Suppression of Cardiac Myocyte Hypertrophy by Conjugated Linoleic Acid

ROLE OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS \(\alpha\) AND \(\gamma\)*

Caroline P. Albin, Melanie A. Kopilas, and Hope D. I. Anderson

From the Faculty of Pharmacy, University of Manitoba and the Canadian Centre for Agri-Food Research in Health and Medicine, St. Boniface General Hospital Research Centre, Winnipeg, Manitoba R2H 2A6, Canada

Conjugated linoleic acid (CLA) refers to a naturally occurring mixture of positional and geometric isomers of linoleic acid. Evidence suggests that CLA is a dietary constituent and nutraceutical with anti-cancer, insulin-sensitizing, immunomodulatory, weight-partitioning, and cardioprotective properties. The aim of this study was to evaluate the effects of intervention with CLA on cardiac hypertrophy. In vitro, CLA prevented indicators of cardiomyocyte hypertrophy elicited by endothelin-1, including cell size augmentation, protein synthesis, and fetal gene activation. Similar anti-hypertrophic effects of CLA were observed in hypertrophy induced by angiotensin II, fibroblast growth factor, and mechanical strain. CLA may inhibit hypertrophy through activation of peroxisome proliferator-activated receptors (PPARs). CLA stimulated PPAR activity in cardiomyocytes, and the anti-hypertrophic effects of CLA were blocked by genetic and pharmacological inhibitors of PPAR isoforms \(\alpha\) and \(\gamma\). CLA may disrupt hypertrophic signaling by stimulating diacylglycerol kinase \(\zeta\), which decreases availability of diacylglycerol and thereby inhibits the protein kinase C\(e\) pathway. In vivo, dietary CLA supplementation significantly reduced blood pressure and cardiac hypertrophy in spontaneously hypertensive heart failure rats. These data suggest that dietary supplementation with CLA may be a viable strategy to prevent pathological cardiac hypertrophy, a major risk factor for heart failure.

Cardiac hypertrophy is the increase in myocardial mass provoked by hemodynamic stress or myocardial injury and is a convergence point for many risk factors leading to heart failure. For more than a century, hypertrophy was viewed as a compensatory response that preserves ventricular performance (1, 2), but prolonged hypertrophy is maladaptive and leads to cardiac arrest and/or failure (3, 4). Thus, attenuation of hypertrophy is a promising therapeutic target to prevent heart failure.

Hypertrophy is characterized at the cardiomyocyte level by increases in cell size, protein synthesis, sarcomeric reassembly, and changes in gene expression (5). Re-induction of fetal genes such as brain natriuretic peptide (BNP)\(^2\) is one of the most consistent markers of hypertrophy. Hence, BNP expression and BNP promoter-reporter constructs are used as experimental indicators of hypertrophy, since virtually every hypertrophic stimulus activates the BNP gene (6).

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of linoleic acid (LA), an 18-carbon polyunsaturated fatty acid with cis double bonds at carbons 9 and 12. In CLA double bonds are conjugated and may be cis or trans. Most biological actions have been ascribed to cis-9, trans-11-, and trans-10, cis-12-CLA (7). Humans acquire CLA through diet from dairy and meat products from ruminant animals because monogastric intestinal bacteria do not make CLA (8).

Current studies have reported that some of the non-cardiac benefits of CLA involve activation of peroxisome proliferator-activated receptors (PPARs) (11–13). PPARs constitute a nuclear receptor family of transcription factors that regulate fatty acid and triglyceride metabolism (14). The crystal structure of PPAR ligand binding domains reveals a large binding pocket that may confer promiscuity for several ligands, including fatty acids (15). There are three isoforms: \(\alpha\), \(\beta\), and \(\gamma\). In the heart all PPAR isoforms are expressed in myocytes, and \textit{bona fide} PPAR agonists prevent hypertrophy in vitro (16–20) and in vivo (17, 21).

We determined the effect of CLA on hypertrophy stimulated in neonatal rat ventricular cardiomyocytes. Because CLA activates PPARs and PPAR agonists prevent hypertrophy, we considered whether PPAR activation might underlie anti-hypertrophic effects of CLA. Then we sought to elucidate the mechanism by which CLA attenuates hypertrophic signaling. Finally, we verified the anti-hypertrophic effect of CLA \textit{in vivo} in the spontaneously hypertensive heart failure (SHHF) rat.

