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Conjugated linoleic acid stimulates an anti-tumorigenic protein NAG-1 in an isomer specific manner

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Conjugated linoleic acids (CLAs), naturally occurring fatty acids in ruminant food products, have anti-tumorigenic and pro-apoptotic properties in animal as well as *in vitro* models of cancer. However, the cellular mechanism has not been fully understood. NAG-1 (non-steroidal anti-inflammatory drug-activated gene-1) is induced by several dietary compounds and belongs to a TGF-β superfamily gene associated with pro-apoptotic and anti-tumorigenic activities. The present study was performed to elucidate the molecular mechanism by which CLA stimulates anti-tumorigenic activity in human colorectal cancer (CRC) cells. The *trans*-10, *cis*-12-CLA (t10,c12-CLA) repressed cell proliferation and induced apoptosis, whereas linoleic acid or c9,t11-CLA showed no effect on cell proliferation and apoptosis. We also found that t10,c12-CLA induced the expression of a pro-apoptotic gene, *NAG-1*, in human CRC cells. Inhibition of NAG-1 expression by small interference RNA (siRNA) results in repression of t10,c12-CLA-induced apoptosis. Microarray analysis using t10,c12-CLA-treated HCT-116 cells revealed that activating transcription factor 3 (ATF3) was induced and its expression was confirmed by western analysis. The t10,c12-CLA treatment followed by the overexpression of ATF3 increased NAG-1 promoter activity in HCT-116 cells. We further provide the evidence that t10,c12-CLA inhibited the phosphorylation of AKT and the blockage of GSK-3 by siRNA abolished t10,c12-CLA-induced ATF3 and NAG-1 expression. The current study demonstrates that t10,c12-CLA stimulates ATF3/NAG-1 expression and subsequently induces apoptosis in an isomer specific manner. These effects may be through inhibition of AKT/GSK-3β pathway in human CRC cells.

Abbreviations: AHPN, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid; ATF3, activating transcription factor3; c9,t11-CLA, *cis*-9, *trans*-11 isomer of CLA; CHX, cycloheximide; CLA, conjugated linoleic acid; CRC, colorectal cancer; CREB, cAMP response element binding protein; GSK-3, glycogen synthase kinase-3; IGF-1, insulin-like growth factor-1; LA, linoleic acid; LBD, ligand binding domain; NAG-1, NSAID-activated gene-1; NSAIDs, non-steroidal anti-inflammatory drugs; PI3K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; t10,c12-CLA, *trans*-10, *cis*-12 isomer of CLA.

Introduction

Cancer is second only to heart disease as the leading cause of death in the USA and colorectal cancer (CRC) is one of the most prevalent causes of cancer-related mortality in the Western world (1). Among the strategies to reduce colon cancer risk, chemoprevention by dietary factors has received considerable attention as an effective approach for cancer prevention. A possible correlation between dietary fat intake and cancer incidence has been hypothesized because the large international variation in rates of cancers of the breast, colon and prostate are strongly correlated with apparent per capita fat consumption.

Conjugated linoleic acid (CLA) is composed of isomers of octadecadienoic acid with conjugated double bonds and is found naturally in dairy products and ruminant meats (2). CLA has been known as a novel dietary component for the prevention of increased risk of cancer due to its chemoprotective properties. Dietary CLA inhibits chemically induced development of colon cancer in animal models (3) and inhibits growth rates of human CRC cells *in vitro* (4). CLA inhibits PI3K/AKT and ERK-1/2 signaling pathways (5) and compromises the expression of cyclins required for G1/S progression and the phosphorylation status of Rb *in vitro* (6). In contrast, CLA was not effective when given only during the promotion phase of colon carcinogenesis (7), or in the *Min* mouse model (8). It is very likely that this conflict persists due to the fact that the chemopreventive mechanism of CLAs is isomer specific and the efficacy of the CLA is dependent on cell or tissue context.

Using PCR-based subtractive hybridization, our group identified a non-steroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1) as a pro-apoptotic and anti-tumorigenic protein induced by NSAIDs (9). The human NAG-1 cDNA encodes a secreted protein with homology to members of the TGF-β superfamily and has been previously identified as macrophage inhibitory cytokine-1 (MIC-1), placental transformation growth factor-β (PTGFB), prostate derived factor (PDF), growth differentiation factor-15 (GDF-15) and placental bone morphogenetic protein (PLAB) (9). NAG-1 is highly expressed in mature intestinal epithelial cells, but is significantly reduced in human CRC samples and neoplastic intestinal polyps of *Min* mice (10). In addition, expression of the full-length NAG-1 protein resulted in increased apoptosis in HCT-116 cells and NAG-1 expression results in a decrease of tumor size in the xenograft mouse model and leads to an inhibition of growth on soft agar (9). In prostate cancer cells, DU-145, exposure to NAG-1 resulted in an increased level of caspase-dependent apoptosis (11). We have recently

developed a transgenic mouse that ubiquitously overexpresses a human NAG-1 cDNA. Studies with these mice reveal a decrease in the number of intestinal tumors and aberrant cryptic foci in response to azoxymethane as compared to control littermates (S.J.Baek, R.Okazaki, J.Martinez, J.-S.Kim, S.-H.Lee, K.Yamaguchi, Y.Mishina, D.Martin, A.Shoieb, M.F.McEntee, and T.E.Eling, submitted for publication). These observations strongly support the notion that NAG-1 elicits many of the growth suppressive anti-cancer effects, particularly in colon. NAG-1 expression is upregulated in a prostaglandin-independent manner in human CRC cells by several anti-tumorigenic compounds such as resveratrol (12), genistein (13), catechins (14) and indole-3-carbinol (15) as well as known anti-tumorigenic compounds, peroxisome proliferator-activated receptorγ (PPARγ) ligands (16), 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F-203) (17) and retinoid 6-(3-(1-adamantyl)-4-hydroxyphenyl)-2-naphthalene carboxylic acid (AHPN) (18). While some dietary compounds, including resveratrol and genistein, induce NAG-1 expression through the p53 tumor suppressor protein (12,13), other compounds including NSAIDs and troglitazone induce NAG-1 through early growth response gene-1 (*EGR-1*) (16,19). Moreover, NAG-1 expression is regulated by (PI3K/AKT/GSK-3β) pathway (20) as well as PKC-dependent pathway involving the activation of nuclear transcription factor κB (NF-κB) (21). Thus, NAG-1 is regulated by several signaling pathways.

