Diacylglycerol kinase inhibitor R59022 attenuates conjugated linoleic acid-mediated inflammation in human adipocytes

Kristina Martinez,* Shruthi Shyamasundar,* Arion Kennedy,† Chia Chi Chuang,* Angel Marsh,* Jennifer Kincaid,* Tanya Reid,* and Michael McIntosh†,*

Department of Nutrition,* University of North Carolina at Greensboro, Greensboro, NC; and Department of Molecular Physiology,† Vanderbilt University Medical Center, Nashville, TN

Abstract  Diacylglycerol kinases (DGK) convert diacylglycerol to phosphatidic acid, which has been reported to stimulate calcium release from the endoplasmic reticulum. Based on our published data showing that trans-10, cis-12 conjugated linoleic acid (t10,c12 CLA)-mediated intracellular calcium accumulation is linked to inflammation and insulin resistance, we hypothesized that inhibiting DGKs with R59022 would prevent t10,c12 CLA-mediated inflammatory signaling and insulin resistance in human adipocytes. Consistent with our hypothesis, R59022 attenuated t10,c12 CLA-mediated i) increased gene expression and protein secretion of interleukin (IL)-8, IL-6, and monocyte chemoattractant protein-1 (MCP-1); ii) increased activation of extracellular signal-related kinase (ERK), c-Jun-NH2-terminal kinase (JNK), and cJun; iii) increased intracellular calcium levels; iv) suppressed mRNA or protein levels of peroxisome proliferator activated receptor γ, adiponectin, and insulin-dependent glucose transporter 4; and v) decreased fatty acid and glucose uptake and triglyceride content. Decreased DGK expression was dose-dependently decreased with R59022. Small interfering RNA (siRNA) targeting DGKγ decreased t10,c12 CLA-induced DGKγ, IL-8, and MCP-1 gene expression, as well as activation of JNK and cJun. Taken together, these data suggest that DGKs mediate, in part, t10,c12 CLA-induced inflammatory signaling in primary human adipocytes.—Martinez, K., S. Shyamasundar, A. Kennedy, C. C. Chuang, A. Marsh, J. Kincaid, T. Reid, and M. McIntosh. Diacylglycerol kinase inhibitor R59022 attenuates conjugated linoleic acid-mediated inflammation in human adipocytes. J. Lipid Res. 2013. 54: 662–670.

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Overweight and obesity are global health issues affecting 1.6 billion individuals worldwide (1). One potential strategy for reducing adiposity is consumption of conjugated linoleic acid (CLA), a group of conjugated octadecadienoic acid isomers derived from linoleic acid, a fatty acid (FA) that contains 18 carbons and 2 double bonds in the cis configuration at the 9th and 12th carbons (i.e., cis9, cis12 octadecadienoic acid) (2). CLA is found in ruminant meats and dairy products, as microbes in the gastrointestinal tract of ruminant animals convert linoleic acid into different isomers of CLA through biohydrogenation (2). This process changes the position and configuration of the double bonds, resulting in a single bond between the two double bonds. The major isomers produced include cis9, trans11 (c9,t11) and trans-10, cis-12 (t10,c12) CLA. Food sources of CLA contain ~80% c9,t11 CLA and 10% t10,c12 CLA, and the remaining 10% is composed of other isomers (2). CLA is also produced chemically from linoleic acid for inclusion in supplements and fortified foods, yielding a composition containing ~40% c9,t11 CLA, ~40% t10,c12 CLA isomers, and the remaining 20% other isomers (2).

Abbreviations:  ACC, acetyl-CoA carboxylase; AM-1, adipocyte media; AP-1, activator protein; αP2, adipocyte fatty acid binding protein; apm-1, adiponectin; [Ca2+]i, intracellular calcium; CaM, calmodulin; CaMK, calmodulin kinase; CLA, conjugated linoleic acid; c9,t11 CLA, cis9, trans-11 CLA; t10,c12 CLA, trans-10, cis-12 CLA; COX, cyclooxygenase; DAG, diacylglycerol; DGK, diacylglycerol kinase; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FFA1, free fatty acid receptor 1; GLUT4, insulin-dependent glucose transporter 4; GPCR, G-protein coupled receptor; IBMX, 1-methyl-3-isobutylxanthine; IL, interleukin; IP, inositol phosphate; IRS, insulin receptor substrate; JNK, c-Jun-NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MCP, monocyte chemoattractant protein; MEK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NFκB, nuclear factor kappa B; PA, phosphatidic acid; PC, phosphatidyl choline; PG, prostaglandin; Pt, phosphatidyl inositol; PIP2, phosphatidylinositol-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PPAR, peroxisome proliferator activated receptor; ROS, reactive oxygen species; S6K, S6 kinase; SCD, stearoyl-CoA desaturase; siRNA, small interfering RNA; TG, triglyceride; TNF, tumor necrosis factor.

