



# AICAR Attenuates TNF $\alpha$ -Induced Inappropriate Secretion of Monocyte Chemoattractant Protein-1 and Adiponectin in 3T3-L1 Adipocytes

Keiko Nagahara<sup>1</sup>, Kazushige Dobashi<sup>1</sup>, Takuya Ishikawa<sup>1</sup>, Yuya Nakano<sup>1</sup>, Yoshifusa Abe<sup>1</sup>,  
Daisuke Tanaka<sup>1,2</sup> and Kazuo Itabashi<sup>1</sup>

<sup>1</sup>Department of Pediatrics, Showa University School of Medicine, Tokyo, Japan

<sup>2</sup>Showa University Graduate School of Health Sciences, Kanagawa, Japan

**Aim:** The increase in monocyte chemoattractant protein-1 (MCP-1) and the decrease in adiponectin production from hypertrophic adipocytes are associated with adipose tissue inflammation and its metabolic complications. The aim of this study was to determine whether 5-aminoimidazole-4-carboxamide 1- $\beta$ -D-ribofuranoside (AICAR), an adenosine monophosphate-activated protein kinase (AMPK) activator, modulates these adipocytokine productions in tumor necrosis factor- $\alpha$  (TNF $\alpha$ )-treated adipocytes.

**Methods:** AICAR and/or other reagents were added to the culture medium, and then, TNF $\alpha$  was added to fully differentiated 3T3-L1 adipocytes. The MCP-1 and adiponectin production in the culture supernatant was measured by ELISA. AMPK, phosphatidylinositol 3-kinase (PI3K), and nuclear factor- $\kappa$ B (NF- $\kappa$ B) activities were also assayed.

**Results:** Treatment with TNF $\alpha$  increased MCP-1 and decreased adiponectin secretion dose-dependently in the 3T3-L1 adipocytes, and AICAR significantly inhibited these TNF $\alpha$ -mediated changes. Interestingly, metformin, another AMPK activator, did not have such effects on these adipocytokines. Both the AMPK and PI3K systems in the cells were significantly activated by the AICAR treatment, but the effects of AICAR on adipocytokines were not weakened by the addition of dorsomorphin, an AMPK inhibitor, or LY294002, a PI3K inhibitor. Pyrrolidine dithiocarbamate (PDTC), an NF- $\kappa$ B inhibitor, showed protective effects similar to those as AICAR. AICAR, but not metformin, significantly inhibited the TNF $\alpha$ -stimulated activation of NF- $\kappa$ B, and dorsomorphin did not change AICAR's effect.

**Conclusion:** AICAR attenuates the TNF $\alpha$ -induced secretion of MCP-1 and adiponectin in 3T3-L1 adipocytes. The observed effects of AICAR seem to be mainly due to the inhibition of NF- $\kappa$ B activation rather than the activation of the AMPK pathway, at least in TNF $\alpha$ -treated adipocytes.

**Key words:** Adipocytokines, AMP kinase, PI3 kinase, Nuclear factor-kappa B, Metformin

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## Introduction

Adipocytes have now been recognized as not only energy storage cells but also endocrine cells that secrete various physiologically active substances, collectively called adipocytokines or adipokines<sup>1-3</sup>. The expansion

of adipose tissue observed in obesity is mainly by enlarged adipocytes. Hypertrophic adipocytes gradually change in their own intracellular metabolism. The production of almost all the adipocytokines [leptin, tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin-6 (IL-6), plasminogen-activator inhibitor-1, retinol binding protein-4, etc.] is increased in obese condition<sup>1-3</sup>. On the other hand, the secretion of adiponectin is decreased with obesity<sup>2-4</sup>. Adiponectin is termed as a "beneficial adipocytokine" because of its antiatherogenic, insulin-mimetic, and lipid-oxidation-activating actions<sup>2, 3</sup>. Altered secretions of adipocytokines are considered to

Address for correspondence: Kazushige Dobashi, Department of Pediatrics, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-Ku, Tokyo, 142-8666, Japan

E-mail: kdobashi@med.showa-u.ac.jp

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play central roles in obesity-related complications such as dyslipidemia, hypertension, and insulin resistance<sup>2-4</sup>.

Recently, obesity has been considered to be associated with a state of chronic, low-grade inflammation<sup>1</sup>. Hypertrophic adipocytes produce high amounts of monocyte chemoattractant protein-1 (MCP-1). The increased MCP-1 levels induce macrophage infiltration into adipose tissue via the MCP-1 receptor, CC chemokine receptor-2. The activated macrophages secrete proinflammatory molecules including TNF $\alpha$ , which stimulate further production of MCP-1 from adipocytes. Hypertrophic adipocytes and activated macrophages synergistically elicit inflammation at least around the adipose tissue, leading to more inappropriate production of adipocytokines. Therefore, MCP-1 production from enlarged adipocytes seems to be the first step in obesity-related complications. However, little is known regarding the regulatory mechanism of MCP-1 secretion in obese condition.

The adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway is important for the regulation of both the cellular and whole-body energy balance, mainly by suppressing anabolic ATP-consuming pathways and stimulating catabolic ATP-generating pathways<sup>5</sup>. Recently, regulating carbohydrate and lipid metabolism by AMPK activation has been proposed to be a therapeutic target for obesity-related insulin resistance and type 2 diabetes mellitus<sup>5</sup>. Although the role of the AMPK system in the liver and muscles has been extensively studied, the role in adipose tissue is less understood. The activation of AMPK may be beneficial for protection against the dysfunction of adipocytes in high cytokine-production conditions.

