Free Radical Research, February 2010; 44(2): 128–134

informa
healthcare

Involvement of mTOR in globular adiponectin-induced generation of reactive oxygen species

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(Received date 29 July 2009; revised 11 September 2009)

Abstract

Globular adiponectin (gAd) induces the generation of reactive oxygen species (ROS) and nitric oxide (NO) in the murine macrophage cell line RAW 264. This study investigated the role of the mammalian target of rapamycin (mTOR) in gAd-induced ROS and NO generation. gAd stimulation induced phosphorylation of mTOR, which peaked at 20 min and dissolved rapidly. Inhibition of phosphatidylinositol 3-kinase activity with wortmannin suppressed gAd-induced phosphorylation of Akt and mTOR. Administration of rapamycin partially reduced gAd-induced generation of intracellular and mitochondrial ROS, but not release of NO. To further confirm the role of mTOR in gAd stimulation, the effect of the activators of AMP-activated protein kinase (AMPK) on gAd-induced mTOR phosphorylation was examined. Pre-treatment with three kinds of AMPK activators, AICAR, 2-deoxy-D-glucose and A-769662, suppressed gAd-induced mTOR phosphorylation. Furthermore, these AMPK activators significantly reduced gAd-evoked intracellular and mitochondrial ROS generation and NO release.

Keywords: *Adiponectin, mTOR, reactive oxygen species, nitric oxide, AMPK*

Introduction

Adiponectin, predominantly secreted from mature adipocytes, is widely studied concerning its role in the regulation of carbohydrate and lipid metabolism, insulin sensitivity, carcinogenesis, cardiovascular diseases and inflammation [1–4]. Most recent studies indicate that higher adiponectin levels are associated with a lower risk of type 2 diabetes, consistent with a dose–response relationship across diverse populations [5]. Adiponectin exists in five configurations and six forms: a globular form, full-length monomer, a low-molecular isoform (trimer), a mid-molecular isoform (hexamer), a high-molecular isoform (an octamer or upper two hexamers) and a serum albumin-bound, low-molecular isoform [6]. Although multiple data from basic and clinical studies have shown anti-inflammatory, anti-atherogenic and anti-diabetic properties of adiponectin, increased local/systemic adiponectin levels are also present in chronic inflammatory and autoimmune diseases such as rheumatoid arthritis and type 1 diabetes [7]. These conflicting actions of adiponectin may suggest that the immunomodulating effects of adiponectin are dependent on its systemic and/or local concentrations and/or the isoforms present [6].

The mammalian target of rapamycin (mTOR) serine-threonine kinase is a master regulator of intracellular signalling for cell proliferation, autophagy, mRNA translation and metabolism [8,9]. mTOR exists in two functionally distinct complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 is composed of mTOR, RAPTOR, LST8 and PRAS40, and its major functions include activation by phosphorylation of p70 ribosomal protein S6 kinase (S6K1) and inactivation of eIF4E-binding protein 1 (4E-BP1), which initiates translation and cell proliferation.

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ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.3109/10715760903348328

Akt activates mTORC1 indirectly by phosphorylating and inactivating TSC2, leading to the suppression of Rheb GTPase, an activator of mTORC1. Akt also directly activates mTORC1 by phosphorylating PRAS40 [8]. mTORC2 contains LST8, RICTOR, SIN1 and functionally mediates the insulin-induced phosphorylation of Akt [10]. Rapamycin, a macrolide antibiotic possessing potent immunosuppressant and anti-cancer activity, forms a drug-receptor complex with FKBP12 and specifically inhibits mTORC1, but not mTORC2, by disrupting the interaction between RAPTOR and mTOR. However, recent reports suggest that long-term administration of rapamycin blocks mTORC2 assembly [11].

Here we investigated the involvement of mTOR activation via the PI3K/Akt signalling pathway in gAd-induced reactive oxygen species (ROS)/nitric oxide (NO) generation in the mouse RAW 264 macrophage cell line. We also examined the role of AMP-activated protein kinase (AMPK) on gAd-induced mTOR phosphorylation.

