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Diacylglycerol kinase alpha regulates globular adiponectin-induced reactive oxygen species

NORIAKI KAMIO[1,2], SUMIO AKIFUSA[1] & YOSHIHISA YAMASHITA[1]

[1]*Section of Preventive and Public Health Dentistry, Division of Oral Health, Growth and Development, Faculty of Dental Science, Kyushu University, Fukuoka, Japan, and* [2]*Department of Oral Health Sciences, Nihon University School of Dentistry, Tokyo, Japan*

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Abstract:
It has previously been reported that the globular form of adiponectin (gAd), mature adipocyte-derived cytokine, induced generation of reactive oxygen species (ROS) and nitric oxide (NO) in the murine macrophage cell line RAW 264. This study investigated whether diacylglycerol kinases (DGKs), enzymes functioning in sub-cellular signalling pathways, had a role on gAd-induced ROS generation in RAW 264 cells. Administration of R59022, a specific inhibitor for DGK, reduced gAd-induced ROS generation and NO release. RAW 264 cell expressed DGKα mRNA. Depression of DGKα mRNA by RNA interference significantly reduced the ROS generation in response to gAd treatment. Interestingly, transfection with the DGKα-specific small interfering RNA attenuated the expression level of Nox1 mRNA in gAd-treated RAW 264 cells. In addition, the DGKα knockdown with siRNA suppressed gAd-induced NO release.

Keywords: *Adiponectin, diacylglycerol kinase, reactive oxygen species, nitric oxide*

Introduction

Adiponectin is an adipocyte-derived hormone that enhances insulin sensitivity and promotes lipid metabolism [1,2]. It shares sequence homology with a family of proteins characterized by an amino-terminal collagen-like region and a carboxy-terminal complement factor C1q-like globular domain [3]. Adiponectin exists in two forms: full-length adiponectin and globular-domain adiponectin (gAd), which is produced by proteolytic cleavage of the full-length form by leukocyte elastase [4]. In addition, there are full-length adiponectin isoforms of low, middle and high molecular weights [5]. Reduced adiponectin secretion is associated with obesity [6]. Obesity-linked down-regulation of adiponectin is a mechanism by which obesity can cause insulin resistance and diabetes [5]. The anti-inflammatory effects of adiponectin involve regulation of lipopolysaccharide-stimulated NF-κB activation and cytokine production [7–10]. However, stimulation of adiponectin alone induces pro-inflammatory responses in immune and epithelial cells [11–13]. High-molecular-weight adiponectin and gAd activate NF-κB in monocytic cells, whereas middle- and low-molecular-weight adiponectin do not [14]. In our previous studies, we showed that gAd evoked the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are synthesized by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) and inducible nitric oxide synthase (iNOS), respectively, in the murine macrophage-like cell line RAW 264 [15].

Diacylglycerol kinases (DGKs) are members of a unique and conserved family of intracellular lipid kinases that phosphorylate diacylglycerol, catalysing its conversion to phosphatidic acid (PA) [16]. Diacylglycerol regulates a wide variety of cellular functions [17]. By regulating local diacylglycerol signalling,

Correspondence: Sumio Akifusa, Section of Preventive and Public Health Dentistry, Division of Oral Health, Growth and Development, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan. Fax: +81 92 642 6354. Email: akifusa@dent.kyushu-u.ac.jp

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DGK plays a central role in many biological responses such as cell proliferation, differentiation, survival and apoptosis [18]. PA regulates a number of signalling proteins such as RasGAP [19], Raf-1 kinase [20], mammalian target of rapamycin [21] and $p47^{phox}$, which is a cytosolic sub-unit required for activation of Nox2 [22].

In the present study, we analysed the role of DGKs in gAd-induced ROS generation in RAW 264 cells.

Materials and methods

Reagents

The DGK inhibitor 6-{2-{4-[(*p*-fluorophenyl) phenylmethylene]-1-piperidinyl}ethyl}-7-methyl-5H-thiazolo(3,2-a)pyrimidine-5-one (R59022) was purchased from Calbiochem (La Jolla, CA). MitoSOX™ and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Molecular Probes (Eugene, OR). Griess-Romijn nitrite reagent was purchased from WAKO (Osaka, Japan).