* This work was supported by the Canadian Institutes of Health Research, the Heart and Stroke Foundation of Manitoba, Dairy Farmers of Canada, and the Manitoba Medical Service Foundation and a fellowship from the Manitoba Health Research Council (to C. P. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: CCARM, St. Boniface General Hospital Research Centre, 351 Taché Ave., Winnipeg, Manitoba R2H 2A6, Canada. Tel.: 204-235-3587; Fax: 204-237-4018; E-mail: handerson@sbrc.ca.

2 The abbreviations used are: BNP, brain natriuretic peptide; LA, linoleic acid; CLA, conjugated LA; PPAR, peroxisome proliferator-activated receptor; SHHF, spontaneously hypertensive heart failure; ET-1, endothelin-1; PE, phenylephrine; AngII, angiotensin II; FGF, fibroblast growth factor; SD, Sprague-Dawley; PKC, protein kinase C; PA, phosphatidic acid; DGK, diacylglycerol kinase; LVPWd, end-diastolic left ventricular posterior wall thickness; LVPWw, end systolic left ventricular posterior wall thickness; LVIDd, end-systolic left ventricular internal diameter; SBP, systolic blood pressure; DBP, diastolic blood pressure; PPRe, peroxisome proliferator response element; DAG, diacylglycerol; wt, wild type; dn, dominant negative.
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**A.** Myocytes were co-transfected with −1595 human hBNP-luciferase (0.5 μg/well) and Renilla luciferase reporter gene (0.5 μg/well). 24 h post-transfection cells were serum-deprived for 24 h and pretreated with vehicle or increasing concentrations of CLA (3–30 μM) for 1 h followed by addition of ET-1 (0.1 μM) for 48 h. Cells were lysed, and luciferase activity was measured. Results are presented as percent of luciferase activity in vehicle-treated controls. **, p < 0.01; ns, not significant, n = 4.

**B.** Myocytes were serum-deprived for 24 h and pretreated with vehicle or CLA (30 μM) for 1 h followed by the addition of ET-1 (0.1 μM) for 48 h. During the last 4 h of intervention, cells were also pulsed with [3H]leucine (1 μCi/ml). Cells were processed, and radioactivity was measured. Results are presented as percent of [3H]leucine incorporation in vehicle-treated controls. *, p < 0.05, **, p < 0.01, n = 3. C, myocytes deprived of serum were pretreated with vehicle or CLA followed by the addition of ET-1 (0.1 μM) for 24 h. Cells were immunostained with anti-rat sarcomeric α-actin and visualized by fluorescence microscopy. Representative images for each treatment are shown. DMSO, Me2SO, D cell surface areas of individual cells were quantified as described under “Experimental Procedures.” Results are presented as percent of myocyte size (μm²) of vehicle-treated controls. **, p < 0.01, n = 3 sets of 30 cells analyzed. E, myocytes were transfected and treated as in Fig. 1A, except cells were pretreated with vehicle or LA (30 μM) for 1 h. Cells were processed and results are presented as in Fig. 1A. *, p < 0.05; ns, not significant, n = 5.

**EXPERIMENTAL PROCEDURES**

**Materials**—The CLA used in our experiments contained ~80% c9,t11 and t10,c12 CLA at an approximate 1:1 ratio as well as trace amounts of other isomers. In addition to the putatively bioactive forms of the molecule (39.1% c9,t11 and 40.7% t10,c12 CLA), the reported composition of this preparation included the following trace isomers: 1.8% c9,c11 CLA; 1.3% c10,c12 CLA; 1.9% t9,t11 and t10,t12 CLA; 1.1% c9,c12 linoleic acid; and 14.1% remainder. Both CLA and LA were from NuChek Prep, Inc.

**Cell Size**—Myocytes were cultured on collagen I-coated Flex plates (Flexcell International Corp.). After transfection, cells were serum-deprived for 24 h, then subjected to cyclical strain (60 Hz) on the FX3000 unit (Flexcell International Corp.) to promote a calculated increment in surface area of ~20% at the point of maximal tension on the culture surface (23).

**Protein Synthesis**—Myocytes were cultured in 12-well plates (1 × 10⁶ cells/well), serum-deprived for 24 h, pretreated with vehicle or CLA (30 μM) for 1 h, then stimulated by addition of ET-1 (0.1 μM; 1 h). Myocyte size was assessed by immunofluorescence microscopy, and computer-assisted planimetry as previously described (24). Pixel values were converted to surface area (μm²) by multiplying by scale factors of the x and y axes.