## Aim

The aim of this study was to determine whether 5-aminoimidazole-4-carboxamide 1- $\beta$ -D-ribofuranside (AICAR), an AMPK activator<sup>5</sup>, was able to attenuate the MCP-1 and adiponectin production induced by TNF $\alpha$  in 3T3-L1 adipocytes.

## Methods

### Reagents and Cell Culture

AICAR (CAS No. 2627-69-2, PubChem CID: 17513) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Recombinant mouse TNF $\alpha$  (expressed in *Escherichia coli*), dimethylbiguanide hydrochloride (metformin), LY294002, and pyrrolidine dithiocarbamate (PDTC) were from Sigma Chemical Co. (St. Louis, MO, USA). Dorsomorphin (compound C) was purchased from Selleck Chemicals

(Houston, TX, USA).

3T3-L1 cells (European Collection of Cell Cultures, Salisbury, Wiltshire, UK, No. 86052701), derived from the mouse embryonic fibroblast cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% bovine serum (Gibco BRL, Rockville, MD, USA) and were differentiated as described previously<sup>6-10</sup>. The cells were checked for the degree of differentiation microscopically. Well-differentiated (>90%) 3T3-L1 adipocytes were exposed to TNF $\alpha$  (1–100 ng/ml) for 24 h. AICAR and/or other reagents were added 1 h before the TNF $\alpha$  treatment. As controls, 3T3-L1 adipocytes were cultured without any treatments for the same length of time.

### MCP-1 and Adiponectin Production

Twenty four hours after each treatment, the culture medium was collected. The MCP-1 and adiponectin concentrations in each medium sample were measured using a mouse CCL2/MCP-1 ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) and a mouse adiponectin ELISA kit (Otsuka Pharmaceuticals, Tokyo, Japan), respectively. The concentration of these proteins was calculated from the standard curve and the total quantity of production during 24 h, expressed as per mg of the total cell protein content.

### Protein Concentration in Cells

After each treatment, the culture medium was removed. Then the cells were collected and homogenized using CellLytic M (Sigma), a detergent solution designed for whole-cell protein extraction from cultured mammalian cells. The protein concentration of the cells was determined by the method of Bradford<sup>11</sup>.

### AMPK Activity

Sixteen hours after the treatment with 50 ng/ml of TNF $\alpha$ , with or without 2 mM of AICAR, the cells were washed and collected. The AMPK activity in each cell lysate sample was immunoassayed using an AMPK Kinase Assay Kit (CycLex Co., Ltd., Ngano, Japan). The assay plate was precoated with a serine 789 in mouse insulin receptor substrate-1, which was phosphorylated by AMPK. The amount of phosphorylated substrate was measured by binding with the antibody that specifically detected only the phosphorylated form of the serine residue on substrate-peptide complex, followed by binding with horseradish-peroxidase-conjugated anti-mouse IgG. The colorimetric signal (450 nm) was quantitated by spectrophotometry.

### Phosphatidylinositol-3-Kinase (PI3K) Activity

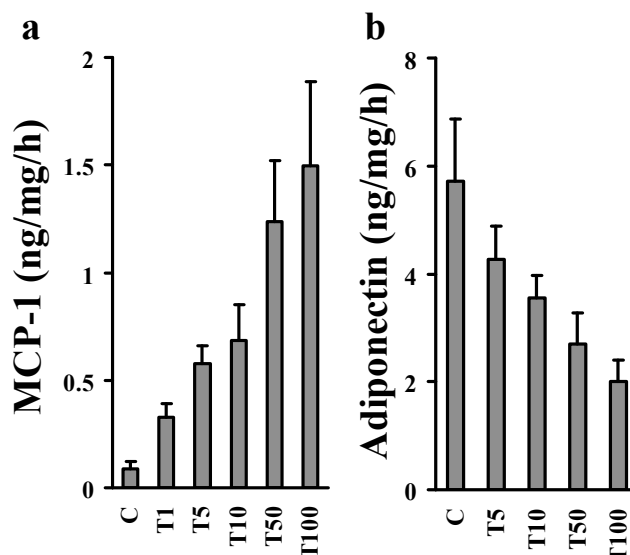
Sixteen hours after each treatment, the cells were washed and collected. The PI3K activity was measured using PI3-Kinase Activity ELISA (Echelon Biosciences Inc., Salt Lake City, UT, USA). Each cell lysate sample was incubated with phosphatidylinositol 4, 5-bisphosphate (PI(4,5)P<sub>2</sub>), the substrate of PI3K, for 1 h at 37°C. Then, the products were added to the phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P<sub>3</sub>)-coated microplate. PI(3,4,5)P<sub>3</sub> is the product of the class I PI3K phosphorylation of PI(4,5)P<sub>2</sub>. PI(3,4,5)P<sub>3</sub> antibody was then added for competitive binding. A peroxidase-conjugated secondary antibody and colorimetric detection (450 nm) was used to detect PI(3,4,5)P<sub>3</sub> antibody binding to the plate.

### Preparation of Nuclear Extracts

The preparation of nuclear extracts was performed using a Nuclear Extract Kit (Active Motif North America, CA, USA)<sup>7-10</sup>. Thirty minutes after the TNF $\alpha$  treatment, the cells were rinsed twice with ice-cold phosphate-buffered saline containing phosphatase inhibitors and were collected by centrifugation. The pellet was then resuspended in ice-cold hypotonic buffer containing 0.5% Nonidet P-40 and was subsequently incubated for 15 min on ice and then centrifuged at 14000  $\times g$  for 30 s at 4°C. The supernatants (cytoplasmic fractions) were removed. The nuclear pellets were resuspended in ice-cold lysis buffer containing protease inhibitors. After 30 min of incubation on ice, the lysates were centrifuged for 10 min at 14000  $\times g$ , and the supernatant (nuclear fractions) were stored at -80°C.

