion of HUVECs might induce intercellular adhesion molecule 1 (ICAM-1) expression via STAT3 signaling cascade and enhance monocyte adhesion to endothelial cells [17], [21]-[23]. In this study, we observed that the treatment of ENERGI-F704 resulted in a 50% decrease in IL-6 secretion, suggesting ENERGI-F704 may modulate high glucose induced monocyte adhesion. Chronic vascular inflammation in diabetes may lead to cardiovascular diseases [10]-[24]. The earliest event of vascular inflammation is the monocyte adhesion to blood vessel. Several reports indicated that AMPK activators such as metformin and AICAR inhibited cytokine induced monocyte adhesion to endothelial cells [25] and [26]. We demonstrated herein ENERGI-F704 suppressed high glucose induced monocyte adhesion (Fig. 4). More importantly, this inhibitory effect of ENERGI-F704 was fully blocked in the present of AMPK inhibitor, compound C, suggesting the suppression of monocyte adhesion by ENERGI-F704 was achieved via AMPK activation.

In conclusion, the present study demonstrates that a novel AMPK activator, ENERGI-F704, suppressed high glucose induced IL-6 secretion and monocyte adhesion to endothelial cells. ENERGI-F704 may have anti-vascular inflammatory activity and might be considered as potential drug for vascular inflammatory related diseases.

REFERENCES


Han-Min Chen was born in Taipei, Taiwan in 1971. He obtained his Ph.D. degree from the department of agriculture chemistry, National Taiwan University, in 1997. His major expertise is enzymology and proteomic technique development. He has served as post-doctoral fellow in Children Hospital Los Angeles from 2000-2002, and as the director in the proteomic department of DigiGenomics, Taiwan. Currently, he is the full-time professor in the department of Life Science of Catholic Fu-Jen University, Taiwan. He has also been the director of institute of Applied Science and Engineering and the associate dean of the college of Science and Engineering since 2012. He has more than 40 high-impact publications to date.

His major interest focuses on the development of techniques which improves the reproducibility and stringency of proteomic experiments. For examples, his laboratory has established the standard protocol for quantitating protein samples for two-dimensional electrophoresis (2-DE) experiments (2008 Proteomics), as well as establishing an algorithm for estimating the optimal condition for isoelectric focusing experiment (2009 Journal of Proteome Research). He also evaluated the applicability of using zinc-imidazole reverse protein stain for various proteomic experiments (2009 Proteomics). Furthermore, his laboratory invented a Snell’s based device that can use for exciting fluorescent signals in gels and transparent matrix (2008 Proteomics). Recently, his laboratory also utilized –omics techniques as well as systems biology approaches to decipher biological phenomena. Using the abovementioned approaches, they found that nanogold particles may induce endoplasmic reticulum (ER) stress and apoptosis in cancer cells (2012 ACSNano). Another new research interest emerges as the modulation of AMP kinase (AMPK), which is responsible for energy homeostasis and aging process in cells. They have identified a metabolite for activating AMPK, namely ENERGI-F704.

Professor Han-Min Chen is currently the associate editor of Journal of Integrated Omics. He has been received the distinguished research award of Catholic Fu-Jen University in 2012.