Materials and methods

Reagents

All cell culture reagents, wortmannin, 2-deoxy-D-glucose (2-DG), 23,27-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclohentriacontine (rapamycin) and N^1-(β-D-ribofuranosyl)-5-aminoimidazole-4-carboxamide (AICAR), were purchased from Sigma (St. Louis, MO). AMPK-specific activator (A-769662) was obtained from Dundee University [12]. MitoSOX™, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Molecular Probes (Carlsbad, CA).

Cell culture

The murine macrophage-like cell line RAW 264 (RCB0535; RIKEN Cell Bank, Ibaragi, Japan) was maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum (Thermo Scientific, Waltham, MA), 2 mM L-glutamine and 50 μg/ml gentamycin at 37°C in an atmosphere of 5% CO_2/95% air.

Purification of recombinant protein

A glutathione S-transferase (GST) fusion vector, pGEX-6P-1 (GE Healthcare, Amersham Biosciences, Uppsala, Sweden), encoding the globular domain of mouse adiponectin (gAd) was provided by Dr. I. Shimomura (Osaka University, Japan). Recombinant gAd was prepared as described previously [13]. Briefly, GST–gAd protein was produced in *Escherichia coli* BL21 and purified using glutathione Sepharose 4B (GE Healthcare, Amersham Biosciences). GST was cleaved from the GST–gACRP30 fusion protein using PreScission protease (GE Healthcare, Amersham Biosciences). The purified recombinant gAd was applied to an Affi-Prep polymyxin column (Bio-Rad Laboratories, Hercules, CA) to remove endotoxin contaminants.

Analysis of cellular redox status

To detect intracellular and mitochondrial ROS formation, measurements of H_2DCFDA and MitoSOX fluorescence were performed using flow cytometry. Cells were incubated for the times indicated at 37°C under 5% CO_2/95% air; phosphate-buffered saline (PBS) containing 5 μM H_2DCFDA and 5 μM MitoSOX was then added and the cells were incubated for another 30 min. The labelled cells were washed twice with PBS and then suspended in PBS for analysis by flow cytometry. The fluorescence intensity of H_2DCFDA was measured at an excitation wavelength of 488 nm and emission wavelength of 580 nm and MitoSOX was measured at excitation and emission wavelengths of 510 and 580 nm, respectively. The resultant histograms were analysed with the Expo32 program (Beckman Coulter, Fullerton, CA) after subtracting the background fluorescence.

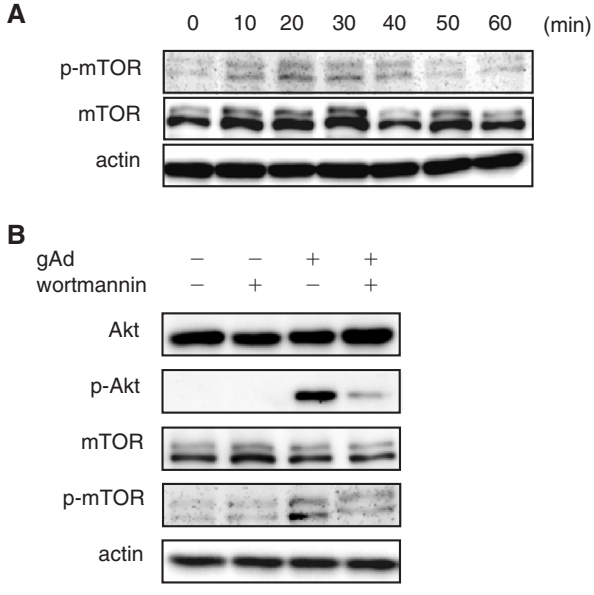

Figure 1. Effect of gAd stimulation on mTOR phosphorylation in RAW 264 cells. (A) Time dependency of gAd-induced mTOR phosphorylation. The protein levels of mTOR and phsopho-mTOR (p-mTOR) were detected by Western blotting. (B) The effect of wortmannin on phosphorylation of both Akt and mTOR. RAW 264 cells were pre-treated with 100 nM wortmannin for 30 min. After being washed with PBS, cells were cultured with or without 10 μg/ml gAd for an additional 24 h. Each experiment was performed three times and all experiments showed similar results.