α-actinin antibody, and β-actin antibody were from Sigma-Aldrich. MK886 and GW9662 were from Biomol. [3H]Leucine was from PerkinElmer Life Sciences. Lipofectin was from Invitrogen. Texas Red-conjugated horse anti-mouse antibody and VECTASHIELD mounting medium containing 4’-6-diamidino-2-phenylindole were from Vector Laboratories. Protein kinase Cε (PKCε) antibody was from Upstate Biotechnology. Diacylglycerol kinase ζ (DGKζ) antibody was from Santa Cruz Biotechnology. ortho-[32P]Phosphate was obtained from GE Healthcare. Dulbecco’s modified Eagle’s medium (DMEM) labeling kit was from Millipore. Baker Si250 TLC plates were from VWR. Phosphatidic acid (PA) standards were from Avanti Polar Lipids.

**Cell Culture of Neonatal Rat Ventricular Cardiomyocytes**—Ventricular cardiomyocytes were isolated from 1-day-old neonatal Sprague-Dawley (SD) rats by digestion of ventricles with several cycles of 0.05% trypsin and mechanical disruption as previously described (22). Cells were cultured on gelatin-coated plates in DMEM containing 10% cosmic calf serum (Hyclone) for 18–24 h before experimentation.

**Mechanical Strain**—Cells were cultured on collagen I-coated Flex plates (Flexcell International Corp.). After transfection, cells were serum-deprived for 24 h, then subjected to cyclical strain (60 Hz) on the FX3000 unit (Flexcell International Corp.) to promote a calculated increment in surface area of ~20% at the point of maximal tension on the culture surface (23).
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Myocytes were cultured in 10-cm plates (15 x 10^6 cells/plate), serum-deprived for 24 h, then treated with vehicle or CLA (30 μM; 1–4 h). Cell lysates were prepared in radioimmune precipitation assay buffer and clarified by centrifugation, and DGKζ was detected by conventional Western blotting. Membranes were stripped and reprobed with β-actin antibody to account for loading variations among lanes.

Labeling of Phospholipids with ortho-[32P]Phosphate and Measurement of PA Formation—Myocytes cultured in 6-well plates (2 x 10^6 cells/well) were deprived of serum in phosphate-free DMEM for 24 h. Cells were incubated with 40 μCi/ml ortho-[32P]phosphate in phosphate-free DMEM for 3 h. Excess [32P] was washed out, cells were stimulated with vehicle or CLA in phosphate-free DMEM, and reactions were terminated.

Cells were scraped into PBS, and lipids were extracted and separated by thin layer chromatography as previously described (25). [32P]PA in each sample was normalized by total [32P]phospholipid.

In Vivo Experiments—7-Week-old male SD (control) and SHHF rats were obtained from Charles River Canada. Animals were maintained at 20 °C, 50% humidity and a 12-h light-dark cycle and allowed free access to water and food. After 2 weeks of acclimatization, a standard diet or one supplemented with 0.5% CLA was offered. Total fat percentage was controlled in diets by adjusting soybean oil content. After 8 weeks, rats were weighed, blood pressure and echocardiographic measurements were performed, and then rats were sacrificed by pentobarbital overdose.

Echocardiography—Rats were anesthetized with isoflurane (initial, 5%; maintenance, 2%); the anterior chest was shaved, and echocardiography was performed using a Sonos 5500 ultrasound system (Agilent Technologies) and a 12 MHz (s12) transducer. The two-dimensional parasternal, short-axis view was used to image the heart at the papillary muscle level. M-mode recordings were analyzed at a sweep speed of 150 mm/s. Measurements included end-diastolic and end-systolic left ventricular posterior wall thickness (LVPWd and LVPWs, respectively). Relative wall thickness was calculated as (LVIDd/LVPWd)³ - LVIDd^3 - LVIDd) x 1.04 x 0.8 + 0.14 g. Fractional shortening was calculated as 100 x (LVIDd - LVIDs)/LVIDd.