Activating transcription factor 3 (*ATF3*) is a member of the ATF/CREB (cAMP response element binding protein) subfamily of the basic-region leucine zipper (bZIP) family and its expression is dramatically induced in response to a variety of stress conditions in many different tissues (22). Recently, *ATF3* has been postulated to be a tumor suppressor gene because *ATF3* coordinates the expression of genes that may be linked to cancer (23,24). Moreover, the expression of *ATF3* was repressed in human colorectal tumors compared to normal adjacent tissue (25). Interestingly, it has been reported that both *ATF3* and *NAG-1* were co-induced by the treatment of the anti-tumorigenic compound indole-3-carbinol (15), 5F-203 (17) as well as by sulindac (25,26). However, the molecular basis for the induction of *ATF3* and the scope of its target genes are not well understood.

In this report, we speculated that the induction of apoptosis by CLA might be related to increase the *ATF3/NAG-1* expression in colon cancer cells. Here we report that *trans*-10, *cis*-12-CLA (t10,c12-CLA) stimulates *ATF3* and *NAG-1* expression, probably mediated by AKT/GSK-3β pathway in human CRC cells.

Materials and methods

Materials

Human colorectal adenocarcinoma cells, HCT-116 and HT-29, were purchased from American Type Culture Collection (Manassas, VA). Antibodies for p53 and ATF3 were purchased from Santa Cruz (Santa Cruz, CA). CREB, phospho-AKT (Ser^{473}) and total AKT antibodies were purchased from Cell Signaling (Beverly, MA). IGF-1 was purchased from BD Biosciences (Bedford, MA). Linoleic acid (LA) was purchased from NuChek Prep (Elysian, MN), and c9,t11-CLA and t10,c12-CLA were purchased from Matreya (Pleasant Gap, PA). Four copies of a Gal4 binding site (MH100x4-TK-LUC) and chimeric receptors (pCMX-Gal-mPPARα-LBD, pCMX-Gal-mPPARγ-LBD and pCMX-Gal-mPPARδ-LBD) were generously provided by Dr R. Evans (Howard Hughes Medical Institute, CA). The pCG-ATF3 construct and the β-catenin constructs were generously provided by Dr T. Hai (Ohio State University, Columbus, OH) and by Dr Barth (Standford University), respectively. *Cyclin D1* promoter constructs were previously described (27). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

Cell culture and treatment

HCT-116 and HT-29 cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) and 10 μg/ml of gentamycin. To examine the effect of CLA, cells were plated in 60-mm culture dishes and incubated until cells were 80–90% confluent. CLA (5.6 mg) was dissolved in 1 ml of 0.1 M KOH (50°C for 10 min) and incubated with 9 ml of 1.25 mM fatty acid-free BSA solution (Roche, Indianapolis, IN) overnight at 4°C (2 mM stock solution, pH 7.2). Fatty acid-bovine serum albumin (BSA) complex were filter-sterilized using 0.22 μm filter (Millipore, Billerica, MA) before use and BSA solution without CLAs was used as vehicle. Cells were washed and fresh serum-free medium containing CLA isomers was replaced.

Cell proliferation and apoptosis with flow cytometric analysis

The effect of the CLA on cell proliferation was investigated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). Briefly, cells were seeded at the concentration of 1000 cells/well in 96-well tissue culture plates in six replicates and maintained overnight. The cells were then treated with 50 μM of LA, c9,t11-CLA, or t10,c12-CLA. At 0, 1, 2, 4, 5 and 6 days after treatment, 20 μl of CellTiter96® Aqueous One solution was added to each well and the plate was incubated for 1 h at 37°C and absorbance at 490 nm was recorded in an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek Instruments, Winooski, VT). Apoptosis was measured using PI/Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA). Briefly, cells were plated in six-well tissue culture dishes and incubated with 50 μM of LA, c9,t11-CLA or t10,c12-CLA for 72 h. Both attached and floating cells were harvested, washed with PBS and stained with Annexin V-FITC, according to the manufacturer's instructions. Early and late apoptosis were quantified by flow cytometry using Beckman Coulter Epixs XL equipped with ADC and ModFit LT software.

Transient transfections

Transient transfections were performed using the Lipofectamine (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. HCT-116 cells were plated in 12-well plates at the concentration of 1×10^5 cells/well. After growth for 18 h, plasmid mixtures containing 0.5 μg of *NAG-1* promoter linked to luciferase and 0.05 μg of pRL-null vector were transfected for 5 h. The transfected cells were cultured in the absence or presence of 50 μM CLA isomers for 24 h. The cells were harvested in 1X luciferase lysis buffer and luciferase activity was normalized to the pRL-null luciferase activity using a dual luciferase assay kit (Promega). For the ATF3 co-transfection experiment, 0.25 μg of *NAG-1* promoter and 0.25 μg of ATF3 expression vector (pCG-ATF3) were co-transfected with 0.05 μg of pRL-null vector. For the PPAR activation study, MH100x4-TK-LUC plasmid (0.25 μg) and pCMX-Gal-mPPARα-LBD, pCMX-Gal-mPPARγ-LBD, or pCMX-Gal-mPPARδ-LBD (0.25 μg each) were co-transfected with pRL-null vector (0.05 μg) into HCT-116 cells and treated with vehicle, LA or CLAs for 24 h as described above. For transfection of constitutively active β-catenin mutant, the cells were transfected with 0.5 μg of control vector (pcDNA3.1-LacZ) or pEGFP-β-catenin-ΔN90 for 5 h and subsequently treated with 50 μM of CLAs for 24 h.

Western analysis

Cells were washed with PBS and cell lysates were isolated in RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS] supplemented with protease inhibitors (1 mM PMSF, 5 μg/ml aprotinin and 5 μg/ml leupeptin) and phosphatase inhibitors (1 mM Na_3VO_4 and 1 mM NaF) and centrifuged at 12,000 r.p.m. for 5 min at 4°C. Protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL) using BSA as the standard. The proteins (30 μg) were separated on SDS-PAGE and transferred to nitrocellulose membranes (Osmonics, Minnetonka, MN). The membranes were incubated with a specific primary antiserum in Tris buffered saline (TBS) containing 0.05% Tween-20 (TBS-T) and 5% non-fat dry milk at 4°C overnight. After three washes with TBS-T, the blots were incubated with peroxidase-conjugated IgG for 1 h at room temperature, visualized using ECL (Amersham Biosciences, Piscataway, NJ) and quantified by Scion Image Software (Scion, Frederick, MD).