† To whom correspondence should be addressed.
e-mail: mkmcinto@uncg.edu

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Consuming a mixture of c9,t11 and t10,c12 CLA isomers or consuming t10,c12 CLA alone reduces body fat mass in rodents, particularly mice, and in some humans (3). However, the isoform-specific mechanism by which CLA reduces adiposity is unclear. Proposed antiobesity mechanisms of t10, c12 CLA include regulation of i) energy metabolism, ii) adipogenesis, iii) lipid metabolism, iv) inflammation, and v) adipocyte apoptosis (reviewed in Ref. 3). However, direct linkage of these potential mechanisms to body fat loss, especially inflammation, is unclear. We have demonstrated that activation of extracellular signal-regulated kinase (ERK) (4) and nuclear factor kappa B (NFκB) play a role in t10,c12 CLA-mediated delipidation and insulin resistance (5). We have also shown that t10,c12 CLA-mediated activation of ERK, c-Jun N-terminal kinase (JNK), NFκB, and production of reactive oxygen species (ROS) was dependent on accumulation of intracellular calcium levels (6). Additionally, we demonstrated that TMB-8, an inhibitor of calcium release from the endoplasmic reticulum (ER), prevents t10,c12 CLA-mediated NFκB binding to promoters of interleukin (IL)-8 and cyclooxygenase (COX)-2 (6). Moreover, activated NFκB and ERK (10–12) induce markers of inflammation and antagonize peroxisome proliferator activated receptor (PPAR)γ activity, thereby causing insulin resistance. These data suggest that t10,c12 CLA mediates inflammatory signaling that antagonizes adipogenic processes in adipocytes. However, the upstream signals responsible for t10,c12 CLA-mediated increases in intracellular calcium levels, inflammatory signaling, insulin resistance, and reduced triglyceride (TG) content in human adipocytes are unknown.

Diacetylgllycerol kinases (DGK) are a family of kinases that phosphorylate diacetylglcerol (DAG), resulting in the conversion of DAG into phosphatidic acid (PA). DAG and PA act as second messengers that activate an array of target proteins, resulting in significant changes in cellular signaling (reviewed in Ref. 13). For example, DAG activates conventional protein kinase C (cPKC), Unc-13, and protein kinase D, whereas PA activates atypical PKC, phosphatidylinositol (PI)-4-phosphate 5-kinase, and mammalian target of rapamycin (mTOR), RasGAP, and Raf-1 kinase (reviewed in Ref. 13). Therefore, DGKs are critical in terminating DAG signaling and initiating PA signaling. In addition to this well-characterized function, DGKs act as scaffolding proteins and regulate subcellular signaling via endosomal and nuclear transport. To date, 10 different DGK isoforms have been identified. Each of the DGKs has up to three PKC-like C1 domains and a catalytic region. DGKs are grouped into five different types, based on their structural and functional features. For example, type I DGKs, which include DGK α, β, and γ, contain recoverin homology domains and EF-hand motifs that serve as calcium-binding domains (reviewed in Ref. 13). Thus, these DGKs are activated in part by calcium binding. Type II DGKs, including DGK δ, η, and κ, contain pleckstrin homology domains, sterile α motif domain, and a separated catalytic region. Type III DGKs, including DGK ε, contain no additional functional domains different than other DGK isoforms. Type IV DGKs, including DGK ξ and ζ, contain a nuclear localization signal, a myristoylated alanine-rich C kinase substrate phosphorylation domain, and four ankyrin repeats. Type V DGKs, including DGK θ, contain three C1 domains, a Gly/Pro-rich domain, and a PH-domain-like region (reviewed in Ref. 13). DGKs also display tissue-specific expression. DGKs are highly expressed in the brain, thymus, and muscle (13). However, DGK expression in adipose tissue or primary human adipocytes is poorly defined. Thus, DGKs are a complex family of kinases, and little is known regarding their potential function in adipose tissue.

Several lines of evidence support the involvement of DGKs in t10,c12 CLA-mediated inflammation and insulin resistance. First, DGK-generated PA levels activate mTOR and S6 kinase (S6K) in HEK 293 cells (14); and we reported that t10,c12 CLA activated these two proteins in primary human adipocytes (15). Second, mTOR and S6K activation have been implicated in the development of insulin resistance, a side effect of CLA supplementation (16, 17). Third, DGK-mediated PA production has also been shown to increase calcium release from the ER (18). This finding could provide a mechanism by which t10,c12 CLA increases intracellular calcium levels in newly differentiated primary human adipocytes (6). Moreover, DGKs has been reported to regulate ERK activation, which we have found to be necessary, in part, for t10,c12 CLA-mediated adipocyte cholesterol accumulation (19). Yasuda et al. (19) found that DGKα facilitated the transport of c-Raf to the plasma membrane, upstream of MEK/ERK activation in response to epidermal growth factor treatment in HeLa cells. Therefore, it is tempting to speculate that t10,c12 CLA-mediated activation of MEK/ERK may involve similar signaling mechanisms. Additionally, DGKs has been shown to regulate tumor necrosis factor (TNF)α-mediated NFκB activation (20), which we have reported to be activated by t10,c12 CLA treatment in adipocytes (5). Finally, a mixture of CLA isomers has been shown to increase expression of DGKγ and increase PA levels in cardiomyocytes (21). Taken together, there are several interesting findings in the literature that suggest a potential role for DGKs in t10,c12 CLA-mediated signaling in primary human adipocytes.