Cell culture

The murine macrophage-like cell line RAW 264 (RCB0535; RIKEN Cell Bank, Ibaraki, Japan) was maintained in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% foetal bovine serum (Thermo Trace Ltd., Melbourne, Australia) and 50 μg/mL gentamicin. The cultures were maintained at 37°C under 5% CO_2.

Purification of recombinant gAd

The glutathione *S*-transferase (GST) fusion vector pGEX-6P-1 (GE Healthcare, Piscataway, NJ) containing the globular domain (gAd) of the mouse adiponectin gene was provided by Dr. I. Shimomura (Osaka University, Osaka, Japan). Recombinant gAd was prepared as described previously [8]. Briefly, GST-gAd protein was produced in *Escherichia coli* BL21 and purified using glutathione Sepharose 4B (GE Healthcare). The GST tag was cleaved using PreScission Protease (GE Healthcare). The isolated protein was then mixed with Affi-Prep Polymyxin Support (Bio-Rad Laboratories, Hercules, CA) to remove endotoxin contaminants.

Real-time RT-PCR

Total RNA was isolated from treated cells using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). The RNA samples were reverse transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The following primers were designed from recently published sequences: DGK*α*, forward 5′-GATGCAGGCACCC TGTACAAT-3′ and reverse 5′-GGACCCATAAGC ATAGGCATCT-3′ (product size: 63 bp) [23]; and *β*-actin, forward 5′-GGTCAGAAGGACTCCTAT GTGG-3′ and reverse 5′-TGTCGTCCCAGTTGGT AACA-3′ (product size: 103 bp) [10]. Amplification was performed using a StepOne Real-Time PCR System (Applied Biosystems) with 2 × QuantiTect SYBR Green PCR Master Mix (Qiagen) in a total volume of 20 μL. Primer and fluorescent probe sets for *β*-actin (Mm00607939_s1), Nox1 (Mm00549170_m1) and Nox2 (Mm01287742_m1) were purchased from Applied Biosystems. Amplification and detection of cDNA were performed using a StepOne Real-Time PCR System (Applied Biosystems) with TaqMan Fast Universal PCR Master Mix (Applied Biosystems) in a total volume of 20 μL.

Determination of nitric oxide levels

The secretion of NO by cultured macrophages was measured by a microplate assay method as described previously [24]. Each culture supernatant (100 μL) was mixed with an equal volume of Griess-Romijn nitrite reagent and the absorbance at 540 nm was measured in a microplate reader (SpectraMax 340PC^{384} spectrometer; Molecular Devices, Sunnyvale, CA). $NaNO_2$ was used for calibration.

Analysis of cellular redox status

To detect intracellular and mitochondrial ROS formation, we determined H_2DCFDA and MitoSOX fluorescence by flow cytometry. Cells were incubated for the indicated lengths of time at 37°C and in 5% CO_2. Phosphate-buffered saline (PBS) containing 5 μM H_2DCFDA (DCF) and 5 μM MitoSOX was then added and the cells incubated for another 30 min. The labelled cells were washed with PBS and suspended in PBS for analysis by flow cytometry. The fluorescence intensity of DCF was measured using an excitation wavelength of 488 nm and emission wavelength of 580 nm. MitoSOX fluorescence was measured with excitation at 510 nm and emission at 580 nm. The resultant histograms were analysed using the Expo32 program (Beckman Coulter, Fullerton, CA) after subtraction of background fluorescence.