Blood Pressure—Systolic and diastolic blood pressures (SBP and DBP, respectively) were measured using tail-cuff method.
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FIGURE 3. CLA induces activation of PPAR isomers. Myocytes were co-transfected with PPRE$_r$-TK-LUC (1.0 μg/well) and Renilla luciferase reporter gene (0.5 μg/well). Subgroups were also co-transfected with a wtPPARα (0.001 μg/well), wtPPARδ (1.0 μg/well), or wtPPARγ (0.1 μg/well) plasmid. 24 h after transfection myocytes were serum-deprived for 24 h, then treated with vehicle or CLA (30 μM; 48 h). Cells were lysed, and luciferase activity was measured. Identical experiments were performed with specific PPAR agonists (10 μM fenofibrate, 1 μM GW501516, and 1 μM troglitazone, respectively). Results are presented as percent of luciferase activity in vehicle-treated controls. *, p < 0.05; **, p < 0.01; ***, p < 0.001; n = 4.

RESULTS

CLA Inhibits ET-1-induced Markers of Cardiomyocyte Hypertrophy—We first determined the effects of CLA on ET-1-induced increases in BNP gene activation, which is a reliable indicator of hypertrophy in the cardiac myocyte (6). ET-1 (0.1 μM; 48 h) increased BNP promoter activity (516 ± 138% versus control, p < 0.01), and this was dose-dependently attenuated by CLA (3–30 μM; Fig. 1A).

[3H]Leucine incorporation into acid-insoluble protein is a measure of protein synthesis that is commonly used as evidence of hypertrophy (24). [3H]Leucine incorporation was increased by ET-1 (0.1 μM; 48 h; 128 ± 6% versus control, p < 0.05), and this was inhibited by CLA (30 μM; 107 ± 8% versus control; Fig. 1B).

We also examined changes in myocyte size, which was measured directly by computer-assisted planimetry. ET-1 treatment (0.1 μM; 24 h) caused enlargement of myocytes (147 ± 7% versus control, p < 0.01) and CLA-attenuated ET-1-induced cell size augmentation (30 μM; 110 ± 4% versus control; Figs. 1, C and D).

We next determined whether the effect of CLA on hypertrophy is specific or an artifactual effect of fatty acids by examining the effect of LA on ET-1-induced BNP promoter activity. LA differs from CLA in that the double bonds are unconjugated (i.e. separated by two, rather than one, single bonds). Before stimulation with ET-1 (0.1 μM; 24 h), myocytes were treated with vehicle or LA (30 μM, 1 h). In myocytes, pretreatment with LA did not have an inhibitory effect on ET-1-induced BNP promoter activity (298 ± 62% versus control, p < 0.05; Fig. 1E).

CLA Inhibits BNP Promoter Activation Induced by Multiple Hypertrophic Stimuli—Several other hypertrophic stimuli activated the BNP promoter (Fig. 2), including mechanical strain (15%, 48 h; 353 ± 17% versus control, p < 0.01), AngII (0.1 μM, 48 h; 253 ± 54% versus control, p < 0.05), FGF (20 ng/ml, 48 h; 329 ± 88% versus control, p < 0.05), and PE (10 μM, 48 h; 157 ± 446.0 versus control, p < 0.01). CLA attenuated BNP promoter activation elicited by mechanical strain (73 ± 10% versus control), AngII (89 ± 37% versus control), and FGF (166 ± 45% versus control) but not PE (1443 ± 362% versus control).

PPARα and PPARγ Mediate the Anti-hypertrophic Effects of CLA—Activated PPARs bind to peroxisome-proliferator response elements (PPREs) to modulate target gene expression. To measure PPAR activation, myocytes were transfected with PPRE$_r$-TK-LUC, a reporter construct containing three copies of the acyl-CoA oxidase PPRE upstream of the thymidine kinase promoter driving luciferase expression (29). As shown in Fig. 3, CLA (30 μM, 48 h) stimulated significant increases in...
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**FIGURE 4.** PPARα and PPARγ mediate the inhibitory effects of CLA on ET-1-induced BNP promoter activity. A–D, myocytes were transfected as in Fig. 1A; subgroups were also transfected with dnPPARα (B, 0.1 μg/well), dnPPARδ (C, 0.1 μg/well), or dnPPARγ (D, 0.01 μg/well) plasmid. 24 h after transfection, cells were serum-deprived for 24 h and pretreated with vehicle or CLA (30 μM, 1 h) followed by the addition of ET-1 for 48 h. Cells were lysed, and luciferase activity was measured. Results are expressed as percent of luciferase activity in vehicle-treated controls. **p < 0.01; ***p < 0.001; ns, not significant, n ≥ 7. E and F, myocytes were transfected as in Fig. 1A. 24 h after transfection, cells were serum-deprived for 24 h, then pretreated with vehicle, MK886 (E, 1 μM, 1 h), or GW9662 (F, 1 μM, 1 h). Subgroups were subsequently treated with CLA as indicated (30 μM, 1 h) followed by the addition of ET-1 for 48 h. Cells were lysed, and luciferase activity was measured. Results are expressed as a percent of luciferase activity in vehicle-treated controls. *p < 0.05; **p < 0.01; ns, not significant, n ≥ 6.