TranSignal™ Human TF cDNA array

To determine the transcription factors activated by t10,c12-CLA treatment, microarray analysis was performed with the TranSignal™ Human TF cDNA array according to manufacturer's instructions (Panomics, Redwood, CA). Briefly, 10 μg of total RNA was used as a template for cDNA synthesis and the cDNA probe was labeled with biotin-dUTP during reverse-transcription. Then, the reaction was stopped, incubated at 68°C for 2 min and neutralized by adding 30 μl of 2× neutralization buffer. The resulting labeled cDNA probe

was applied to a prehybridized TranSignal™ Human TF cDNA membrane array. The hybridization was performed at 42°C for 12 h in a hybridization oven. After two-step washing at 42°C, the membrane was blocked and treated with streptavidin–horseradish peroxidase (HRP) conjugate and finally exposed to Luminol enhancer and peroxidase solution. Signals were detected by exposure to X-ray film.

Isolation and analysis of RNA

Total RNA was isolated with Trizol Reagent (Invitrogen), according to the manufacturer's instructions. Subsequently, 10 μg of total RNA were fractionated on 1.4% agarose-2.2 M formaldehyde gels and transferred to positively-charged nylon membrane. The cDNA probe for NAG-1 was synthesized using the Biotin random prime kit (Pierce). The probes used were full-length NAG-1 fragments (28). Hybridization and chemiluminescent signal detection were performed using the North2South chemiluminescent detection kit (Pierce) according to the manufacturer's instructions. Autoradiographs were scanned and images were analyzed using Scion Image software.

RNA interference

HCT-116 cells were transfected with the GSK-3α/β small interference RNA (siRNA; Cell Signaling) at a concentration of 100 nM or negative control siRNA (Ambion, Austin, TX), using TransIT-TKO transfection reagent (Mirus, Madison, WI), as described previously (20). The NAG-1 siRNA vector (pSuper-retro-puro-si NAG-1) was constructed and described previously (21). HCT-116 cells were transfected with 2 μg of siRNA NAG-1 using Lipofectamine 2000 (Invitrogen). After 24 h transfection, the cells were treated with vehicle or t10,c12-CLA (50 μM) for 24 and 72 h for Western blot and apoptosis analysis, respectively.

Statistical analysis

Statistical analysis was performed with Student's unpaired *t*-test, with statistical significance set at *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Results

t10,c12-CLA inhibits cell proliferation and induces apoptosis in HCT-116 cells

To investigate the effect of CLAs on the growth of CRC cells in culture, HCT-116 cells were incubated with different isoforms of CLA for several days and cell proliferation was measured using Cell Proliferation Assay kit (Promega). Since the physiological levels of CLA isomers have been reported to be in the range of 20–70 μM (29), 50 μM of CLA was used in this experiment. As shown in Figure 1A, HCT-116 cells treated with t10,c12-CLA reduced the cell growth rate by 20, 34 and 40% on Day 4, 5 and 6, respectively. However, LA and c9,t11-CLA did not significantly influence cell growth (Figure 1A). Similar data were obtained by cell counting and trypan blue exclusion (data not shown). Apoptosis was also measured using flow cytometry analysis. As shown in Figure 1B, 2-fold increase of apoptotic cells was observed in HCT-116 cells treated with 50 μM of t10,c12-CLA for 3 days, compared to vehicle-treated cells. Taken together, these results indicate that t10,c12-CLA induces apoptosis and cell growth arrest in HCT-116 cells in an isomer specific manner.

t10,c12-CLA stimulates NAG-1 expression in HCT-116 cells

NAG-1 is linked to cell growth arrest and apoptosis and is responsible for chemoprevention by several dietary compounds in a variety of carcinoma models (12–18). To investigate whether CLA-induced apoptosis is associated with *NAG-1* expression, HCT-116 cells were grown and treated with different isomers of CLA. *NAG-1* and other growth related genes were measured by Western analysis. As shown in Figure 2A, *NAG-1* expression was increased by ~7.2, 8.5 and 10.2-fold in HCT-116 cells treated with 25, 50 and 100 μM of t10,c12-CLA, respectively. NAG-1 protein levels were not significantly changed by the addition of LA or c9,t11-CLA in all doses tested. It has been shown that the anti-tumorigenic effects of CLA may be mediated by the p53 protein (6) and NAG-1 expression was regulated by p53 protein at the transcriptional level (12,13). Therefore, the same blot was hybridized with p53 antibody, but the p53 expression was not altered by CLAs and LA treatment, suggesting that NAG-1 induction by t10,c12-CLA may not be associated with p53 protein expression in HCT-116 cells (p53 wild-type). Other cell cycle regulated genes including *cyclin D1* and *p27* were not altered by CLA treatment in HCT-116 cells (Figure 2A).

The β-catenin signaling is an important pathway affecting colorectal tumorigenesis. To investigate whether β-catenin activation affects CLA-induced NAG-1 expression, HCT-116 cells were transfected with constitutively active form of β-catenin (ΔN90 mutant) (30) and treated with either c9,t11-CLA or t10,c12-CLA for 24 h. As shown in Figure 2B, NAG-1 was induced in HCT-116 cells treated with t10,c12-CLA, while overexpression of β-catenin mutant did not modulate t10,c12-CLA-induced NAG-1 expression. It is notable that the molecular weight of exogenous β-catenin is bigger than endogenous β-catenin due to inclusion of 26 kDa of enhanced green fluorescent protein (EGFP). This construct has been used to activate β-catenin signaling (31) and we also confirmed the enhancement of β-catenin activity using TOP Flash reporter gene as well as *cyclin D1* promoter containing