Based on the close similarity between pathways activated by DGK and t10,c12 CLA, we hypothesized that DGKs play an important role in t10,c12 CLA-mediated inflammation, insulin resistance, and delipidation. To test this hypothesis, we employed the chemical DGK inhibitor R59022 and siRNA targeting DGKα. In this study, we demonstrated that DGKs, particularly DGKα, may be involved in the regulation of t10,c12 CLA-mediated inflammatory signaling, insulin resistance, and delipidation in primary human adipocytes.

MATERIALS AND METHODS

Materials

All cell cultureware was purchased from Fisher Scientific (Norcross, GA). Lightning chemiluminescence substrate was purchased from Perkin Elmer Life Science (Boston, MA). Immunoblotting buffers and precast gels were purchased from Invitrogen by Life Technologies (Carlsbad, CA). Polyclonal antibodies for anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Beta
(β)-actin, and monoclonal antibody for anti-PPARγ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibody for DGKγ was purchased from Abcam (Cambridge, MA). Anti-total and anti-phospho (P) ERK, JNK, and P-cJun antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Hyclone fetal bovine serum was purchased from Fisher Scientific. Isomers of CLA (+98% pure) were purchased from Matreya (Pleasant Gap, PA). Flu-o-3 acetyloxymethyl ester (Fluo-3 AM), pluronic F-127, and probenecid were purchased from Invitrogen by Life Technologies. Thapsigargin and ionomycin were purchased from Calbiochem-EMD Biosciences, Inc. (La Jolla, CA). The cell permeable DGK inhibitor R59022 (6-[2-[4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl]ethyl]-7-methyl-5H-1H-benzo[d][1,3]oxazolo(3,2-d)pyrimidin-5-one) was purchased from EMD Chemicals (Gibbstown, NJ). This inhibitor functions by inhibiting DAG phosphorylation, which is more specific to the type I, calcium-sensitive DGKs (22). Dharmacon DGKγ, GAPDH, and nontargeting ON-TARGETplus siRNA, and Dharmafect 1 transfection reagent were purchased from ThermoScientific (Lafayette, CO). All other reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Culturing of human primary adipocytes

Abdominal white adipose tissue was obtained with consent from the Institutional Review Boards at the University of North Carolina at Greensboro and the Moses Cone Memorial Hospital during elective abdominoplasty of nondiabetic Caucasian and African American females between the ages of 20 and 50 years with a body mass index ≤32.0. These selection criteria allowed for reduced variation in gender, age, and obesity status. Tissue was digested using collagenase; stromal vascular cells were isolated as previously described (4). Stromal vascular cells were differentiated with adipocyte media (AM-1) containing 1 µM rosiglitazone and 250 µM 1-methyl-3-isobutylxanthine for three days, which yielded cultures containing ~30–50% adipocytes. On days 7–14, cells were pretreated with 0.1, 1, or 10 µM R59022 dissolved in DMSO for 30 min and subsequently treated with 50–150 µM t10,c12 CLA or BSA (BSA) vehicle control for 5 min to 48 h depending on the experimental outcome measured. All cultures were normalized to contain the same amount of BSA and DMSO vehicles. Each independent experiment was repeated at least twice using a mixture of cells from three subjects, unless otherwise indicated.

FA preparation

t10,c12 and c9,t11 CLA were delivered as free FAs complexed to 7.5% FA-free BSA (Sigma A7030, lot #040M1649) at a 1:1 molar ratio as previously described (4). This BSA was chosen based on its decreased capacity to induce inflammatory gene expression compared with other BSA samples tested (unpublished data). For measuring [Ca\(^{2+}\)]\(_i\) levels, t10,c12 CLA dissolved in a 0.1M KOH solution was used.

TG content

TG levels were determined with a modified commercially available TG assay as previously described (23).

\(^3\)H-2-deoxyglucose uptake

Cultures of human adipocytes were supplemented with low-glucose DMEM on day 10. The following day, cultures were pre-treated with 0.1, 1, or 10 µM R59022 for 30 min and subsequently treated with BSA vehicle or 50 µM t10,c12 CLA for 48 h. Cultures were stimulated for 10 min with 100 nM insulin and treated with 4 nmo l \(^3\)H-2-deoxyglucose for 90 min. The amount of \(^3\)H-2-deoxyglucose was measured via scintillation counting as described previously (23).