PPREα-TK-LUC activity in the presence of wild type PPARα (0.001 μg/ml; 327 ± 27% versus control, p < 0.001; Fig. 3A), PPARδ (1.0 μg/ml; 179 ± 18% versus control, p < 0.01; Fig. 3B), and PPARγ (0.1 μg/ml; 294 ± 44% versus control, p < 0.001; Fig. 3C). Overexpression of PPARs was also required for stimulation of the PPRE reporter in response to the PPARα agonist, fenofibrate (10 μM, 48 h; 457 ± 150% versus control, p < 0.05; Fig. 3A), the PPARδ agonist, GW501516 (1 μM, 48 h; 1190 ± 194.7% versus control, p < 0.001; Fig. 3B), and the PPARγ agonist, troglitazone (1 μM, 48 h; 442 ± 119% versus control, p < 0.01; Fig. 3C).

We next determined whether PPAR isoforms participate in the anti-hypertrophic actions of CLA. Dominant negative PPARα (Fig. 4B) and dominant negative PPARγ (Fig. 4D) abolished the inhibitory effect of CLA on ET-1-stimulated BNP promoter activity, whereas dominant negative PPARδ had no significant effect (Fig. 4C). This finding was confirmed pharmacologically using MK886 and GW9662, which selectively antagonize PPARα (30) and γ, respectively. Both MK886 (Fig. 4E) and GW9662 (Fig. 4F) abolished the anti-hypertrophic actions of CLA. None of the dominant negative constructs or pharmacological inhibitors affected ET-1-induced BNP promoter activity alone.

**CLA inhibits ET-1-induced hypertrophic signaling—Production of diacylglycerol (DAG) and subsequent translocation and activation of PKC isoforms, especially ε, are key early signaling events stimulated through the ETA receptor (31, 32). Therefore, we examined the effect of CLA on ET-1-induced translocation of PKCε. ET-1 increased the ratio of particulate to total PKCε (0.1 μM, 5 min; 247 ± 58% versus control, p < 0.01), and this translocation of PKCε to the particulate fraction was abolished by CLA (98 ± 17% versus control; Fig. 5).

DGKζ may have a role in the regulation of cardiac hypertrophy (33). DGKζ phospholipid DAG to produce PA. The resultant decrease in availability of DAG attenuates translocation and activation of PKC. Thus, we sought to determine whether CLA activates DGKζ. We observed that CLA (30 μM, 1 h) increases protein expression of DGKζ compared with vehicle-treated controls (304.6 ± 43.8 versus control, p < 0.05; Fig. 6A), an event that was sustained over 2- and 4-h time points. Additionally, MK886 (1 μM, 1 h) or GW9662 (1 μM, 1 h) each blocked the ability of CLA to increase DGKζ protein expression, suggesting a role for PPARα and/or γ in the activation of DGKζ by CLA (Figs. 6, B and C). We also measured [32P]PA formation in 32P-labeled myocytes in the presence of CLA. We were unable to detect a difference in
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FIGURE 5. CLA attenuates ET-1-induced translocation of PKCε. Myocytes deprived of serum for 24 h were pretreated with vehicle or CLA (30 μM, 1 h) followed by the addition of ET-1 (0.1 μM, 5 min). Cells were lysed, and particulate and soluble fractions were separated by ultracentrifugation. Proteins in each fraction were subjected to Western blotting (WB) analysis using an anti-PKCε antibody. Results are presented as percent of particulate/total PKCε in vehicle-treated controls, where total PKCε equals soluble plus particulate fractions. *, p < 0.05; **, p < 0.01, n = 4.

[32P]PA formation between myocytes treated with vehicle or CLA for 1 h (Fig. 6D). However, treatment with CLA for 2 h induced a significant increase in [32P]PA formation, which indicates that CLA stimulates DGK activity (Fig. 6D).