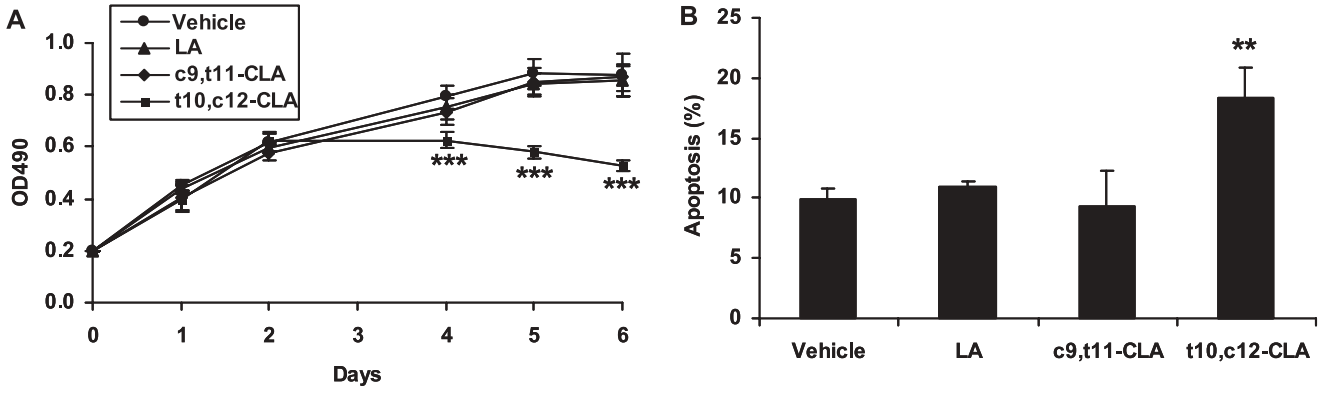

Fig. 1. Growth arrest and apoptosis in CLA-treated HCT-116 cells. (**A**) HCT-116 cells were treated with vehicle, LA (50 μM) or different CLAs (50 μM each) for several days. Cell growth was measured using the CellTiter96 Aqueous One Solution Cell Proliferation Assay. Values are expressed as mean ± SD of six replicates. ***, $P < 0.001$ versus vehicle-treated cells (**B**) HCT-116 cells were treated with vehicle, LA (50 μM) or different CLAs (50 μM each) for 72 h. Apoptosis was analyzed using PI/Annexin V staining as described in Materials and methods. Values are expressed as mean ± SD of three replicates. **, $P < 0.01$ versus vehicle-treated cells.

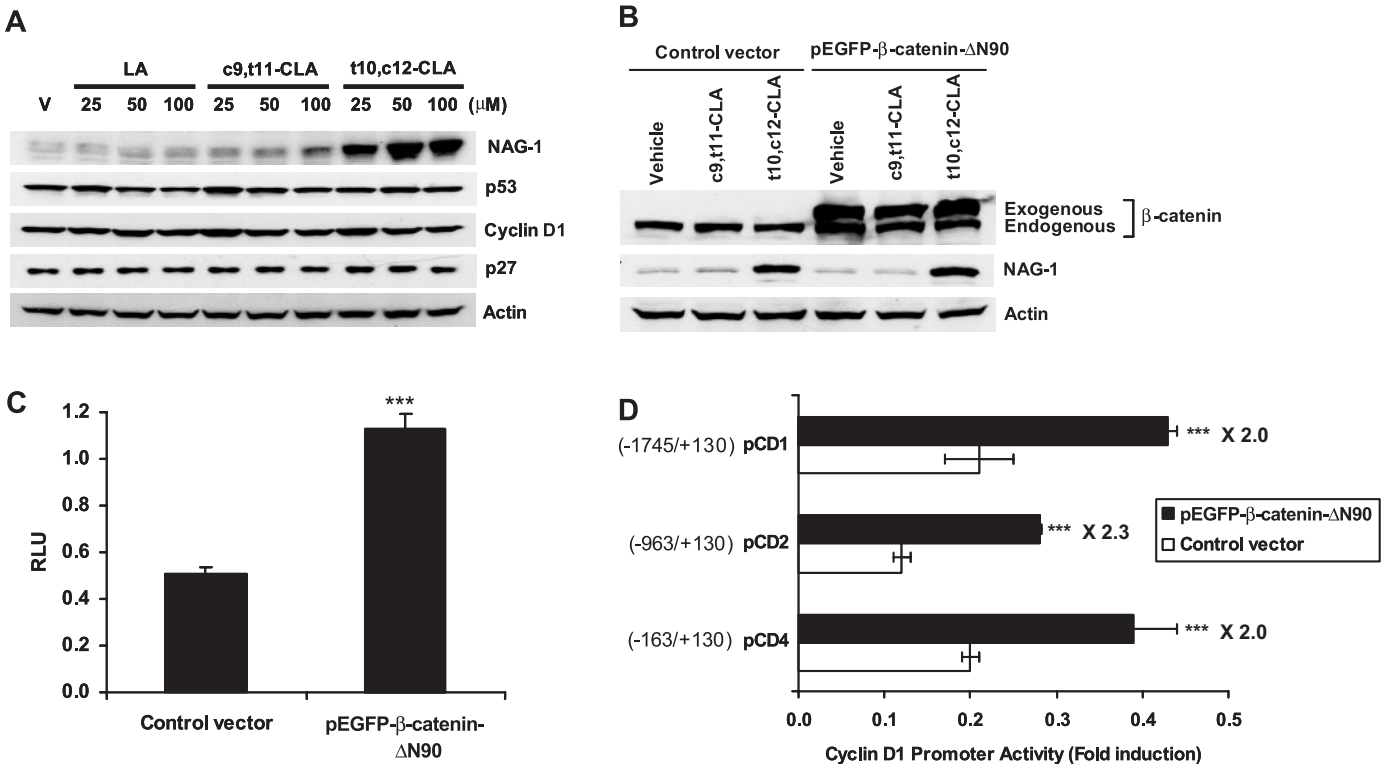

Fig. 2. Dose–response of NAG-1 protein levels in CLA-treated HCT-116 cells. (**A**) HCT-116 cells were treated with vehicle (V), LA or different CLAs at various concentrations for 24 h. Subsequently, 30 μg of total cell lysates were subjected to 14% SDS–PAGE. NAG-1, p53, cyclin D1, p27 and actin antibodies were probed. (**B**) HCT-116 cells were transfected with constitutively active form of β-catenin (ΔN90 mutant conjugated with EGFP) (30) and treated with either 50 μM of c9,t11-CLA or t10,c12-CLA for 24 h. NAG-1, β-catenin and actin expression were measured as described in (A). (**C**) TOP flash constructs containing six copies of TCF binding site (0.25 μg) were co-transfected into HCT-116 cells with control (0.25 μg) or pEGFP-β-catenin-ΔN90 (0.25 μg) using lipofectamine and luciferase activity was measured. (**D**) *Cyclin D1* promoters (0.25 μg) containing TCF-binding sites (32) were co-transfected into HCT-116 cells with control (0.25 μg) or pEGFP-β-catenin-ΔN90 (0.25 μg) using lipofectamine and luciferase activity was measured. Values are expressed as mean ± SD of three replicates. ***, $P < 0.001$ versus control group.