\(^14\)C-oleic acid uptake

Cultures of human primary adipocytes were supplemented with low-glucose DMEM on day 10. The following day, cultures were pre-treated with 0.1, 1, or 10 µM R59022 for 30 min and subsequently treated with BSA vehicle or 30 µM t10,c12 CLA for 48 h. Cultures were treated with 12.5 nM \(^14\)C-oleic acid (0.2 µCi; specific activity = 40-60 mCi/mmol) for 120 min. The amount of \(^14\)C-oleic acid oleic acid was measured via scintillation counting as described previously (23).

Immunoblotting

After experimental treatments, cultures were washed once with ice-cold HBSS. The cells were then solubilized by the direct addition of a lysis buffer containing PBS (pH 7.5), 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM Na\(\text{VO}_3\) 20 mM β-glycerophosphate, 10 mM NaF, and a protease inhibitor mixture (Calbiochem) including 500 µM AEBSF, 1 µg/ml aprotinin, 1 µM E-64, 500 µM EDTA, and 1 µl leupeptin. Monolayers were scraped and transferred to prechilled microfuge tubes. Cell lysates were then sonicated three times for 5 s and stored on ice for an additional 20 min. Cell debris was pelleted by centrifugation at 14,000 rpm at 4°C, and the resulting supernatant was collected for analysis. The protein concentration of each sample was determined using a bichinchoninic acid assay (Pierce). Subsequently, 20 µg of protein from each sample was prepared with Nu-Page LDS sample buffer (Life Technologies) for denaturing gel electrophoresis using 4–12% NuPage precast gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes that were next blocked with 5% milk in TBST for 1 h and washed three times in TBST for 5 min. Blots were incubated overnight at 4°C with primary antibodies targeting DGKγ, P-ERK, P-JNK, P-cJun, and total cJun at a dilution of 1:1,000, and then subsequently incubated in the respective horseradish peroxidase-conjugated secondary antibody at a dilution of 1:5,000 at room temperature for 1 h. Primary and secondary antibodies targeting GAPDH were used at a 1:5,000 dilution. Primary and secondary antibodies targeting PPARγ were used at dilutions 1:200 and 1:2,000, respectively. After washing, blots were treated with chemiluminescence reagent for 1 min, and film was exposed using a SRX-101A Konica Minolta film developer. To quantify treatment differences, densitometry was performed using a Kodak Image Station 440. Data were normalized to β-actin loading control.

RNA isolation and PCR

Total RNA was isolated from the cultures using TRI Reagent purchased from Molecular Research Center (Cincinnati, OH), according to manufacturer’s protocol. For quantitative real-time PCR, 1.0 µg total RNA was converted into first-strand cDNA using high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed in an Applied Biosystems 7500 FAST real-time PCR system using Taqman gene expression assays. To account for possible variation in cDNA input or the presence of PCR inhibitors, the endogenous reference gene GAPDH was simultaneously quantified for each sample, and these data were normalized accordingly. Due to RNA interference of GAPDH in Fig. 6, TATA box binding protein (TBP) was used as the endogenous reference gene. The relative standard curve method using seven 2-fold dilutions ranging from 100 to 1.56 ng RNA was used to check primer efficiency and linearity of each transcript according to Applied Biosystems’s Guide to Performing Relative Quantification of Gene Expression Using Real-Time Quantitative PCR.

Secretion of IL-6, IL-8, and MCP-1

The concentrations of interleukin (IL)-6, IL-8, and monocyte chemoattractant protein (MCP)-1 were determined using the Bio-Plex suspension array system from Bio-Rad (Hercules, CA).
following the manufacturer’s protocol. Briefly, media was collected from cultures that were pretreated with 30 µM R59022 for 30 min, and subsequently treated with 50 µM t10,c12 CLA or BSA for 24 h. This time point was based on previous time course studies showing that the maximum level of cytokine secretion occurred after 24 h of t10,c12 CLA treatment (5). The media was centrifuged at 13,200 g for 10 min at 4°C to clear the samples of cellular debris. Samples and standards were run in duplicate. Based on the manufacturer’s report Bio-Plex Pro Human Cytokine, Chemokine, and Growth Factor Assays - Bulletin 5828, the intra-assay percentage coefficient of variation for IL-6, IL-8, and MCP-1 are 7, 9, and 9%, respectively. The interassay percentage CVs are 11, 4, and 7%, respectively.