RNA interference

A non-specific scrambled control small interfering RNA (siRNA) and siRNA against DGK*α* were purchased from Invitrogen (Carlsbad, CA). RAW 264 cells were transfected with siRNA (100 pmol) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

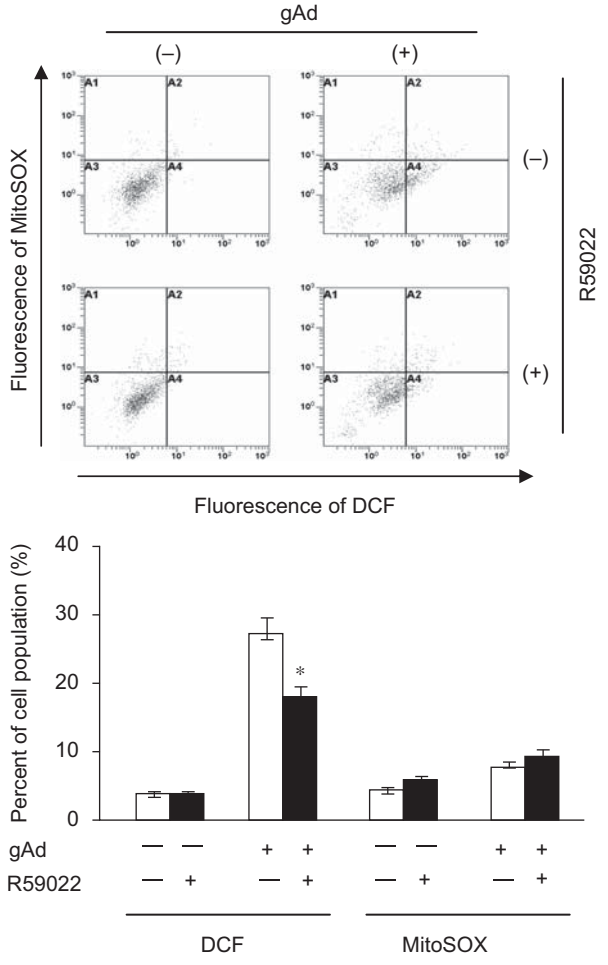

Figure 1. Effect of DGK inhibitor on gAd-induced ROS. RAW 264 cells were pre-treated with or without 10 μM R59022 and stimulated with 10 μg/mL gAd for 24 h. DCF-positive and MitoSOX-positive cell populations indicate intracellular and mitochondrial ROS, respectively, as measured by flow cytometry as described in Materials and methods. Sums of population in A2 + A4 and A1 + A2 plotted area were designated DCF- and MitoSOX-positive population, respectively. Values are means ± SD, $n = 3$, $^{*}p < 0.01$ compared to cells treated without R59022.

Statistical analysis

The significance of differences was determined using Student's *t*-test or one-way analysis of variance with Newman-Keul or Bonnferroni post-hoc testing. Values of $p < 0.05$ were taken to indicate statistical significance.

Results

DGK inhibition suppresses gAd-induced ROS and NO generation

Previously, we reported that gAd stimulation induces ROS generation and NO release in RAW 264 cells [15]. To investigate the role of DGK, we examined the effect of R59022, a specific inhibitor of DGK, on ROS/NO generation in gAd-treated RAW 264 cells. We assessed the generation of ROS in the intracellular and mitochondrial compartments of gAd-treated RAW 264 cells by measuring DCF and MitoSOX fluorescence, respectively. As shown in Figure 1, gAd stimulation increased the number of cells positive for DCF (sum of the populations in the A2 and A4 plotted areas). Pre-treatment with 10 μM R59022 significantly reduced intracellular ROS generation in gAd-treated RAW 264 cells. Furthermore, we examined whether R59022 could inhibit gAd-induced NO production. The quantity of NO released was measured, after its conversion to nitrite, using the Griess method. As shown in Figure 2, pre-treatment with 10 μM R59022 markedly reduced NO release from gAd-treated RAW 264 cells (13.9 ± 1.5 μM $NaNO_2$) compared with that from cells pre-treated with medium alone (35.5 ± 0.4 μM $NaNO_2$).

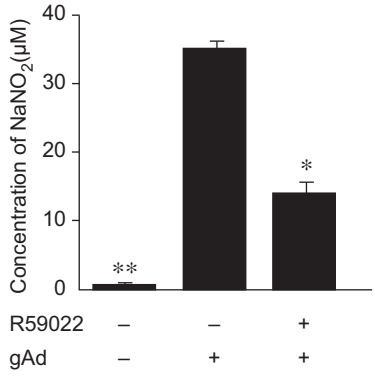

Figure 2. Effect of DGK inhibitor on gAd-induced NO release. RAW 264 cells were pre-treated with or without 10 μM R59022 and exposed to 10 μg/mL gAd for 24 h. The amount of reduced NO was measured by the Griess method as described in Materials and methods. Values are means ± SD, $n = 3$, $^{*}p < 0.05$; $^{**}p < 0.01$.