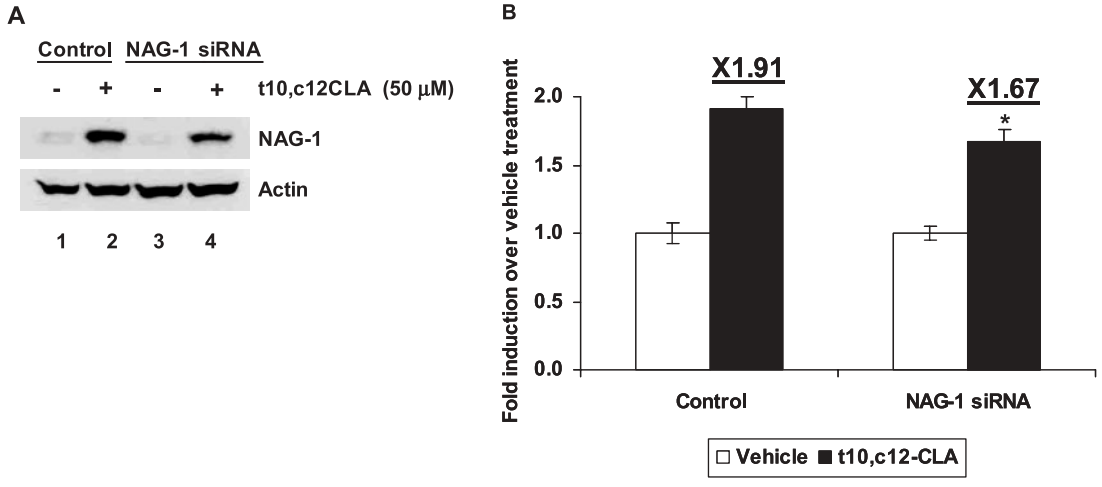

Fig. 3. Inhibition of NAG-1 using NAG-1 siRNA alters t10,c12-CLA-induced apoptosis. (**A**) HCT-116 cells were transfected with control vector (pSuper-retro-puro) or NAG-1 siRNA (pSuper-retro-puro-siNAG-1) (21) and treated with t10,c12-CLA (50 μM) or vehicle for 24 h. Western analysis was performed for NAG-1 and actin. (**B**) HCT-116 cells were transfected with control vector or NAG-1 siRNA construct. Apoptosis was analyzed at 72 h after treatment using PI/Annexin V staining as described in Materials and methods.

several TCF binding sites (27,32) (Figure 2C and D).

NAG-1 expression mediates at least in part CLA-induced apoptosis

To examine whether NAG-1 induction has any relevance to the induction of apoptosis by t10,c12-CLA, we transfected NAG-1 siRNA into HCT-116 cells and total lysates were subjected to western blot analysis for NAG-1. As shown in Figure 3A, the expression of NAG-1 siRNA partially blocked t10,c12-CLA-induced NAG-1 expression by 40% in the presence of CLA (Figure 3A, lanes 2 and 4). Duplicated samples were measured for apoptosis using flow cytometry. Fold induction of apoptosis by t10,c12-CLA in NAG-1 siRNA-transfected cells was lower than that of control vector-transfected cells (1.91

versus 1.67; $P = 0.015$), suggesting the inhibition of NAG-1 expression results in the suppression of t10,c12-CLA-induced apoptosis in HCT-116 cells.

NAG-1 induction by t10,c12-CLA is independent on PPAR transactivation

The activation of PPARγ by its ligands has been shown to have beneficial effects on several types of cancers including colon (33) and NAG-1 is one of PPARγ target genes (34). Thus, we examined whether LA, c9,t11-CLA or t10,c12-CLA binds to PPAR as a ligand using a luciferase system. HCT-116 cells were plated in 12-well plates and transfected with four copies of a Gal4 binding site (MH100x4-TK-LUC) and chimeric receptors (pCMX-Gal-mPPARα-LBD, pCMX-Gal-mPPARγ-LBD, or pCMX-Gal-mPPARδ-LBD). In this system, when a compound binds to the ligand binding domain (LBD) from PPARα, γ or δ of the chimeric receptor, the DNA binding domain of the yeast Gal4 binds to co-transfected Gal4 binding site and initiates transcription of the firefly luciferase (LUC). After the cells were treated with LA, c9,t11-CLA or t10,c12-CLA (50 μM) for 24 h, luciferase activities were measured to assess the transactivation for each PPAR receptor. As shown in Figure 4, c9,t11-CLA and t10,c12-CLA showed 3.5 and 4.0-fold increase of transactivation to PPARα, whereas LA showed 1.7-fold increase. LA, c9,t11- and t10,c12-CLA showed 1.6, 1.8 and 2.1-fold increase of transactivation to PPARγ, respectively. However, PPARδ showed no significant increase in activity with any treatment (Figure 4). Since LA and CLAs are the PPARα and γ ligands, these data do not provide the molecular mechanism by which only the t10,c12-CLA induces NAG-1 expression. Thus, the t10,c12-CLA-induced NAG-1 expression may not result from PPAR activation. This is consistent with previous reports, showing NAG-1 induction by other PPARγ agonists in PPARγ-independent manner (35).