**Measuring intracellular [Ca^{2+}] levels**

[Ca^{2+}] levels were determined using the calcium sensitive fluorescent dye Fluo-3 AM. Briefly, cells were preloaded with 5 µM Fluo-3 AM and an anionic detergent, 10% Phloronic F-127, at 37°C for 30 min in the dark. Next, cells were washed with HBSS containing CaCl_2 and probenecid, which prevents Fluo-3 AM leakage from cells. Cells were pretreated with R59022 or DMSO vehicle control for 10 min. Subsequently, baseline fluorescence was measured using a synergy multidetection microplate reader (BioTek Inc., Winooski, VT) for 1 min at 10 s intervals. Cells were then treated with 5 µM thapsigargin (positive control), 1 µg/ml ionomycin (positive control), or 150 µM t10,c12 CLA, and fluorescence was monitored at 20 s intervals for 7 min. Excitation wavelength was 485 nm, and fluorescence was collected at 528 nm. Changes in the ratio of calcium-dependent fluorescence to prestimulus background fluorescence (F/F_0) were plotted over time. For simplicity, single representative experiments are shown.

**siRNA-mediated knockdown of DGKβ**

Transfection of human primary adipocytes with DGKβ siRNA was conducted on day 7 of differentiation in 35 mm cell culture plates. Cells were seeded and differentiated as previously described. Cultures were supplemented with either 50 nM DGKβ siRNA, GAPDH siRNA, or nontargeting siRNA complexed with Dharmafect reagent 1 (2 µl/ml) from Dharmaco for 72 h. Transfection reagent and undelivered siRNA were removed 24 h posttransfection by removing the media and replacing with complete adipocyte media (AM-1, Zen-Bio Inc., Research Triangle Park, NC).

**Statistical analyses**

Data are expressed as the means ± SE. Data were analyzed using one-way ANOVA followed by Student’s tests for each pair for multiple comparisons. Alternatively, data were analyzed using two-way ANOVA with interaction followed by Tukey’s honestly significant difference test. Differences were considered significant if P < 0.05. All analyses were performed using JMP version 9 (SAS Institute, Cary, NC).

**RESULTS**

**DGK inhibitor R59022 attenuates t10,c12 CLA-mediated suppression of glucose, FA uptake, and TG content**

It has been previously reported that DGKs increase [Ca^{2+}]_i levels via PA-mediated secretion from the ER (18). Because we previously found that t10,c12 CLA-mediated inflammatory signaling was dependent on increased [Ca^{2+}]_i, we hypothesized that DGKs played an important role in t10,c12 CLA-mediated inflammation, insulin resistance, delipidation. To determine the extent to which t10,c12 CLA-mediated delipidation involves DGK activation, cultures of primary human adipocyte were pretreated with 3, 10, or 30 µM of the DGK inhibitor R59022 for 30 min and subsequently treated with 50 µM t10,c12 CLA for 24 h. The t10,c12 CLA-mediated reduction in TG content was attenuated with 30 µM R59022 (Fig. 1A).

Consistent with these data, t10,c12 CLA-mediated suppression of FA (Fig. 1B) and glucose uptake (Fig. 1C) was attenuated with 10 µM R59022 (Fig. 1B, C).

**R59022 attenuates t10,c12 CLA-mediated suppression of PPARγ and its target genes**

Due to the role of PPARγ in promoting FA and glucose uptake, we hypothesized that R59022 would also prevent t10,c12 CLA from decreasing PPARγ protein levels. Consistent with our hypothesis, 30 µM R59022 attenuated t10,c12 CLA-mediated reduction in PPARγ protein levels (Fig. 2A). Consistently, R59022 pretreatment partially prevented t10,c12 CLA-mediated suppression of lipogenic genes, including PPARγ, FA binding protein (aP2), insulin-dependent glucose transporter (GLUT4), and acetyl CoA carboxylase (ACC)-1 (Fig. 2B). These data suggest that DGKs are involved in CLA-mediated delipidation and insulin resistance.

**R59022 attenuates t10,c12 CLA-mediated inflammatory gene expression and protein secretion**

We previously demonstrated that t10,c12 CLA-induced inflammation leads to a suppression of insulin signaling and sensitivity (4–6). To demonstrate the involvement of DGKs in t10,c12 CLA-mediated inflammatory signaling, inflammatory gene expression and cytokine/chemokine release were measured in cultures pretreated with R59022 and subsequently treated with BSA vehicle control or 50 µM t10,c12 CLA for 18 or 24 h, respectively. R59022 attenuated t10,c12 CLA-induced IL-8, IL-6, and MCP-1 gene expression (Fig. 3A) and protein secretion (Fig. 3B). These data suggest that DGKs are involved in t10,c12 CLA-mediated inflammatory signaling in primary human adipocytes.