Effects of DGKα siRNA on gAd-induced ROS and NO generation in RAW 264 cells

We further examined whether DGK*α*, one of the best-characterized DGK isoforms, is involved in gAd-induced ROS and NO generation in RAW 264 cells. DGK*α* mRNA is expressed in RAW 264 cells, as determined by RT-PCR (Figure 3). We conducted RNA interference studies to examine the effect of DGK*α* knockdown on ROS generation in gAd-treated RAW 264 cells. DGK*α*-specific siRNA knocked down DGK*α* mRNA levels to < 30% of the levels in cells transfected with control siRNA (data not shown). After transfection with DGK*α*-specific or control siRNA, gAd-induced intracellular and mitochondrial ROS levels were assessed by monitoring DCF and MitoSOX fluorescence. As shown in Figure 4A, knockdown of DGK*α* mRNA markedly reduced gAd-induced intracellular and mitochondrial ROS generation. To confirm the relationship between DGK*α* and NADPH oxidase, we examined the expression level of the major component of NADPH oxidase, Nox1.

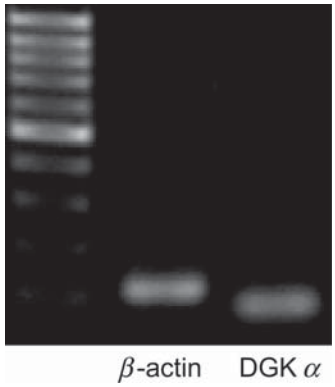

Figure 3. Expression of DGK*α* mRNA in RAW 264 cells. RT-PCR was performed as described in Materials and methods. Products of RT-PCR were electropheresed in 2% agarose gel and then visualized with ethidium bromide staining.

Treatment with gAd induced Nox1 mRNA expression in RAW 264 cells (Figure 4B). Transfection with DGK*α*-specific siRNA reduced gAd-induced Nox1 mRNA expression by 52.8% ± 12.9%, compared with the level in cells transfected with non-specific control siRNA. In addition, as shown in Figure 5, depression of DGK*α* expression by DGK*α*-specific siRNA suppressed gAd-induced NO release (37.0 ± 1.9 μM) compared with that of cells transfected with control scrambled siRNA (46.6 ± 1.0 μM).

Discussion

This study shows that inhibition of DGK*α* activity by a specific inhibitor or siRNA reduces gAd-induced ROS and NO generation, suggesting that DGK*α* is required in the gAd-induced ROS/RNS pathway. Type I DGKs, including DGK*α* [25], DGK*β* [26] and DGKγ [27], have two sets of calcium-binding EF-hand motifs that render them more active in the presence of calcium [28]. We previously demonstrated that gAd-induced intracellular and mitochondrial ROS generation and NO release involve Ca^{2+} signalling [29]. In a recent study, inhibition of DGK with R59022 or through DGK*α* siRNA transfection decreased oxidative stress-induced apoptosis in RIE-1 cells [18].

In the present study, we demonstrated that gAd stimulation induced the expression of Nox1 mRNA and DGK*α* knockdown suppressed the gAd-induced Nox1 mRNA in RAW 264 cells. Members of the Nox family share the capacity to transport electrons across the plasma membrane and to generate superoxide and other downstream ROS [30]. There have been a number of reports regarding the role of Nox proteins in various processes such as cell survival and apoptosis [30]. PA, the reaction product of DGK, regulates $p47^{phox}$, a key cytosolic sub-unit required for activation of phagocyte NADPH oxidase [22]. Nox1 mRNA is most highly expressed in colon epithelia, but it is also expressed at lower levels in endothelial cells,