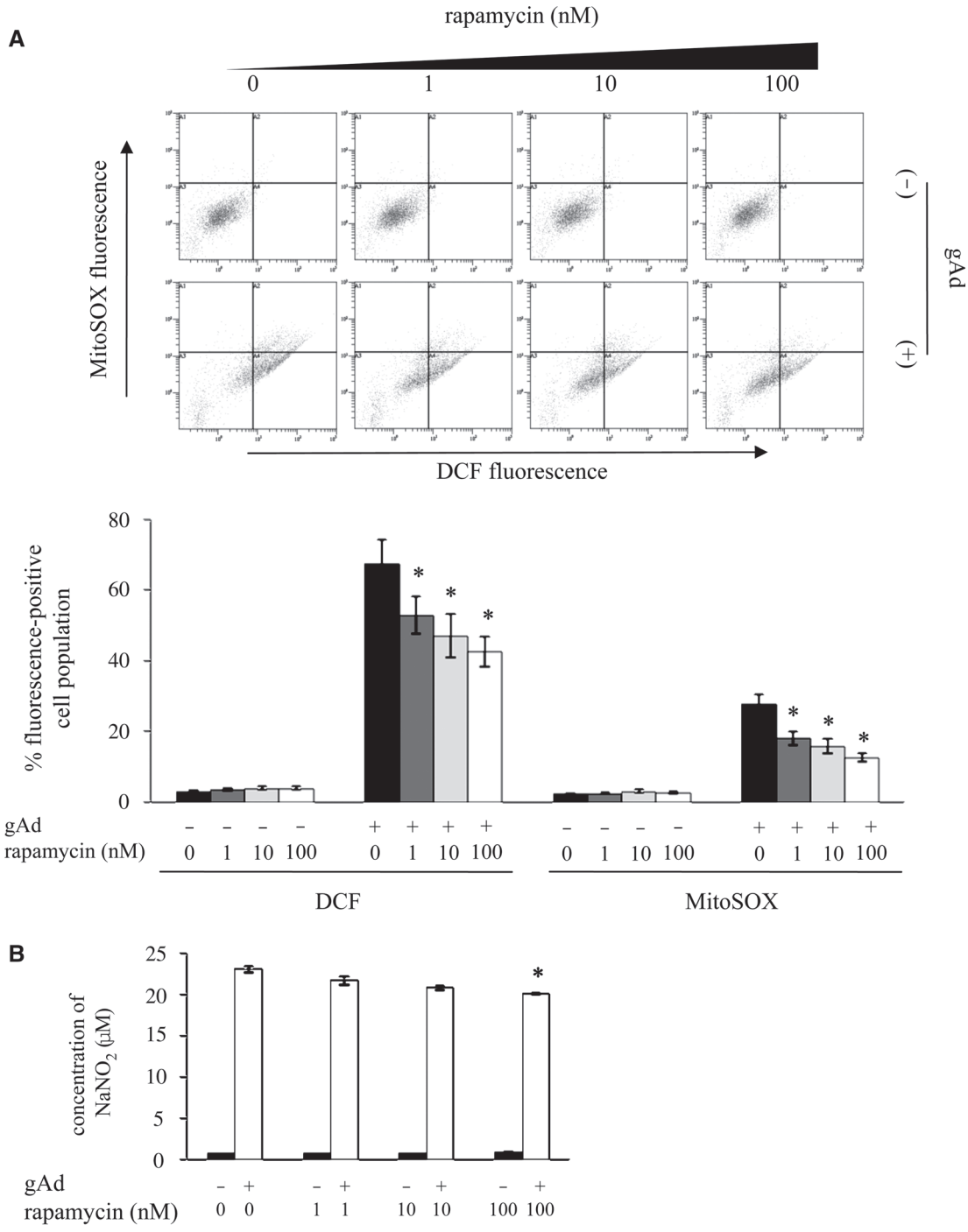

Figure 2. Effect of rapamycin of gAd-induced ROS and NO generation in RAW 264 cells. RAW 264 cells were treated with rapamycin (range 1–100 nM) with or without 10 μg/ml gAd for 24 h. (A) Effect of rapamycin on gAd-induced ROS generation. The intracellular and mitochondrial ROS, assessed by DCF and MitoSOX fluorescence, respectively, were calculated. Error bars indicate SD. (B) Effect of rapamycin on gAd-induced NO release. Amounts of released NO were measured by Griess method as described in the Materials and methods section. $*p<0.05$ compared with cells stimulated with gAd alone.

Western blot analysis

Cell extracts were resolved by SDS-PAGE before being transferred to PVDF membranes (Bio-Rad Laboratories). After blocking, the membranes were incubated with primary and secondary antibodies and then washed thoroughly and examined using ECL Plus (GE Healthcare, Amersham Biosciences). The band densities were scanned and quantified by LAS-1000plus (Fuji Film, Tokyo, Japan). Anti-Akt, phospho-Akt (Ser473), mTOR, phospho-mTOR (Ser2481)-specific antibody (Cell Signaling Technology, Danvers, MA) and anti-actin antibody (MP Biomedicals, Solon, OH) were used for the primary antibodies. HRP-linked anti-rabbit IgG antibodies

(Cell Signaling Technology) and HRP-linked anti-mouse IgG antibodies (Zymed Laboratories, San Francisco, CA) were used as secondary antibodies.

Analysis of nitrite status