### Quantification of Nuclear Factor- $\kappa$ B (NF- $\kappa$ B) Activity

The nuclear extracts were applied to the ELISA-based assay kit (Active Motif North America, CA, USA), and the NF- $\kappa$ B activity was quantified according to the manufacturer's protocol as follows. Five micrograms of protein from the nuclear extracts was incubated for 1 h at 25°C with oligonucleotides containing an NF- $\kappa$ B binding consensus, which was coated to microwells, in the presence of competitive binding with the wild-type or mutated consensus oligonucleotides (the latter has no effect on NF- $\kappa$ B binding). Then, rabbit anti-NF- $\kappa$ B p65 antibodies were added to each well and incubated for 1 h at 25°C, followed by incubation with peroxidase-conjugated goat anti-rabbit IgG. The peroxidase activity was quantitated by spectrophotometry at 450 nm. We have measured the NF- $\kappa$ B activity using this method many times before<sup>7-10</sup>.



**Fig. 1.** Effect of TNF $\alpha$  on MCP-1 and adiponectin production in 3T3-L1 adipocytes

3T3-L1 cells were incubated with 1–100 ng/ml of TNF $\alpha$  (T1, T5, T10, T50, T100) for 24 h and the MCP-1 (Fig. 1a) and adiponectin (Fig. 1b) concentration in the culture medium was assayed. The concentration was standardized with total protein contents of the cells. Data are means  $\pm$  SD of five to seven observations.

a: Control (C) vs T5, T10, T50, or T100:  $p < 0.001$ , T1 vs T10:  $p < 0.05$ , T1 vs T50 or T100:  $p < 0.001$ , T5 vs T50 or T100:  $p < 0.001$ , T10 vs T50 or T100:  $p < 0.001$ , T50 vs T100:  $p < 0.05$ .  
b: C vs T5:  $p < 0.01$ , C vs T10, T50 or T100:  $p < 0.001$ , T5 vs T50:  $p < 0.005$ , T5 vs T100:  $p < 0.001$ , T10 vs T100:  $p < 0.01$ .

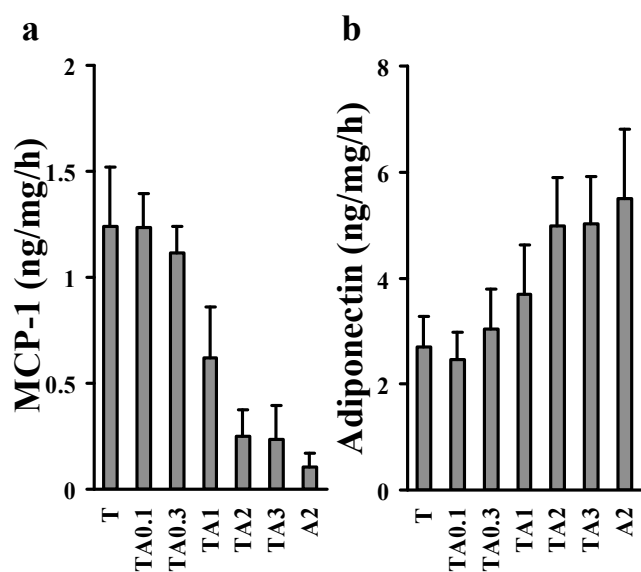
### Statistics

Data are presented as the mean and standard deviation (SD). The statistical analysis of the data was performed using one-way analysis of variance followed by a post hoc test (Fisher's Protected Least Significant Difference test). The values were considered to be statistically significant at  $p < 0.05$ . The statistical analyses were performed in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

## Results

### Effect of TNF $\alpha$ on MCP-1 and Adiponectin Production in 3T3-L1 Adipocytes

Treatment with TNF $\alpha$  (1–100 ng/ml) dose-dependently increased the production of MCP-1 in the fully differentiated 3T3-L1 adipocytes (Fig. 1a). The MCP-1 concentration with 50 ng/ml of TNF $\alpha$  treatment was 1380% of that in the untreated cells ( $p < 0.001$ ). On the other hand, treatment with 5–100 ng/ml of TNF $\alpha$  dose-dependently decreased the production of adiponectin in the 3T3-L1 adipocytes (Fig. 1b). The adiponectin concentration after 50 ng/ml of TNF $\alpha$  treatment was 47.4% of that in the



**Fig. 2.** Effect of AICAR on MCP-1 and adiponectin production in TNF $\alpha$ -stimulated 3T3-L1 adipocytes

3T3-L1 cells were preincubated with 0.1–3 mM of AICAR (A) for 1 h and then treated with 50 ng/ml of TNF $\alpha$  (TA0.1, TA0.3, TA1, TA2, TA3). After 24 h, the MCP-1 (Fig. 2a) and adiponectin (Fig. 2b) concentration in the culture medium was assayed. Data are means  $\pm$  SD of five to seven observations.