CLA Attenuates Hypertrophy in Vivo—M-mode echocardiography showed early cardiac hypertrophy in SHHF rats (Table 1). Hypertrophic parameters including LVPWd, LVPWs, LV mass, and relative wall thickness were augmented in SHHF rats compared with age-matched SD rats. Fractional shortening in SD and SHHF rats were not significantly different, demonstrating that at 17 weeks of age, SHHF rats do not show signs of declining left ventricular function. Experimental control or CLA diets were palatable to the rats and well tolerated, with no incidents of ill health, diarrhea, weight loss, lethargy, or aggression. Dietary CLA (0.5%, 8 weeks) had no effect on body weight in SD (Fig. 7A) or SHHF rats (Fig. 7B) yet had improved cardiac geometry by attenuating thickening of LVPWd and LVPWs, reducing LV mass, and decreasing relative wall thickness. CLA had no effect on cardiac geometry in SD rats (Table 1).

SBP (187 ± 11 versus 114 ± 4 mm Hg in SD rats, p < 0.001), DBP (92 ± 6 versus 55 ± 2 mm Hg in SD rats, p < 0.001), and mean blood pressure (123 ± 7 versus 74 ± 3 mm Hg in SD rats, p < 0.001) were elevated in SHHF rats compared with SD rats. 8-Week treatment with CLA had no effect on blood pressure in SD rats but partially attenuated the development of hypertension in SHHF rats (Table 2). Notably, the anti-hypertrophic effect of CLA reached statistical significance at 3 weeks in terms of reduced thickening of LVPWd and LVPWs before its reducing effect on blood pressure (data not shown).

DISCUSSION

This study shows that CLA suppresses cardiomyocyte hypertrophy through activation of PPAR isoforms α and γ. This action of CLA is applicable to a number of models of hypertrophy, since CLA attenuated BNP promoter activation triggered by mechanical strain, AngII, and FGF. To our knowledge this is the first report that supplementation with a dietary polyunsaturated fatty acid can prevent cardiac hypertrophy in vivo. CLA supplementation to SHHF rats, which would normally exhibit hypertrophic cardiac growth at this age, resulted in reductions in thickening of the left ventricular wall, left ventricular mass, and relative wall thickness as well as blood pressure. It should be noted that in our experiments the anti-hypertrophic effect of CLA in SHHF rats was evident after only 3 weeks of CLA treatment, whereas BP was not yet significantly reduced. Thus, the ability of CLA to prevent cardiac hypertrophy is attributable at least in part to direct actions on the heart rather than improved hypertension, which is consistent with our in vitro data. Thus, in addition to its other cardioprotective attributes such as anti-arrhythmic (10, 34), anti-atherogenic (35–37), and anti-hypertensive (38–40) effects, our study suggests that CLA also protects against the development of cardiac hypertrophy.

In humans, basal plasma levels of CLA are in the μM range (~7 μM) (41). Daily supplementation with CLA in healthy male (3.0 g/day) and female (3.9 g/d) volunteers resulted in 3–4-fold increases in plasma CLA (42). Given the 3–4-fold increase of plasma CLA with supplementation (41–43) and the fact that local tissue concentrations easily reach levels 10-fold greater than plasma concentrations (44, 45), our experimental concentration of CLA (30 μM) should be physiologically attainable and relevant.

Several lines of evidence indicate that CLA inhibited cardiomyocyte hypertrophy by activating PPARs. Previous studies have reported that specific PPAR agonists such as fibrates or thiazolidinediones inhibit hypertrophy in vivo (21, 46–48) and in vitro (18–21, 49) and that CLA activates all three PPAR isoforms in non-cardiac tissues (11, 51–55). We found that CLA activated all PPAR isoforms in myocytes in the presence of overexpressed PPARs. Similarly, the PPARα, -δ, and -γ-specific agonists, fenofibrate, GW501516, and troglitazone, also required overexpression of PPARs to induce PPAR activation. CLA activated PPARα and -γ to similar extents as fenofibrate and troglitazone, respectively. In contrast, CLA weakly stimulated PPARδ compared with GW501516. Weak activation of PPARδ by CLA in these experiments does not preclude an anti-hypertrophic action of activated PPARδ. However, it may explain why CLA prevented hypertrophy in response to ET-1, AngII, FGF, and mechanical strain but failed to block PE-induced hypertrophy. Lack of CLA effect on PE-induced growth may be due to insufficient activation of PPARδ by CLA, since the selective PPARδ agonist, 1-165041, inhibits PE-induced hypertrophy (18).