NO release in cultured macrophages was measured using a microplate assay method, as described previously. To measure nitrite (NO^{2-}), 100 μl of macrophage culture supernatant was mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride, 2.5% H_3PO_4) and incubated for 10 min at room temperature. The NO_2 concentration was measured as the absorbance at 540 nm using a SpectraMax 340PC^{384} spectrometer (Molecular Devices, Sunnyvale, CA) after external calibration with $NaNO_2$.

Statistical analysis

Statistical analysis was performed using Student's *t*-test; $p<0.05$ was considered significant.

Results

gAd stimulation induces mTOR phosphorylation

To investigate whether mTOR involves gAd-stimulated intracellular signalling in the murine macrophage-like cell line RAW 264, we assessed the phosphorylation of mTOR by Western blotting. Phosphorylated mTOR was detected at 10 min, peaked at 20 min and rapidly dissolved within 50 min in gAd-treated cells (Figure 1A). Next, to examine the signal transducer for gAd-induced mTOR activation, we tested the effect of phosphatidylinositol 3-kinase (PI3K)-specific inhibitor, wortmannin, on mTOR phosphorylation in gAd-treated RAW 264 cells. RAW 264 cells pre-treated with 100 nM wortmannin were incubated with gAd for 20 min. As shown in Figure 1B, pre-treatment with wortmannin clearly dissolved both gAd-induced Akt and mTOR phosphorylation. These data suggest that phosphorylation of mTOR is involved in the gAd-stimulating signal transducing pathway via PI3K/Akt activation.