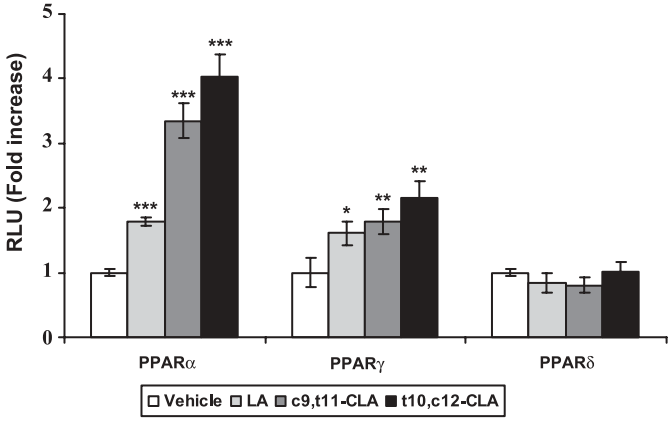

Fig. 4. PPAR activation assays. HCT-116 cells were plated at 10^5 cells/well in 12-well plates and were transiently transfected with four copies of a Gal4 binding site (MH100x4-TK-LUC) and chimeric receptors pCMX-Gal-mPPARα-LBD, pCMX-Gal-mPPARγ-LBD or pCMX-Gal-mPPARδ-LBD. The cells were treated with vehicle, LA (50 μM) or different CLAs (50 μM each) for 24 h and luciferase activity was measured. The data present as relative luciferase activity (firefly luciferase signal/renilla luciferase signal). The results show the mean ± SD of three independent transfections. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ versus vehicle-treated cells.

t10,c12-CLA does not affect RNA stability but requires de novo synthesis on NAG-1 induction

It has been shown that NAG-1 is regulated at the post-transcriptional level by AHPN (18). To determine whether NAG-1 was regulated by t10,c12-CLA at a post-transcriptional level, we have examined its effect on *NAG-1* mRNA stability. HCT-116 cells were treated with either 50 μM of t10,c12-CLA or vehicle for 24 h and then 5 μg/ml of actinomycin D was added at 0, 1, 2, 4 or 8 h. The half-lives of the mRNA were estimated by northern analysis. As shown in Figure 5A, t10, c12-CLA treatment does not affect the half-life of *NAG-1* mRNA compared to vehicle treatment (199 versus 186 min in vehicle versus t10,c12-CLA-treated cells, respectively). These results suggest that t10,c12-CLA may not affect *NAG-1* RNA stability. To investigate whether CLA-induced NAG-1 expression requires *de novo* synthesis, HCT-116 cells were pretreated with or without 10 μg/ml cycloheximide (CHX) for 30 min and then followed by treatment with vehicle or 50 μM of t10,c12-CLA. As shown in Figure 5B, *NAG-1* mRNA was induced by t10,c12-CLA treatment (20.3-fold, Figure 5B, lanes 1 and 2). However, in the presence of CHX, t10,c12-CLA did not increase the same level of *NAG-1* mRNA (2.3-fold, Figure 5B, lanes 3 and 4), suggesting that CLA-induced NAG-1 expression requires, at least in part, *de novo* protein synthesis. CHX treatment resulted in the induction of *NAG-1* mRNA, possibly via accumulation of *NAG-1* mRNA (Figure 5B, lane 3).

ATF3 expression is induced by t10,c12-CLA

The t10,c12-CLA treatment is not involved in the RNA stability or PPARγ activation and NAG-1 expression requires *de novo* synthesis. Therefore, we measured changes in gene expression using a human transcription factor cDNA array as described in Materials and methods (Figure 6A) to investigate the molecular mechanisms responsible for NAG-1 induction by t10,c12-CLA. From the microarray experiment, *ATF3*, *E2F1* and *ER*β were identified as upregulated genes, whereas *SMAD1*, *SMAD4* and *RAR* were downregulated by treatment with t10,c12-CLA in HCT-116 cells. In this report, we decided to focus on *ATF3* because it has been shown to be induced by several compounds including NSAIDs and dietary compounds, indole-3 carbinol and catechins (14,15,26). To confirm the microarray data, ATF3 protein levels were measured by western blot analysis. ATF3 expression was increased in a dose-dependent manner in HCT-116 cells treated with t10,c12-CLA, whereas LA and c9,t11-CLA did not induce ATF3 expression. We also measured CREB protein levels because the consensus binding site for ATF3 is similar to the CRE consensus sequence. The protein level of CREB was not altered in the presence of LA or CLA isomers (Figure 6B). Finally, other human CRC cells, HT-29 cells (p53 mutated and COX-2 expressing cells), were also treated with t10,c12-CLA at various concentrations. As shown in Figure 6C, the t10,c12-CLA treatment induced NAG-1 and ATF3 expressions in HT-29 cells, indicating that NAG-1 induction by t10, c12-CLA was seen not only in HCT-116 cells, but also in other CRC cells. Taken together, ATF3 expression may associate with the t10,c12-CLA-induced NAG-1 expression.

Promoter activity of NAG-1 by CLAs and ATF3 overexpression

To address whether the expression of ATF3 is connected with NAG-1 expression, time course expression of both ATF3 and