Dominant negative (dn) mutant PPARδ failed to abrogate the growth inhibitory action of CLA. We did not determine expression levels of endogenous wild type (wt) PPARδ nor dn PPARδ mutant, so we cannot comment on the stoichiometric ratio of wt:dnPPARδ. However, we did test a range of dnPPARδ con-

[Image 117x699 to 194x716]

[Image 118x665 to 195x686]
centrations (0.01 (not shown) – 0.1 (Fig. 4C) µg/well); none of these blocked CLA. In contrast, dn mutants of PPARα or -γ abolished the inhibitory effect of CLA on ET-1-induced hypertrophy, as did pharmacologically antagonizing PPARα or -γ. Therefore, we provide direct evidence that prevention of hypertrophy by CLA is mediated by PPARα or γ, but probably not δ.

In this study we suggest that CLA attenuates cardiomyocyte hypertrophy via activation of DGKζ, leading to inhibition of PKCe translocation and activation (illustrated in Fig. 8). PKCe activation is an early event of ET-1 signaling. Binding of ET-1 to the ETα receptor triggers activation of Gαq/α11, resulting in DAG production and accumulation in the sarcolemmal membrane. In cardiomyocytes, PKCe and to a much lesser degree, PKCδ8, rapidly bind to DAG in the sarcolemma and are activated (31, 32, 56). Recent studies by Takeishi et al. suggest that the PKC pathway may be attenuated by DGKζ (33). DGKζ phosphorylates DAG, producing PA, thus decreasing available DAG and subsequent PKCe translocation and activation. In neonatal rat cardiomyocytes, adenoviral overexpression of DGKζ abolished ET-1-induced translocation of PKCe but had no effect on PKCeα or -δ subcellular localization (57). Arimoto et al. (58) showed a similar effect in vivo in transgenic mice overexpressing cardiac-specific DGKζ. In wild type mice, infusion of AngII- or PE-induced translocation of PKC isoforms to the plasma membrane, whereas in DGKζ transgenic mice, PKC translocation was abolished (58). Additionally, levels of DAG were elevated in response to PE in wild type mice but not in transgenic mice, suggesting that attenuation of PKC may be a result of DGKζ regulating DAG levels (58). We report here that CLA attenuated ET-1-induced PKCe translocation to the particulate or membrane fraction. We also found that CLA induced an increase in the protein expression of DGKζ and stimulated the production of PA, which suggests increased activity of DGKζ. Despite observing an

![FIGURE 6. CLA induces DGKζ protein expression and activation. A–C, myocytes deprived of serum for 24 h were pre-treated with vehicle (A; 1 h), MK886 (B; 1 µM, 1 h), or GW6622 (C; 1 µM, 1 h), then stimulated with vehicle or CLA (30 µM, 1–4 h). Cells were lysed, and samples were subjected to Western blotting (WB) analysis using DGKζ antibody. Membranes were stripped and reprobed with β-actin antibody to account for loading variation. Results are presented as percent of vehicle-treated controls. A, *p < 0.05, n = 3; B, p = ns, n = 4; C, p = ns, n = 5. D, myocytes deprived of serum for 24 h in phosphate-free DMEM were labeled with ortho[32P]phosphate in phosphate-free DMEM for 3 h. Excess label was washed out, and cells were stimulated with vehicle or CLA (30 µM, 1–2 h). Reactions were terminated, lipids were extracted, and along with a PA standard, separated by TLC. Radiolabeled PA and total phospholipid in each sample were quantified by liquid scintillation counting. Results are expressed as ratio of (PA/total PL) × 10 000. *p < 0.05, n = 4.](image)

**TABLE 1**

Cardiac geometry

Dietary CLA prevented hypertrophy in the SHHF rat. 9-Week-old rats were fed control (0.5% soybean oil) or CLA (0.5% CLA) diets ad libitum. Experimental diets were maintained for 8 weeks. Hypertrophy was measured by M-mode echocardiography as normalized left ventricular posterior wall and relative wall thickening as well as left ventricular mass. BW, body weight, n = 5.