Rapamycin inhibits gAd-evoked ROS and NO generation in RAW 264 cells

We previously reported that gAd stimulation induced intracellular and mitochondrial ROS generation and NO release in RAW 264 cells [14]. To examine the role of mTOR, we studied the effect of mTOR-specific inhibitor, rapamycin, on ROS/NO generation in gAd-treated RAW 264 cells. We assessed the generation of intracellular and mitochondrial ROS by the populations of DCF- and MitoSOX-positive cells, respectively, using flow cytometry. As shown in Figure 2A, administration of rapamycin (1 nM) significantly reduced DCF- and MitoSOX-positive cell populations (52.9% ± 6.7% and 18.0% ± 1.6%, respectively), compared to medium alone (67.5% ± 7.5% and 27.7% ± 2.6%, respectively), in gAd-treated RAW 264 cell. The administration of rapamycin (range 1–100 nM) had no effect on cell viability as assessed by MTT methods (data not shown). On the contrary, the administration of rapamycin had less effect on gAd-induced NO. There was no significant inhibitory effect of 1 nM rapamycin treatment on gAd-induced NO release (Figure 2B). However, the amount of gAd-induced NO slightly lower in 100 nM rapamycin-treated cells (20.1 ± 0.4 μM of $NaNO_2$) compared to medium alone (23.1 ± 0.3 μM of $NaNO_2$).

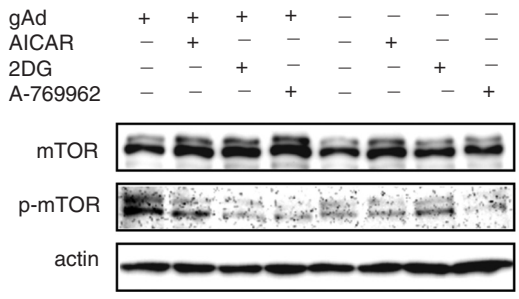

Figure 3. Effect of AMPK activators on gAd-induced mTOR phosphorylation. AICAR (1 mM), 2-DG (5 mM) or A-769662 (200 μM) was administrated with gAd (10 μg/ml) in culture medium. The protein levels of mTOR and phospho-mTOR (p-mTOR) were detected by Western blotting. Each experiment was performed three times and all experiments showed similar results.

Activation of AMPK suppresses gAd-induced mTOR phosphorylation, followed by ROS/NO generation

Recent evidence has demonstrated that the activity of mTOR is negatively controlled by AMP-activated protein kinase (AMPK) as an Akt/PI3K-independent pathway [8]. To investigate the effect of AMPK activation on gAd-induced mTOR phosphorylation, we examined three kinds of AMPK activators, AICAR, 2-DG and A-769662, in gAd-treated RAW 264 cells. As shown in Figure 3, administration of AMPK activators apparently reduced the level of phospho-mTOR. Next, we analysed the effect of AMPK activators on gAd-evoked ROS/NO generation. Each activator clearly reduced gAd-evoked intracellular ROS generation (Figure 4A). While AICAR and 2-DG increased mitochondrial ROS, A-769662 critically suppressed gAd-induced mitochondrial ROS generation (Figure 4A). As shown in Figure 4B, the amounts of NO released from gAd-treated RAW 264 cells were significantly lower after treatment with AICAR, 2-DG and A-769662 (13.2 ± 0.4,