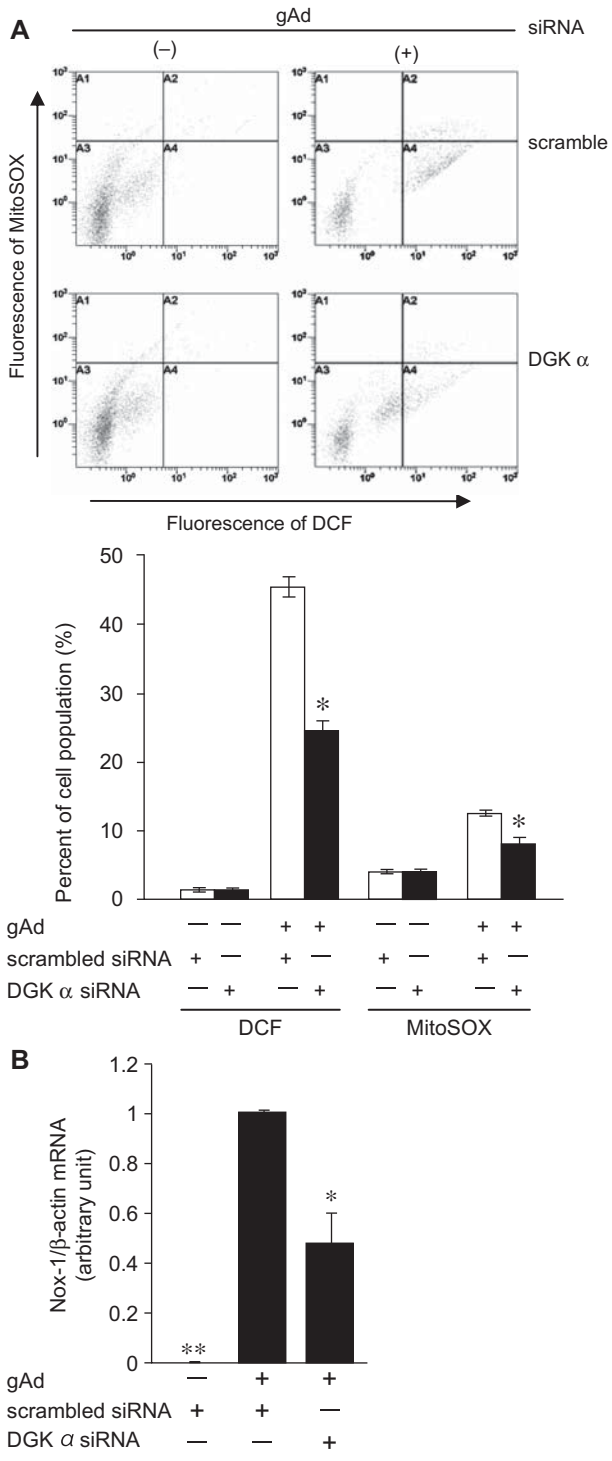

Figure 4. Effect of DGKα siRNA on gAd-induced ROS generation in RAW 264 cells. RAW 264 cells were transfected with DGK*α*-specific or non-specific scrambled siRNA, as described in Materials and methods. (A) The transfected RAW 264 cells were stimulated with 10 μg/mL gAd for 24 h. DCF-positive and MitoSOX-positive cell populations indicate intracellular and mitochondrial ROS, respectively, as measured by flow cytometry as described in Materials and methods. Values are means ± SD, $n = 3$, $^{*}p < 0.01$ compared to control transfectant with gAd. (B) The transfected RAW 264 cells were exposed to 10 μg/mL gAd for 4 h. The amount of Nox1 mRNA was then measured by real-time RT-PCR. Values are means ± SD, $n = 3$. $^{*}p < 0.05$; $^{**}p < 0.01$ compared to control transfectants with gAd.