**R59022 attenuates t10,c12 CLA-mediated activation of MAPK and c-Jun and [Ca^{2+}]_i levels**

We have previously shown that t10,c12 CLA-induced inflammation is dependent on MAPK activation (3) and [Ca^{2+}]_i accumulation (6). Therefore, we examined the role of DGKs in t10,c12 CLA-mediated activation of MAPK (i.e., ERK and JNK) and activator protein (AP)-1 (i.e., c-Jun), due to their role in upregulating inflammatory gene expression. Indeed, 30 µM R59022 attenuated t10,c12 CLA-mediated ERK, JNK, and c-Jun phosphorylation (Fig. 4A). These data suggest that DGKs are involved in CLA-mediated MAPK and c-Jun phosphorylation. Due to the involvement of [Ca^{2+}]_i in t10,c12 CLA-mediated inflammatory signaling, the role of DGKs in elevating [Ca^{2+}]_i levels by t10,c12 CLA was determined. Cultures were pretreated with increasing doses of R59022 for 10 min and subsequently treated with t10,c12 CLA, after which [Ca^{2+}]_i levels were measured using the fluorescent calcium indicator Fluo-3-AM. As expected, t10,c12 CLA increased [Ca^{2+}]_i levels within 1 min, which were decreased by R59022 (Fig. 4B). To better understand how R59022 decreases [Ca^{2+}]_i accumulation, [Ca^{2+}]_i levels were measured in cultures treated with...
ionomycin, which causes calcium influx from outside the cell, and thapsigargin, which inhibits calcium-ATPases on the ER causing calcium release from the ER, in the presence or absence of R59022. Interestingly, R59022 completely blocked ionomycin-mediated calcium and partially attenuated thapsigargin-mediated calcium accumulation (Fig. 4C). These data suggest that DGKs may be involved in t10,c12 CLA-mediated increase in \([\text{Ca}^{2+}]_i\) from both intra- and extracellular stores.

t10,c12 CLA increases DGK\(\eta\) and DGK\(\delta\) expression, which is blocked by R59022

To determine the DGK isoform responsible for these effects, we analyzed basal gene expression of several DGK isoforms, including DGK\(\alpha\), \(\beta\), \(\gamma\), \(\eta\), and \(\zeta\). These isoforms were chosen based on microarray analyses of all DGK isoforms (data not shown). DGK\(\alpha\) was the most highly expressed isoform (Fig. 5A). DGK\(\delta\) and DGK\(\eta\) were expressed at similar levels, whereas DGK\(\gamma\) and DGK\(\zeta\) were the least abundant isoforms (Fig. 5A). To assess the effect of t10,c12 CLA on DGK expression, cultures were treated with 50 \(\mu\)M t10,c12 CLA from 3 to 24 h. DGK\(\eta\) was induced by t10,c12 CLA treatment at 6, 12, and 24 h, and DGK\(\delta\) was modestly induced by t10,c12 CLA treatment after 12–24 h. DGK\(\zeta\) was induced after 24 h of treatment (Fig. 5B). There was no effect of t10,c12 CLA on DGK\(\alpha\) or DGK\(\gamma\) expression at any time point (data not shown). Treatment with R59022 for 18 h decreased t10,c12 CLA-induced DGK\(\eta\) and DGK\(\delta\), but not DGK\(\alpha\) mRNA levels (Fig. 5C).

Depletion of DGK\(\eta\) by RNA interference attenuates t10, c12 CLA-induced inflammatory gene expression and P-cJun activation

Preliminary experiments investigating DGK activity via translocation to the plasma membrane were conducted to evaluate the isoform likely responsible for t10,c12 CLA-mediated inflammation and insulin resistance. Of each isoform examined, including DGK\(\alpha\), \(\beta\), \(\gamma\), \(\eta\), and \(\zeta\), only results examining DGK\(\eta\) suggested translocation to the plasma membrane (data not shown). Based on t10,c12 CLA-mediated induction of DGK\(\eta\) and suppression by R59022 and preliminary data suggesting DGK\(\eta\) translocation to the plasma membrane by t10, c12 CLA, we targeted DGK\(\eta\) as the candidate DGK isoform responsible for t10,c12 CLA-induced inflammation and insulin resistance. To identify a specific role of DGK\(\eta\) in t10,c12 CLA-mediated inflammatory signaling, we employed RNA interference to selectively deplete DGK\(\eta\) before treatment with t10,c12 CLA. DGK\(\eta\) siRNA modestly decreased DGK\(\eta\) protein levels without affecting GAPDH or \(\beta\)-actin protein levels (Fig. 6A). Successful knockdown was supported by using GAPDH siRNA as a positive control, which decreased GAPDH protein levels without affecting \(\beta\)-actin levels. Densitometry revealed a 20 and 24% knockdown of DGK\(\eta\) and GAPDH protein levels, respectively (Fig. 6A). To confirm these results, mRNA levels of DGK\(\eta\) and GAPDH positive control were measured. Treatment with DGK siRNA resulted in a 50% knockdown of DGK\(\eta\) mRNA levels compared with the siRNA control (Fig. 6B). GAPDH siRNA selectively decreased GAPDH mRNA levels by 70% (Fig. 6B). Next, cultures were pretreated with 50 nM DGK\(\eta\) siRNA for 72 h and subsequently treated with t10,c12 CLA.
for 18 h. Treatment with DGK\(_{\beta}\) siRNA modestly decreased CLA-mediated c\(\text{jun}\) and JNK phosphorylation (Fig. 6C). To determine whether DGK\(_{\beta}\) specifically played a role in t10,c12 CLA-mediated inflammatory signaling, inflammatory gene expression was measured. Notably, siRNA targeting DGK\(_{\beta}\) attenuated t10,c12 CLA-induced IL-8, and DGK\(_{\beta}\) expression (Fig. 6D), as well as calmodulin kinase (CaMK)\(\beta\) expression (data not shown). These data suggest that DGK\(_{\beta}\) is involved in the upregulation of inflammatory genes and may also be self-regulating at the transcriptional level.