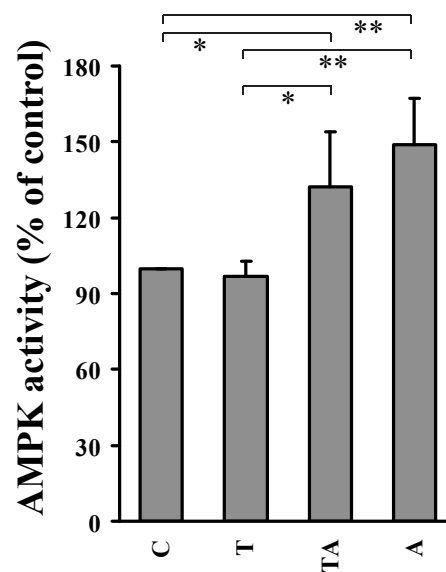
a: T, TA0.1 or TA0.3 vs TA1, TA2, TA3 or A2:  $p < 0.001$ , TA1 vs TA2 or TA3:  $p < 0.005$ .

b: T or TA0.1 vs TA2, TA3 or A2:  $p < 0.001$ , TA0.1 vs TA1:  $p < 0.05$ , TA0.3 vs TA2 or TA3:  $p < 0.05$ , TA0.3 vs A2:  $p < 0.001$ , TA1 vs TA2 or TA3:  $p < 0.05$ .

untreated cells ( $p < 0.001$ ).

### Effect of AICAR on MCP-1 and Adiponectin Production in TNF $\alpha$ -Treated 3T3-L1 Adipocytes

The TNF $\alpha$ -stimulated production of MCP-1 dose-dependently decreased with preincubation with AICAR (0.1–3 mM, **Fig. 2a**). After pretreatment with 2 mM of AICAR, the MCP-1 level decreased to 20.3% of that in the cells treated with 50 ng/ml of TNF $\alpha$  ( $p < 0.001$ ). Treatment with 2 mM of AICAR alone did not significantly alter the MCP-1 production in 3T3-L1 adipocytes. The decreased adiponectin production caused by the treatment with 50 ng/ml of TNF $\alpha$  dose-dependently increased with preincubation with 0.1–3 mM of AICAR (**Fig. 2b**). Pretreatment with AICAR at a concentration of 2 mM induced an increase in the adiponectin level to 184.0% of that in the 50 ng/ml TNF $\alpha$ -treated cells ( $p < 0.001$ ). AICAR alone, at a concentration of 2 mM, did not significantly alter the adiponectin production in 3T3-L1 adipocytes.



**Fig. 3.** Effect of TNF $\alpha$  and AICAR on AMPK activity in 3T3-L1 adipocytes

3T3-L1 cells were incubated with 50 ng/ml of TNF $\alpha$  (T), a combination of 2 mM of AICAR with 50 ng/ml of TNF $\alpha$  (TA), or 2 mM of AICAR alone (A). After 16 h, AMPK activity was assayed. The activity was expressed as percent of the controls  $\pm$  SD ( $n = 5$ ). \*:  $p < 0.005$ . \*\*:  $p < 0.001$ .

### Effect of TNF $\alpha$ and AICAR on AMPK Activity in 3T3-L1 Adipocytes

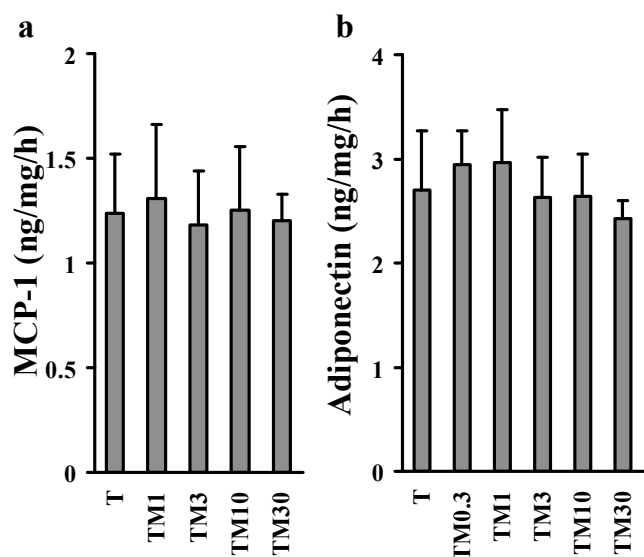
Treatment with 50 ng/ml of TNF $\alpha$  did not alter the AMPK activity, whereas treatment with 2 mM of AICAR significantly increased this activity in 3T3-L1 adipocytes (**Fig. 3**). The AMPK activity in the cells treated with 2 mM of AICAR was 148.7% of that in the untreated control cells ( $p < 0.001$ ). AICAR (2 mM) activated the AMPK system even when TNF $\alpha$  (50 ng/ml) was present ( $p < 0.005$ ).

### Effect of Metformin on MCP-1 and Adiponectin Production in TNF $\alpha$ -Treated 3T3-L1 Adipocytes

The metformin treatments (0.3–30 mM) did not significantly attenuate the inappropriate production of MCP-1 (**Fig. 4a**) and adiponectin (**Fig. 4b**) induced by the addition of 50 ng/ml of TNF $\alpha$ .

### Effect of Dorsomorphin on MCP-1 and Adiponectin Production in TNF $\alpha$ -Treated 3T3-L1 Adipocytes with or without AICAR

Treatment with 1, 3, or 10  $\mu$ M of dorsomorphin, an AMPK inhibitor, did not significantly modify the level of MCP-1 (**Fig. 5a**) or adiponectin (**Fig. 5b**) secretion induced by TNF $\alpha$  (50 ng/ml) in the 3T3-L1 adipocytes. In addition, treatment with dorsomorphin did not change the effects of AICAR on the adipocyte-



**Fig. 4.** Effect of metformin on MCP-1 and adiponectin production in TNF $\alpha$ -stimulated 3T3-L1 adipocytes

3T3-L1 cells were preincubated with 0.3–30 mM of metformin for 1 h and then treated with 50 ng/ml of TNF $\alpha$  (TM0.3, TM1, TM3, TM10, TM30). After 24 h, the MCP-1 (Fig. 4a) and the adiponectin (Fig. 4b) concentration in the culture medium was assayed. Data are means  $\pm$  SD of five to seven observations.

tokines. The treatment with 10  $\mu$ M of dorsomorphin alone did not increase or decrease the MCP-1 and adiponectin concentration.