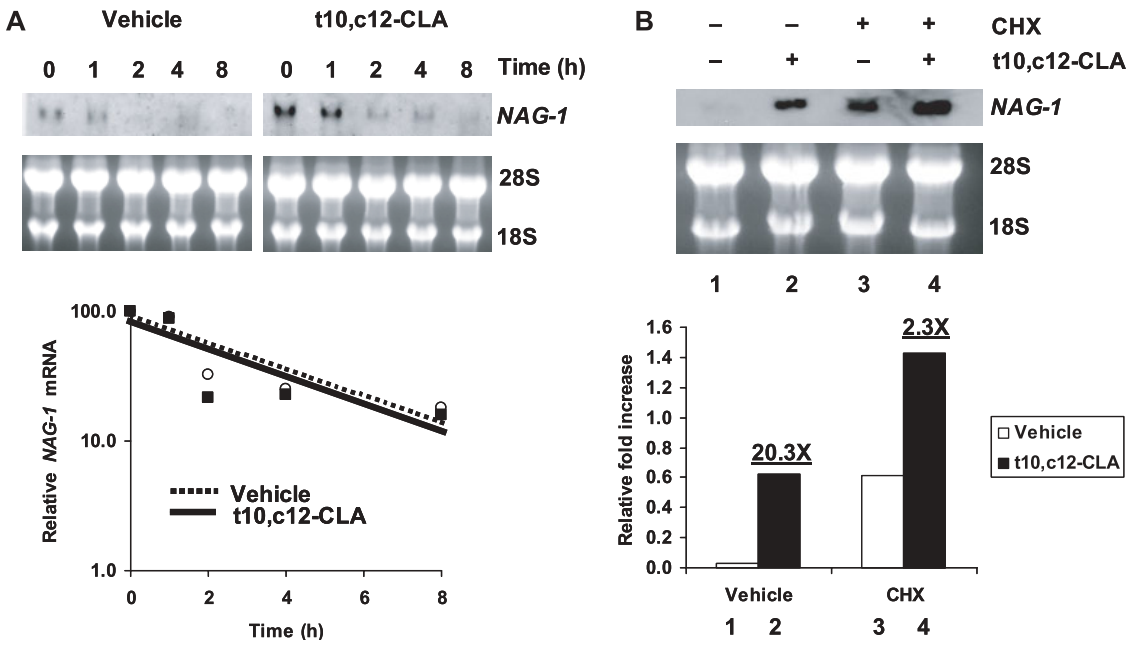

Fig. 5. CLA-induced NAG-1 expression requires *de novo* synthesis. (**A**) HCT-116 cells were treated with t10,c12-CLA (50 μM) or vehicle for 24 h and subsequently with actinomycin D (5 μg/ml). At the indicated times, total RNAs were isolated and examined by northern blot analysis with a biotin-labeled probe for human NAG-1. Both 28S and 18S bands were used for the equal loading control. Hybridization signals were quantified with Scion Image software. The relative level of NAG-1 mRNA (relative to the level of 18S) was calculated and the results plotted as the percentage of the mRNA level present at time zero of actinomycin D treatment. (**B**) CHX (10 μg/ml) or vehicle was added 30 min before HCT-116 cells were treated with t10,c12-CLA (50 μM). After 24 h, total RNA was isolated and examined by northern blot analysis for *NAG-1* mRNA expression. 18S bands were used for the equal loading control. The intensity of bands were quantified and represented as a fold induction over vehicle-treated sample at the bottom.

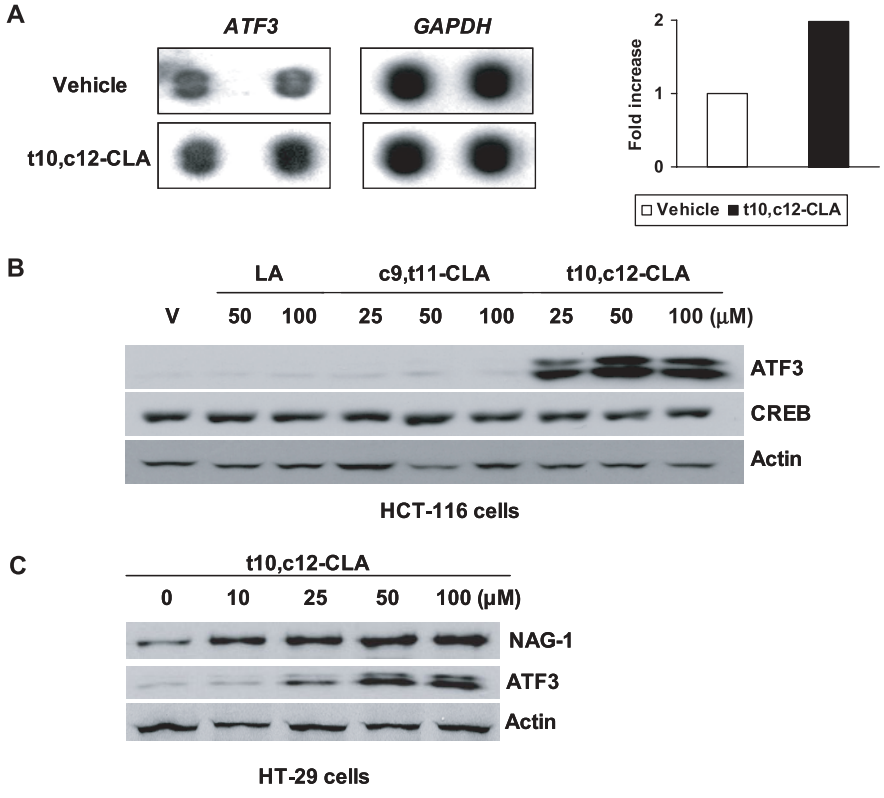

Fig. 6. ATF3 induction by t10,c12-CLA. (**A**) HCT-116 cells were treated with either vehicle or t10,c12-CLA (50 μM) for 24 h and then microarray analysis was performed according to the manufacture's protocol. The TranSignal™ Human TF cDNA array was used and ATF3 and GAPDH were indicated as duplicated spots. The intensities were measured by Scion Image and represented as a fold induction over vehicle-treated sample. (**B**) HCT-116 cells were treated with vehicle, LA or different CLAs for 24 h. Subsequently, 30 μg of total proteins were loaded and Western analysis was performed using ATF3, CREB and actin antibodies. (**C**) HT-29 cells were treated with t10,c12-CLA at indicated concentration. Cell lysates were subjected to western analysis and the expressions of NAG-1, ATF3 and actin were examined.