Lastly, to determine whether DGK\(_{\beta}\) contributes to t10,c12 CLA-mediated insulin resistance, insulin-stimulated glucose uptake was measured in cultures treated with DGK\(_{\beta}\) siRNA. Treatment with siRNA targeting DGK\(_{\beta}\) did not attenuate t10,c12 CLA-mediated suppression of insulin-stimulated glucose uptake (data not shown). Collectively, these data suggest that DGKs, particularly DGK\(_{\beta}\), play a role in CLA-mediated inflammation, but the specific role of DGK\(_{\beta}\) in t10,c12 CLA-mediated insulin resistance is unclear.

**DISCUSSION**

Consistent with our hypothesis, the DGK inhibitor R59022 attenuated t10,c12-mediated suppression of TG levels, radiolabeled oleic acid uptake, insulin-stimulated glucose uptake, and PPAR\(\gamma\) protein levels and target gene expression. Additionally, R59022 attenuated t10,c12 CLA-induced inflammatory gene and protein secretion, MAPK and c\(\text{jun}\) phosphorylation, and \([\text{Ca}^{2+}]_{\text{i}}\) accumulation. In further support of our hypothesis, gene silencing of DGK\(_{\beta}\) with siRNA also attenuated t10,c12 CLA-induced c\(\text{jun}\) and JNK phosphorylation and induction of inflammatory genes. Taken together, these data suggest that DGKs play role in t10,c12 CLA-mediated induction of inflammation and insulin resistance in cultures of human adipocytes.

As with most chemical inhibitors, the efficacy and specificity of R59022 has been questioned (22). Because we were unsure which DGK isoform might be involved in CLA-mediated inflammatory signaling in adipocytes, we decided to use R59022 rather than the more specific type.
I DGK inhibitor R59949. In preliminary experiments using R59949, we found it had little effect on reducing inflammatory gene expression and did not significantly attenuate t10,c12 CLA-mediated suppression of insulin-stimulated glucose uptake (data not shown). These findings suggest that type I DGKs may not play a role in t10,c12 CLA signaling and provide another reason why the type II DGKs were targeted instead of other DGK isoforms. Based on the finding that DGKα displayed the highest level of expression, it is possible that DGKα may have important functions in adipocytes, but perhaps not specifically related to inflammatory signaling induced by t10,c12 CLA. Gene silencing

Fig. 5. R59022 (R5) attenuates trans-10, cis-12 (t10,c12) CLA-induced DGK gene expression. (A) Basal expression of DGKα, η, δ, ε, and γ was measured in cultures of newly differentiated human adipocytes on day 8. (B) Cultures were treated with BSA vehicle control or 50 µM t10,c12 CLA (CLA) for 3–24 h or (C) pretreated with 5, 10, or 30 µM R59022 (R5) and subsequently treated with BSA vehicle control or 50 µM t10,c12 CLA (CLA) for 18 h. mRNA levels were measured via RT-qPCR. Data were normalized to GAPDH endogenous controls. Data are representative of two (A, B) to three (C) independent experiments. Means ± SEM (n = 2–3) not sharing a common superscript differ significantly (P < 0.05).

Fig. 6. Silencing DGKη attenuates trans-10, cis-12 (t10,c12) CLA-mediated phosphorylation of cJun and JNK and induction of inflammatory gene expression. Cultures were treated with no treatment (NT) or 50 nM siRNA targeting DGKη (D), GAPDH (G), or scrambled siRNA (S) using 2 µl/ml of Dharmafect Reagent 1 for 72 h. Cultures were then (A) harvested and immunoblotted for DGKη, GAPDH, and β-actin, (B) harvested for mRNA analysis of DGKη and GAPDH, or (C, D) treated with BSA vehicle control or t10,c12 CLA (CLA) for 18 h and harvested for P-cJun, P-JNK, GAPDH, and B-Actin (C) or mRNA levels of IL-8 and MCP-1 (D). Densitometry was performed to quantify treatment differences (A, C). Data were normalized to β-actin (A, C) or TBP endogenous control (B, D). Data are representative of two (A, C) to three (B, D) independent experiments. Means ± SEM (n = 2–4) not sharing a common superscript differ significantly (P < 0.05).
studies are needed to confirm a role of candidate DGK isoforms in t10,c12 CLA-mediated inflammation. Based on data presented here, we speculate that the type II DGK DGK\(\eta\) may be involved in CLA-mediated inflammatory signaling.