#### Effect of TNF $\alpha$ and AICAR on PI3K Activity in 3T3-L1 Adipocytes

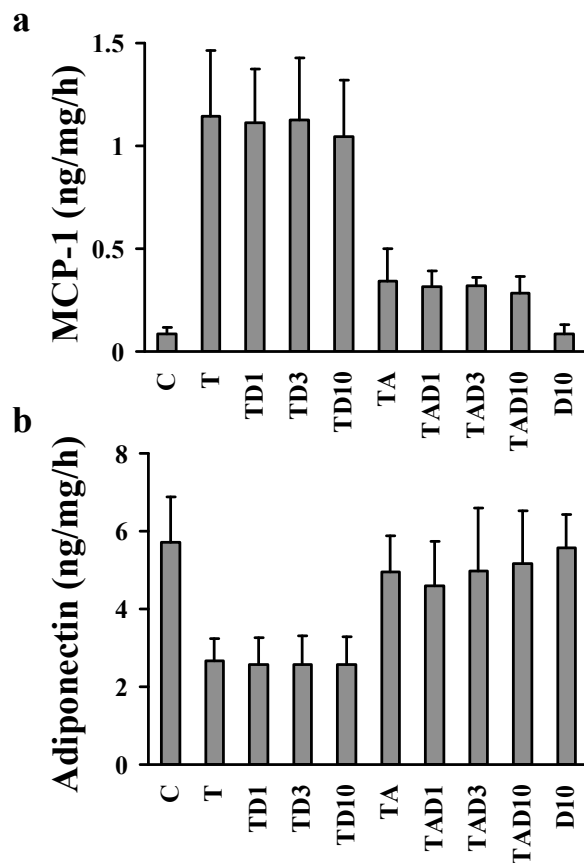
Treatment with 50 ng/ml of TNF $\alpha$  activated the PI3K system in the 3T3-L1 adipocytes ( $p < 0.01$ , **Fig. 6**). The combination treatment of TNF $\alpha$  (50 ng/ml) with AICAR (2 mM) activated PI3K to a greater extent than TNF $\alpha$  did alone. The PI3K activity in the cells treated with TNF $\alpha$  with AICAR was 434.1% of that in the control cells ( $p < 0.001$ ) and 185.4% of that in the TNF $\alpha$ -treated cells ( $p < 0.001$ ).

#### Effect of LY294002 on the AICAR-Induced Effects on MCP-1 and Adiponectin Secretion

AICAR (2 mM) significantly inhibited the TNF $\alpha$ -induced increase in MCP-1 and TNF $\alpha$ -induced decrease in adiponectin. These effects of AICAR on MCP-1 and adiponectin secretion were not modulated by the treatment with 1–30 mM of LY294002, a PI3K inhibitor (**Fig. 7**).

#### Effect of PDTC on TNF $\alpha$ -Treated MCP-1 and Adiponectin Production in 3T3-L1 Adipocytes

The TNF $\alpha$ -mediated changes in MCP-1 and adiponectin were dose-dependently attenuated by pre-



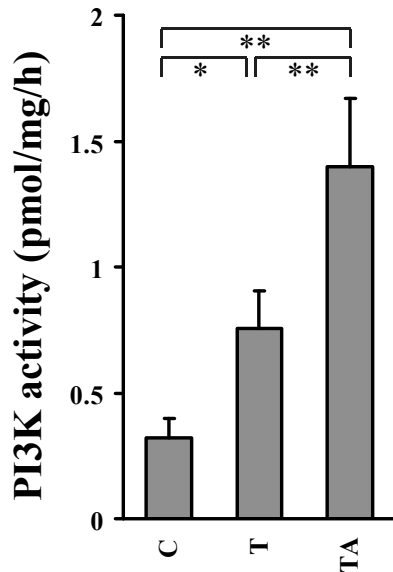
**Fig. 5.** Effect of dorsomorphin on MCP-1 and adiponectin production in TNF $\alpha$ -stimulated 3T3-L1 adipocytes with or without AICAR

3T3-L1 cells were preincubated with 1–10  $\mu$ M of dorsomorphin (Compound C) with or without 2 mM of AICAR for 1 h and then treated with 50 ng/ml of TNF $\alpha$  (TD1, TD3, TD10, and TAD1, TAD3, TAD10). After 24 h, the MCP-1 (Fig. 5a) and adiponectin (Fig. 5b) concentration in the culture medium was assayed. Data are means  $\pm$  SD of five observations. D10: Only dorsomorphin treated cells.

a: Control (C) vs T, TD1, TD3 or TD10:  $p < 0.001$ , T, TD1, TD3 or TD10 vs TA, TAD1, TAD3, TAD10 or D10:  $p < 0.001$ , TA vs C or D10:  $p < 0.05$ .

b: C or D10 vs T, TD1, TD3 or TD10:  $p < 0.001$ , T vs TA or TAD3:  $p < 0.005$ , T vs TAD1:  $p < 0.01$ , T vs TAD10:  $p < 0.001$ , TD1, TD3 or TD10 vs TA, TAD3 or TAD10:  $p < 0.001$ , TD1, TD3 or TD10 vs TAD1:  $p < 0.005$ .

incubation with 0.1–100  $\mu$ M of PDTC. The MCP-1 level was decreased to 19.4% of that in the cells treated with 50 ng/ml of TNF $\alpha$  when preincubated with 10  $\mu$ M of PDTC ( $p < 0.001$ , **Fig. 8a**) and the adiponectin level was increased to 184.8% ( $p < 0.001$ , **Fig. 8b**).