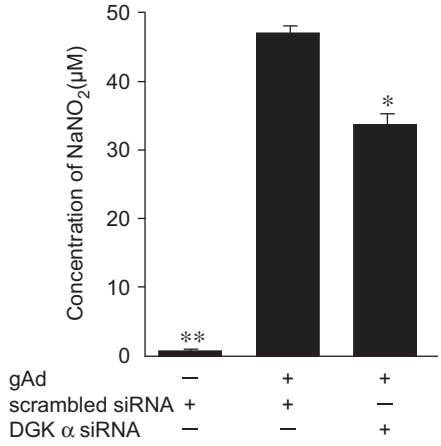

Figure 5. Effect of DGK*α* siRNA on gAd-induced NO release in RAW 264 cells. RAW 264 cells were transfected with DGK*α*-specific or non-specific scrambled siRNA, as described in Materials and methods. The transfected RAW 264 cells were exposed to 10 μg/mL gAd for 24 h. The amount of reduced NO was measured by the Griess method as described in Materials and methods. Values are means ± SD, $n = 3$, $^{*}p < 0.05$; $^{**}p < 0.01$ compared to control transfectants with gAd.

osteoclasts and microglia [31]. Nox1 becomes activated by forming a complex with cytosolic activators, in a manner similar to Nox2. It can interact with $p47^{phox}$, $p67^{phox}$ and the small GTPase Rac, but it is most highly activated by Nox organizer 1 and Nox activator 1, which are $p47^{phox}$ and $p67^{phox}$ homologues, respectively [31]. Nox1 has been linked to cell death in pancreatic acinar AR42J cells [32] and embryonic kidney 293T cells [33]. In contrast, Nox2 mRNA is continuously expressed in RAW 264 cells (data not shown). Recently, we found that transfection of siRNAs specific for $p47^{phox}$ and $gp91^{phox}$ (Nox2) significantly reduces gAd-induced ROS generation [15]. Nox2 activation involves its association with GTP-Rac, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ [31]. Nox2-derived ROS in macrophages have been reported to induce apoptosis by activating the ASK1-p38 MAP kinase pathway [34]. The reason why the Nox2 mRNA level remains unchanged in gAd-stimulated cells is not clear. It is possible that protein modification, for example phosphorylation or methylation, may alter Nox2 activity following gAd stimulation.

We previously demonstrated that gAd treatment induced mitochondrial ROS generation, as detected by MitoSOX fluorescence and that Bcl-2 over-expression significantly reduced intracellular and mitochondrial ROS generation in gAd-treated RAW 264 cells [35]. Administration of DGK*α* siRNA had no effect on Bcl-2 expression in gAd-treated RAW 264 cells (data not shown). However, given that knocking down DGK*α* through mRNA interference significantly suppressed gAd-evoked mitochondrial ROS generation, there may be a critical relationship between DGK*α* and mitochondrial activity. Additionally, the DGK-specific inhibitor R59022 had no effect on mitochondrial ROS generation in gAd-treated RAW 264 cells (Figure 1). It is unclear why the inhibition of DGK*α* by siRNA, but not by R59022, reduced gAd-induced mitochondrial ROS generation. The fact that treatment with R59022 alone induced a slight increase in mitochondrial ROS levels suggests that R59022 may have unknown effects on mitochondrial activity.

Physiological and sub-physiological concentrations of NO prevent apoptosis induced by trophic factor withdrawal, Fas, TNF*α* and lipopolysaccharide, whereas high concentrations of NO and peroxynitrite can induce apoptosis [36]. Moreover, NO donors can elevate the Bcl-2 mRNA and protein levels, thereby inhibiting apoptotic cell death [37], although NO-induced apoptosis of human myeloid leukaemia U937 cells was associated with down-regulation of Bcl-2 and caspase activation [38]. We previously demonstrated that NO generation is essential for gAd-induced apoptosis in RAW 264 cells [15]. In the present study, R59022 and knockdown of DGK*α* reduced gAd-induced NO release, suggesting that DGK*α* regulates NO production in gAd-treated RAW 264 cells.

In conclusion, R59022 and a DGK*α*-specific siRNA reduced gAd-evoked ROS and NO generation. In addition, DGK*α* knockdown significantly reduced NOX1 mRNA expression in response to gAd treatment. Taken together, these results suggest that the gAd-induced generation of ROS and NO involves DGK*α*.

Declaration of interest

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