A common mechanism by which FAs increase calcium levels is through activation of G-protein coupled receptors (GPCR), such as GPR40. GPR40, coupled to the G-protein subunit \(G_{\text{q/11}}\), activates phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into DAG and inositol phosphates (IP). IPs activate receptors on the ER, triggering calcium mobilization (reviewed in Ref. 24). Intriguingly, Schmidt and colleagues recently reported that CLA increases \([\text{Ca}^{2+}]_i\) and stimulates insulin release from INS-1E pancreatic cells via activation of the cell surface receptor free fatty acid receptor 1 (FFA1)/GPR40 (25). An alternative mechanism has been proposed by Camina and colleagues, whereby PC-specific PLC, activated by a pertussis toxin-sensitive G-protein, generates choline and DAG, which is converted into PA by DGKs. Subsequently, PA triggers calcium mobilization from IP3-independent calcium pools (18, 26–28).

Consistent with these findings, we reported that D609, a phosphatidylinositol (PC)-specific PLC (PC-PLC) inhibitor, prevents t10,c12 CLA-mediated \([\text{Ca}^{2+}]_i\), accumulation, ROS production, and inflammatory gene expression (i.e., IL-8, ATF3, and COX-2) (6). Notably, we have shown that pertussis toxin attenuates t10,c12 CLA-mediated MEK/ERK phosphorylation and suppression of \(^{14}\text{C}\)-oleic acid uptake and insulin-stimulated \(^{1}H\)-2-deoxyglucose uptake, suggesting that t10,c12 CLA activates a G-protein-coupled receptor linked to PLC, which generates choline and DAG, and which is converted into PA by DGKs. Subsequently, PA triggers calcium mobilization from IP3-independent calcium pools (18, 26–28).

Based on the data reported here and reports in the literature, we propose a working model (Fig. 7) in which t10,c12 CLA activates a GPCR linked to PLC, which generates IP3 and DAG. DGKs convert DAG into PA, and together with IP3, stimulate calcium secretion from the ER. Elevated calcium levels activate calcium-sensitive kinases, such as CaMKII, which have been shown to inhibit differentiation upon prostaglandin (PG)\(F_{2\alpha}\) treatment (29). Interestingly, we have shown that CLA treatment inhibits differentiation in primary human preadipocytes (30–33) and increases PG\(F_{2\alpha}\) secretion (31). In addition, KN-62, a CaMKII inhibitor, attenuates t10,c12 CLA-mediated ERK and [NK phosphorylation, ROS production, induction of inflammatory genes, PG\(F_{2\alpha}\) secretion, and suppression of PPAR\(\gamma\) protein levels and insulin-stimulated glucose uptake (6). We found that R59022 decreased CaMKII\(\beta\) gene expression (data not shown), thus providing another mechanism by which DGKs mediate t10,c12 CLA-induced inflammation. Collectively, these findings support mechanisms by which t10,c12 CLA may trigger intracellular calcium accumulation and subsequent activation of inflammatory pathways.

Fig. 7. Working model. Trans-10, cis-12 conjugated linoleic acid (t10,c12 CLA) activates a G-protein coupled receptor linked to PLC. PLC cleaves PIP2 or PC into DAG and IP3 or choline, respectively. IP3 activates the IP3 receptor on the ER, and PA produced by DGKs stimulates Ca\(^{2+}\) release from the ER. Intracellular Ca\(^{2+}\) activates calcium-sensitive kinases, such as CaMKII, leading to ROS production and MAPK activation. AP-1 or NF\(\kappa\)-B suppresses PPAR\(\gamma\) activity, leading to decreased glucose and FA uptake, resulting in delipidation and insulin resistance in adipocytes. Lastly, t10,c12 CLA-mediated induction of DGK\(\eta\) was inhibited with
knockdown of DGKα, suggesting self-regulation in a feed-forward manner. It is unclear how or through which transcription factor DGKα is induced by t10,c12 CLA. Results from the literature show that DGKα is induced by glucocorticoids in DDT1 cells and 12-O-tetradecanoylphorbol-13-acetate in HepG2 cells (34, 35). To our knowledge, these are the only inducers identified to date. Thus, further experiments are needed to determine the mechanism by which t10,c12 CLA induces DGKα gene expression in primary human adipocytes. Interestingly, the induction of DGKα follows a pattern similar to that seen in t10,c12 CLA’s induction of inflammatory genes. Thus, it is tempting to speculate that DGKα induction is mediated by NFκB or AP-1, two transcription factors activated by t10,c12 CLA that induce inflammatory gene expression (5, 6, 31). In summary, these data suggest that DGKs mediate, in part, t10,c12 CLA-induced inflammatory signaling in primary human adipocytes.

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