**Fig. 6.** Effect of TNF $\alpha$  and AICAR on PI3K activity in 3T3-L1 adipocytes

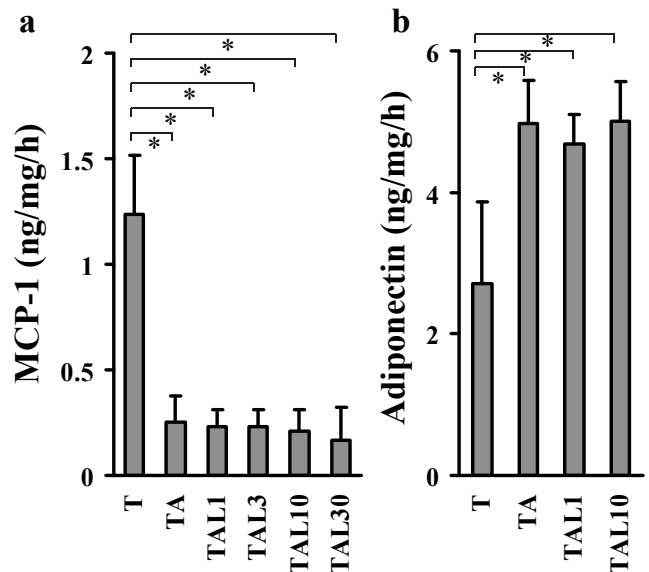
3T3-L1 adipocytes were incubated with 50 ng/ml of TNF $\alpha$  (T) or a combination of 2 mM of AICAR with TNF $\alpha$  (TA). After 16 h, the AMPK activity was assayed. The activity was expressed as percent of the controls  $\pm$  SD ( $n=5$ ). C: control, \*:  $p < 0.01$ , \*\*:  $p < 0.001$ .

### Effect of AICAR, Dorsomorphin, Metformin and PDTC on NF- $\kappa$ B Activity in TNF $\alpha$ -Treated 3T3-L1 Adipocytes

NF- $\kappa$ B activation occurred markedly after the treatment with 50 ng/ml of TNF $\alpha$  (Fig. 9). The activity of NF- $\kappa$ B after the combined treatment with TNF $\alpha$  and AICAR or TNF $\alpha$  and PDTC was 32.0% and 4.6%, respectively, of that in the cells treated with TNF $\alpha$  alone ( $p < 0.001$ ). In contrast, metformin, another AMPK activator, did not show an inhibitory effect similar to that of AICAR, and dorsomorphin, an AMPK inhibitor, did not significantly inhibit the effect of AICAR either. AICAR itself did not affect the NF- $\kappa$ B activity in the 3T3-L1 adipocytes.

## Discussion

The present study revealed that TNF $\alpha$  increased MCP-1 production and decreased adiponectin production in 3T3-L1 adipocytes and that AICAR attenuated these TNF $\alpha$ -induced changes. Many investigators have reported that the MCP-1 production is increased and the adiponectin production is decreased by TNF $\alpha$  treatment in adipocytes<sup>1-3</sup>. Our present results agree with those previous studies. However, to our knowledge, there have been no reports that AICAR modulates MCP-1 or adiponectin secretion in



**Fig. 7.** Effect of LY294002 on MCP-1 and adiponectin production in 3T3-L1 adipocytes treated TNF $\alpha$  with AICAR

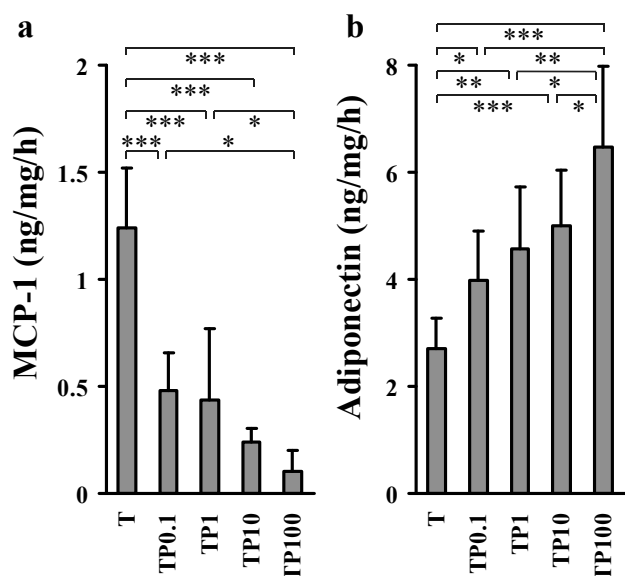
3T3-L1 cells were preincubated with 2 mM of AICAR and 1–30 mM of LY294002 for 1 h and then treated with 50 ng/ml of TNF $\alpha$  (TAL1, TAL3, TAL10, TAL30). After 24 h, the MCP-1 (Fig. 7a) and the adiponectin (Fig. 7b) concentration in the culture medium was assayed. Data are means  $\pm$  SD of five to seven observations. \*:  $p < 0.001$ .

TNF $\alpha$ -treated adipocytes.