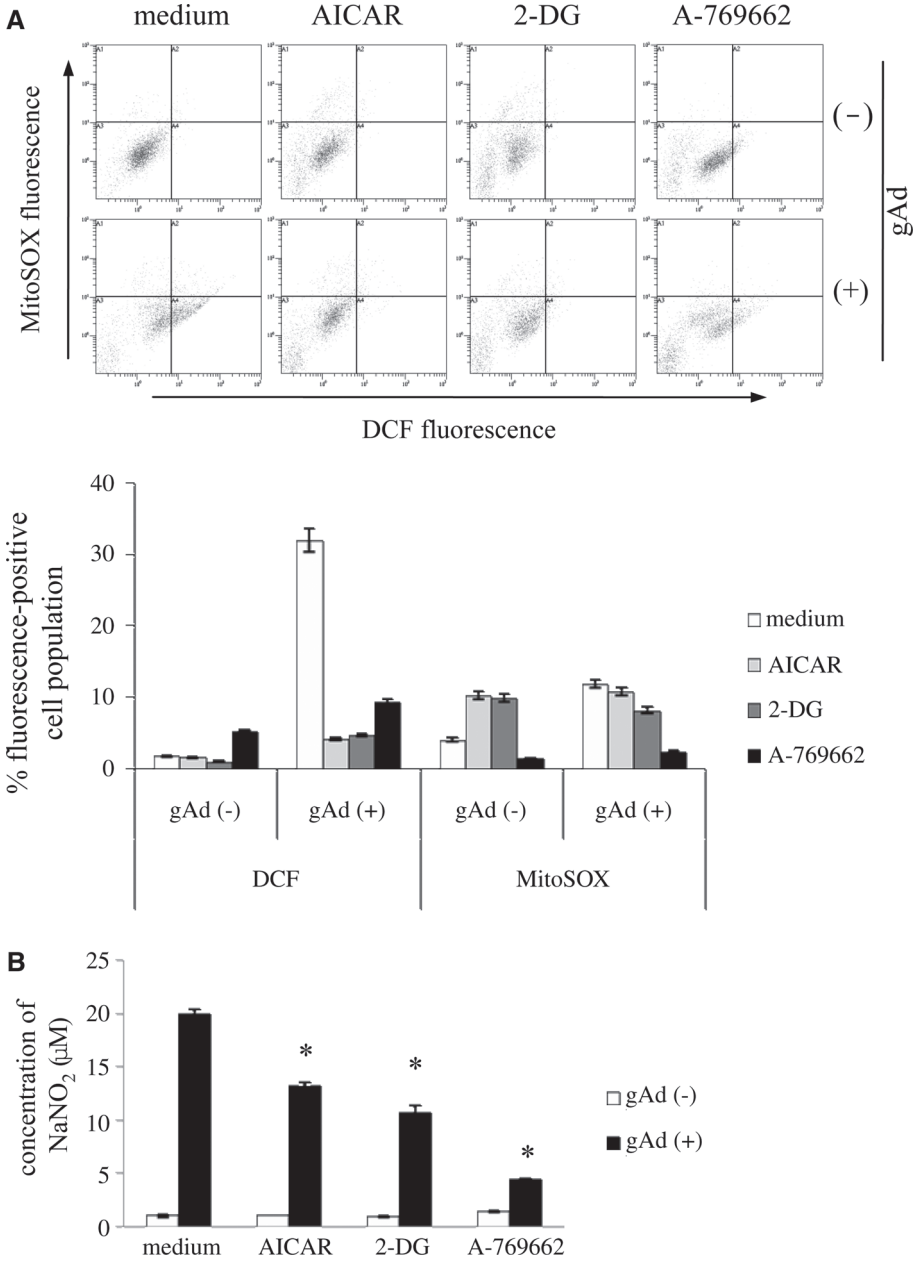

Figure 4. Effect of AMPK activators on gAd-induced ROS and NO generation in RAW 264 cells. RAW 264 cells were cultured with AICAR (1 mM), 2-DG (5 mM) or A-769662 (200 μM). (A) Intracellular and mitochondrial ROS assessed by DCF and MitoSOX fluorescence, respectively, using flow cytometry as described in the Materials and methods section and populations of each fluorescence-positive cell were calculated. (B) Culture media were collected at the end of the culture period and the NO concentration was measured by the Griess method, after conversion to nitrite. $*p<0.05$ compared with cells stimulated with gAd alone.

10.7 ± 0.6 and 4.4 ± 0.1 μM of $NaNO_2$, respectively) compared to medium alone (19.9 ± 0.5 μM of $NaNO_2$).

Discussion

In this study, we demonstrated that gAd stimulation induced phosphorylation of mTOR via the PI3K/Akt signal-transducing pathway and that rapamycin partially reduced gAd-evoked intracellular and mitochondrial ROS generation. The gAd-induced mTOR phosphorylation and sequentially provoked ROS/NO generation were inhibited by AMPK activators in RAW 264 cells.