| Parameter                | SD          | SD + CLA     | SHHF        | SHHF + CLA   |
|--------------------------|-------------|--------------|-------------|--------------|
| LVPWd/BW (1000 × (cm/g)) | 0.54 ± 0.06 | 0.56 ± 0.06  | 0.95 ± 0.09  | 0.73 ± 0.06  |
| LVPWs/BW (1000 × (cm/g)) | 0.66 ± 0.03 | 0.77 ± 0.11  | 1.18 ± 0.11  | 0.90 ± 0.06  |
| LVIdc/BW (1000 × (cm/g)) | 1.30 ± 0.03 | 1.25 ± 0.04  | 1.34 ± 0.13  | 1.58 ± 0.14  |
| LVIdc/BW (1000 × (cm/g)) | 0.67 ± 0.04 | 0.65 ± 0.03  | 0.62 ± 0.12  | 0.74 ± 0.07  |
| Relative wall thickness  | 0.84 ± 0.1  | 0.90 ± 0.11  | 1.46 ± 0.21  | 0.96 ± 0.28  |
| LV mass/BW (100×)       | 0.11 ± 0.01 | 0.11 ± 0.01  | 0.21 ± 0.03  | 0.16 ± 0.01  |
| BW (g)                  | 579 ± 7     | 613 ± 23     | 395 ± 15     | 397 ± 11     |
| Fractional shortening (%) | 48.6 ± 3.2 | 47.5 ± 1.3   | 55.2 ± 4.5   | 52.9 ± 3.7   |

*p < 0.01 vs. SD.

*p < 0.05 vs. SHHF.

*p < 0.05.
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**FIGURE 7.** Dietary CLA does not affect body weight in the SD or SHHF rat. 9-Week old rats were fed control (0.5% soybean oil) or CLA (0.5% CLA) diets ad libitum. Experimental diets were maintained for 8 weeks. n = 5, p = ns.

**TABLE 2**

| Parameter       | SD       | SD + CLA | SHHF    | SHHF + CLA |
|-----------------|----------|----------|---------|------------|
| SBP (mm Hg)     | 114 ± 4  | 122 ± 5  | 187 ± 11| 164 ± 8*   |
| DBP (mm Hg)     | 55 ± 2   | 59 ± 2   | 92 ± 6* | 80 ± 4*    |
| Mean BP (mm Hg) | 74 ± 3   | 80 ± 3   | 123 ± 7*| 106 ± 6*   |
| Pulse pressure  | 59 ± 2   | 63 ± 2   | 96 ± 5* | 85 ± 4*    |

* p < 0.001 vs. SD.
* p < 0.05 vs. SHHF.
* p < 0.01.

**FIGURE 8.** Proposed mechanism by which CLA abrogates ET-1-induced hypertrophic signaling. ET-1 induces accumulation of DAG in the plasma membrane, which causes translocation and activation of PKCe, an early signaling mediator of hypertrophy. CLA may interrupt hypertrophic signaling by activating PPARα and/or γ isoforms, which stimulates DGKζ to phosphorylate DAG, producing PA. The resulting decreased availability of DAG inhibits PKCe recruitment to the plasma membrane.

increase in DGKζ protein expression in myocytes treated with CLA for 1 h, we did not detect DGKζ activity (increase in PA formation) until 2 h of treatment with CLA. It is entirely possi-

ble that small changes in DGKζ activity that elude our detection at 1 h would be enough to disrupt hypertrophic signaling. Additionally, at 2 h of CLA treatment, PA may have accumulated to a level that is measurable by our assay. We also observed that increased expression of DGKζ is dependent on PPARα or -γ. A role for PPAR in the activation of DGK and inhibition of PKC signaling has also been observed in endothelial cells. PPARγ agonists, specifically ciglitazone, troglitazone, and 15d-PGJ2, were each shown to inhibit endothelial cell activation by up-regulating DGKα and disrupting translocation of PKCβ (59).

It should be noted that since we employed a mixed (albeit pure) preparation of CLA, the question arises whether its anti-hypertrophic activity is supported by one or more of the iso-

mers that constitute the preparation. Individual CLA isomers may suffice to prevent hypertrophy. Alternatively, a synergistic interaction(s) between isomers may contribute to certain bi-

ological effects of CLA. Further studies using pure CLA isomers are required to fully elucidate the specificity of different iso-

mers in hypertrophy.

In summary, this study shows that activation of PPARα and γ by CLA prevents cardiac hypertrophy through activation of DGKζ and subsequent inhibition of the PKCe pathway. These findings provide novel support for the role of polyunsaturated fatty acids as cardioprotective dietary elements. In a time when prevention of hypertrophy is viewed as a promising therapeutic target (60) and ~44% of heart failure patients resort to nutrition-based therapies (50), this study may have important implications for effective nutritional intervention toward the pre-

vention of cardiac disease.

Acknowledgments—We are grateful to Lam Dang and Nicole Clement for excellent technical assistance. We also thank Dr. Chris Anderson for critical review of the manuscript.

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