AICAR was reported to decrease the secretion of MCP-1 in unstimulated 3T3-L1 adipocytes<sup>12</sup> and human adipocytes obtained from biopsy<sup>13</sup>. AICAR was also reported to decrease the total adiponectin secretion in untreated 3T3-L1 adipocytes<sup>14,15</sup>. In contrast, Huypens *et al.*<sup>16</sup> reported that AICAR did not significantly alter the adiponectin mRNA expression and protein level in 3T3-L1 adipocytes. In our study, AICAR alone did not affect the MCP-1 and adiponectin production in the 3T3-L1 adipocytes.

AICAR is an AMPK activator and actually activated AMPK in the 3T3-L1 adipocytes in this study. However, metformin<sup>5</sup>, another AMPK activator, did not attenuate the TNF $\alpha$ -induced changes in the secretion of MCP-1 and adiponectin. These results suggest that AICAR may possess some sort of mechanism that metformin lacks. To our knowledge, it has not been reported previously whether metformin improves the TNF $\alpha$ -induced alteration of MCP-1 and adiponectin production in adipocytes.

Metformin has been reported to decrease the MCP-1 gene expression<sup>17</sup> and protein production<sup>18</sup> in unstimulated human adipocytes. Moreno-Navarrete *et al.*<sup>17</sup> reported that metformin decreased the adipo-

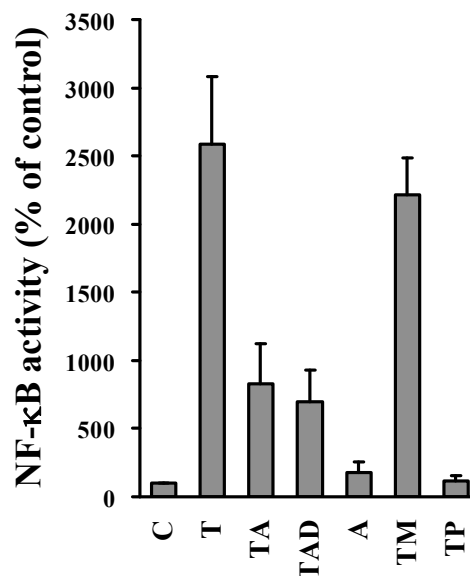


**Fig. 8.** Effect of PDTC on MCP-1 and adiponectin production in TNF $\alpha$ -stimulated 3T3-L1 adipocytes

3T3-L1 cells were pre-incubated with 0.1–100  $\mu$ M of PDTC for 1 h and then treated with 50 ng/ml of TNF $\alpha$  (TP0.1, TP1, TP10, TP100). After 24 h, the MCP-1 (Fig. 8a) and adiponectin (Fig. 8b) concentration in the culture medium was assayed. The concentration was standardized with total protein contents of the cells. Data are Means  $\pm$  SD of 5 to 7 observations. \*:  $p < 0.05$ , \*\*:  $p < 0.005$ , \*\*\*:  $p < 0.001$ .

nectin gene expression in human unstimulated adipocytes, however, Huypens *et al.*<sup>16)</sup> reported that metformin did not affect transcription but reduced the adiponectin protein expression in 3T3-L1 adipocytes. Kanda *et al.*<sup>19)</sup> reported that adiponectin gene and protein production were not altered by metformin treatment in 3T3-L1 adipocytes. In summary, these reports stated that MCP-1 and adiponectin production are decreased or unaffected by AICAR or metformin in unstimulated adipocytes.

Dorsomorphin (compound C) is a well-known AMPK inhibitor<sup>5)</sup> and has been used in many studies. For example, Guo *et al.* reported that treatment with 0.1–1  $\mu$ M of dorsomorphin significantly blocked the AICAR-induced AMPK activation dose-dependently in RAW 264.7 cells<sup>20)</sup>. To understand the association of cellular AMPK system with our observed results after TNF $\alpha$  and AICAR treatments, we investigated the effect of dorsomorphin on MCP-1 and adiponectin production in 3T3-L1 adipocytes. Interestingly, the AMPK inhibitor (dorsomorphin) could neither intensify the effect of TNF $\alpha$  nor weaken the effect of AICAR. These results suggest that the AMPK signal is neither involved in the observed effect of TNF $\alpha$  nor in the protective effect of AICAR either. If this is true, it is make sense that metformin did not attenuate the



**Fig. 9.** Effect of AICAR, dorsomorphin, metformin and PDTC on TNF $\alpha$ -induced NF- $\kappa$ B activity in 3T3-L1 adipocytes.

The nuclear fraction of 3T3-L1 cells was extracted, and assayed for the free NF- $\kappa$ B level by ELISA. The activity was expressed as percent of the controls ( $n=4$ ). C: control, T: 50 ng/ml of TNF $\alpha$ , A: 2 mM of AICAR, D: 10  $\mu$ M of Dorsomorphin, M: 30 mM of metformin, P: 50  $\mu$ M of PDTC. C vs T, TA or TM;  $p < 0.001$ , C vs TAD;  $p < 0.005$ , T vs TA, TAD, A or TP;  $p < 0.001$ , TA vs A;  $p < 0.005$ , TA vs TM or TP;  $p < 0.001$ , TAD vs A;  $p < 0.01$ , TAD vs TM;  $p < 0.001$ .

effects of TNF $\alpha$  in the present study as an AMPK activator. AICAR is considered to have another mechanism of action besides AMPK activation.

AICAR is suggested to activate the PI3K pathway<sup>21)</sup> as well as the AMPK pathway. For this reason, we checked the PI3K activity and showed that TNF $\alpha$  treatment significantly activated PI3K and that AICAR further activated the PI3K system in 3T3-L1 adipocytes. However, the observed effect of AICAR on MCP-1 and adiponectin production in the TNF $\alpha$ -treated cells was not offset by LY294002, a PI3K inhibitor. These results indicate that the PI3K signal is not involved in the effect of AICAR on MCP-1 and adiponectin secretion.