Our previous report indicated that gAd induced iNOS mRNA expression and up-regulated NOS activity in RAW 264 cells. A recent study reported that the administration of rapamycin inhibited lipopolysaccharide (LPS)-induced NO production by iNOS protein degradation in a lactacystin (an inhibitor of 20S proteasome)-sensitive manner in RAW 264.7 cells [15]. According to that report, however, administration of range 1–100 nM of rapamycin had a slight effect and excess concentration of rapamycin (1 μM)

reduced the amount of LPS-induced NO release to about only 50% of the NO released without rapamycin. In this study, the administration of rapamycin (range 1–100 nM) had a little effect on gAd-induced NO release (Figure 2B). In contrast, treatment with a lower dose (1 nM) of rapamycin had a significant inhibitory effect on gAd-evoked ROS generation (Figure 2A). The relationship between ROS and Akt/mTOR is indicated in various stimuli and/or cell lines. A recent report demonstrated that ROS generation in breakpoint cluster region-abelson-transformed cells required PI3K/Akt/mTOR activation [16]. Taken together, mTOR closely related with gAd-induced ROS generation, but is not critical in NO release in RAW 264 cells.

The alternative mTOR regulation mechanism is well established as an Akt-independent energy-sensing pathway related to APMK activation [8]. AMPK is activated when decreases occur in the cellular energy state, as reflected by an increase in the AMP:ATP ratio. Activated AMPK is phosphorylated by two upstream kinases (CaMKK and LKB1). It enhances ATP generation and inhibits enzymes that consume ATP but are not acutely necessary for survival. One target of AMPK in an energy-depleted status is mTOR via TSC2 and RAPTOR phosphorylation. Previous studies in human prostate cancer cells demonstrated that adiponectin promotes the phosphorylation of both AMPK and Akt following mTOR phosphorylation [17]. In contrast, most recent studies in colorectal cancer have indicated that stimulation of adiponectin activated AMPK and suppressed mTOR [18]. The growth of both prostate and colorectal cancer cells are inhibited by adiponectin administration *in vitro*. In addition, in other cell types such as adipocytes, myotubes and osteoprogenitor cell HepG2 cells, adiponectin activates AMPK, but not Akt [17,19]. In RAW 264 cells, gAd activates both AMPK and Akt according to our observations. The conflicting results between different cell types in the involvement of Akt/mTOR activation in the adiponectin-stimulating signal pathway are still unknown. The signal transduction pathways of the sequential agents of adiponectin receptors remain to be confirmed.

Administration of AICAR induces mitochondrial oxygen radical formation via activation of complex I followed by apoptotic cell death in rat pancreatic beta cells [20]. In this study, administration of AICAR and 2-DG induced mitochondrial ROS generation (Figure 4A). However, A-769662 treatment crucially attenuated mitochondrial ROS generation with or without gAd. In addition, A-769662 exerted a stronger inhibitive effect on gAd-induced NO release compared to AICAR and 2-DG. While this indicates that AICAR causes side-effects not related to AMPK activity, the difference between the actions of A-769662 and other AMPK activators on ROS/NO generation is not presently apparent.

We used three kinds of pharmacological agents as AMPK activators, AICAR, 2-DG and A-769662. AICAR and A-769662 directly activate AMPK and 2-DG indirectly activates AMPK by increasing intracellular AMP [8]. These AMPK activators critically inhibited mTOR phosphorylation and ROS/NO production in gAd-treated RAW 264 cells. Recent studies have reported that rosiglitazone (a drug in the thiazolidinedione class that targets insulin resistance) induced AMPK activation reduced glucose-evoked ROS generation via inhibition of PKC, followed by inactivation of NAD(P)H oxidase [21]. We previously reported that gAd-induced ROS generation was suppressed by pre-treatment with the PKC inhibitors Goe 6983 and bisindolylmaleimide I, but not with an AMPK inhibitor, compound C [14]. Taken together, although gAd activates AMPK, AMPK activity may not be critical in gAd-induced ROS generation in RAW 264 cells.

Acknowledgements

Support for this research was provided by a Grant-in-Aid for Scientific Research (C) 20592457 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

***Declaration of interest*:** The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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This paper was first published online on Early Online on 29 October 2009.