We previously reported that PDTC, an NF- $\kappa$ B inhibitor, inhibited the IL-6 and nitric oxide (NO) production, which is increased via the NF- $\kappa$ B pathway, induced by the combination of lipopolysaccharide (LPS) with TNF $\alpha$  in 3T3-L1 adipocytes<sup>22)</sup>. Hence, PDTC treatment was tested instead of AICAR treatment in this study. The PDTC treatment significantly attenuated the TNF $\alpha$ -induced inappropriate secretion of MCP-1 and adiponectin in 3T3-L1 adipocytes, similarly to AICAR.

NF- $\kappa$ B is characterized as an activator of the expression of many genes in various cell types including adipocytes<sup>23</sup>. In the resting state, I- $\kappa$ B forms a complex with NF- $\kappa$ B in the cytosol and masks the nuclear localization signal. LPS and various cytokines activate I- $\kappa$ B kinase, which phosphorylates and degrades I- $\kappa$ B. Free NF- $\kappa$ B, an active form of the molecule, migrates to the nucleus and triggers the transcription of genes. Both MCP-1 and adiponectin genes are reported to be regulated by NF- $\kappa$ B activation in TNF $\alpha$ -treated 3T3-L1 adipocytes<sup>23</sup>.

There have been no reports on whether AICAR inhibits NF- $\kappa$ B activity in adipocytes. Therefore, we studied the NF- $\kappa$ B activity and found for the first time that AICAR, but not metformin, significantly inhibited TNF $\alpha$ -induced NF- $\kappa$ B activation in 3T3-L1 adipocytes. Furthermore, the dorsomorphin treatment did not modify this effect of AICAR, indicating that the AMPK pathway is not involved in the inhibitory effect of AICAR on NF- $\kappa$ B activation.

The effect of AICAR on LPS- or cytokine-activated NF- $\kappa$ B has been studied in several cell types before, except for in adipocytes. AICAR has been reported to inhibit the LPS-induced activation of NF- $\kappa$ B in RAW264.7 cells<sup>20, 24</sup>, rat retina cells<sup>25</sup>, murine bone marrow neutrophils<sup>26</sup>, primary astrocytes<sup>27</sup>, microglial (BV2) cells<sup>27</sup>, and rat microglial cells<sup>28</sup>. AICAR has also been reported to inhibit the TNF $\alpha$ -induced activation of NF- $\kappa$ B in human colon cancer HCT116 cells<sup>29</sup> and human umbilical vein endothelial cells (HUVECs)<sup>30</sup>.

It is not known whether metformin can significantly inhibit NF- $\kappa$ B activation in adipocytes. Łabuzek *et al.*<sup>31</sup> reported that metformin significantly activated AMPK but could not inhibit LPS-stimulated NF- $\kappa$ B activation in rat primary microglial cells. In contrast, treatment with 1–10 mM of metformin has been reported to inhibit the TNF $\alpha$ -induced NF- $\kappa$ B activation in HUVECs<sup>30</sup>. However, Huang *et al.*<sup>32</sup> suggested that the inhibitory effect of metformin was through PI3K activation in HUVECs. Kuo *et al.*<sup>33</sup> reported that AICAR inhibited LPS-induced protein expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), whose genes are upregulated by NF- $\kappa$ B, in macrophages and microglial cells, but metformin did not inhibit the iNOS and COX-2 expression levels despite the significant activation of the AMPK pathway.

Both AICAR and metformin are well-known AMPK activators. However, their mechanisms of AMPK activation are different. AICAR is phosphorylated inside the cells and becomes an AMP mimic, which directly activates AMPK<sup>34, 35</sup>. In contrast, the activation of AMPK by metformin is suggested to be

an indirect mechanism<sup>35</sup>. It was reported previously that metformin is not able to activate the AMPK partially purified from rat liver, despite the success of AMP<sup>35</sup>.

The NF- $\kappa$ B activity in adipocytes is regulated by several different mechanisms. We previously reported that the activation of peroxisome proliferator-activated receptor- $\gamma$  by troglitazone<sup>6</sup>, PKA activation by cAMP<sup>7</sup>, small GTP-binding protein signal by hydroxymethylglutaryl (HMG)-CoA reductase inhibitors<sup>8, 9</sup>, and an antioxidant N-acetylcysteine<sup>10</sup> modulated the cytokine-induced NF- $\kappa$ B activity in 3T3-L1 adipocytes. Łabuzek *et al.* reported that AICAR inhibited the production of inflammatory molecules (IL-6, IL-10, TNF $\alpha$ , iNOS etc.) in LPS-treated microglial cells and also suggested the effects of AICAR are via AMPK-independent pathways in a study using dorsomorphin<sup>31</sup>. The anti-inflammatory AMPK-independent action of AICAR may be more intense than that of metformin at least in TNF $\alpha$ -treated 3T3-L1 adipocytes.

## Conclusion

AICAR, but not metformin, attenuates the TNF $\alpha$ -induced inappropriate secretion of MCP-1 and adiponectin in 3T3-L1 adipocytes. The present study suggests that the NF- $\kappa$ B activation pathway strongly affects cellular dysfunction and that neither AMPK nor PI3K signals are significantly involved in the observed effects of TNF $\alpha$ , particularly in adipocytes. AICAR is able to inhibit TNF $\alpha$ -mediated NF- $\kappa$ B activation and this AMPK-independent mechanism of AICAR could be more intense than that of metformin, at least in adipocytes.

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## Conflicts of Interest

We have no conflicts of interest to declare.

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