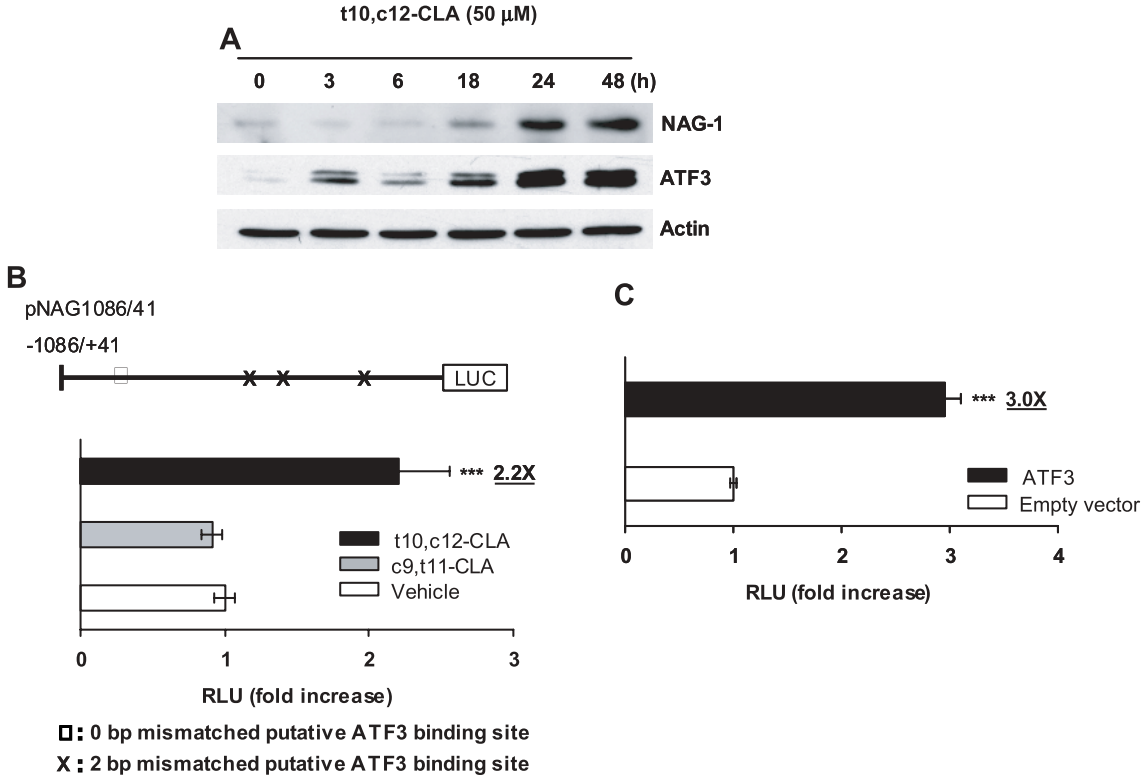

Fig. 7. *NAG-1* promoter assay in CLA-treated HCT-116 cells. (**A**) Western analysis of NAG-1 and ATF3 from HCT-116 cells treated with t10,c12-CLA (50 μM) at different time points. (**B**) There are four potential ATF3 binding sites with point mutation. The pNAG1086/41 construct (0.5 μg) was co-transfected with pRL-null vector (0.05 μg) as described in Materials and methods. The cells were treated with vehicle, or different CLAs (50 μM each) for 24 h and luciferase activity was measured. Fold increase refers to ratio of luciferase activity of CLA-treated cells compared to vehicle-treated cells. The results show mean ± SD of three separate transfections. ***, $P < 0.001$ versus vehicle-treated cells. (**C**) The pNAG1086/41 construct was co-transfected with pCG-ATF3 expression vector and luciferase activity was measured. Fold increase refers to ratio of luciferase activity of pCG-ATF3-transfected cells compared to empty vector-transfected cells. The results show mean ± SD of three separate transfections. ***, $P < 0.001$ versus empty vector-transfected cells.

NAG-1 protein was performed in HCT-116 cells treated with 50 μM of t10,c12-CLA (Figure 7A). NAG-1 expression began to increase at 18 h and reached a plateau at 24 h, while ATF3 expression increased as early as 3 h and reached a plateau at 24 h, suggesting the possibility that the induction of ATF3 plays a role in the expression of NAG-1 in the presence of t10,c12-CLA. To test whether t10,c12-CLA affects transcriptional regulation of the *NAG-1* gene, we measured promoter activity using luciferase construct (pNAG1086/41) of the NAG-1 promoter (28). The pNAG1086/41 construct containing four putative ATF3 binding sites was transfected into HCT-116 cells and treated with 50 μM of CLA isomers for 24 h. As shown in Figure 7B, t10,c12-CLA treatment resulted in the 2.2-fold induction of luciferase, while c9,t11-CLA did not influence luciferase activity. This result is consistent with western analysis, indicating that only t10,c12-CLA induces NAG-1 transcription in HCT-116 cells (Figure 2A). To obtain further evidence in support of the hypothesis that t10,c12-CLA induces NAG-1 through ATF3, we co-transfected the NAG-1 promoter construct and an ATF3 expression vector into HCT-116 cells and subsequently measured luciferase activity. ATF3 transfection resulted in the 3-fold induction of luciferase activity in NAG-1 promoter-transfected cells when compared with empty vector-transfected cells (Figure 7C). This suggests that −1086/+41 region of the *NAG-1* promoter is necessary for t10,c12-CLA-induced NAG-1 expression and ATF3 plays a pivotal role for the t10,c12-CLA-induced NAG-1 expression at the transcriptional level.

t10,c12-CLA inhibits IGF-1-induced activation of AKT pathway

We have reported previously that NAG-1 is induced by the inhibition of PI3K/AKT/GSK-3β pathway (20). CLA has been also shown to negatively affect AKT pathway (5). To determine whether t10,c12-CLA affects AKT pathway, HCT-116 cells were incubated with t10,c12-CLA for 24 h and then stimulated with insulin-like growth factor-1 (IGF-1) for 5 h. Total cell lysates were analyzed by western analysis with phospho-Ser^{473}AKT and total AKT antibodies. The phosphorylation of AKT was not observed without IGF-1 stimulation in HCT-116 cells (Figure 8A). However, the phosphorylation of AKT by IGF-1 was abolished in the presence of t10,c12-CLA (Figure 8A, lanes 3 and 4), indicating that t10,c12-CLA inhibits IGF-1-induced AKT phosphorylation. The increased phosphorylation of AKT that was induced by IGF-1 treatment was completely blocked by a PI3K inhibitor, LY294002 pretreatment (Figure 8A, lane 5). A similar result was seen in IGF-1 treated HT-29 cells (data not shown). As a downstream target of the AKT pathway, the inhibition of GSK-3β abolished NAG-1 expression (20). To determine whether downregulation of *GSK-3* gene using siRNA inhibit t10,c12-CLA-induced NAG-1 and ATF3 expression, HCT-116 cells were transiently transfected with GSK-3 siRNA or control siRNA and followed by treatment with 50 μM of t10,c12-CLA. As shown in Figure 8B, the transfection of HCT-116 cells with GSK-3 siRNA significantly inhibited the induction of ATF3 as well as NAG-1 protein in the presence of

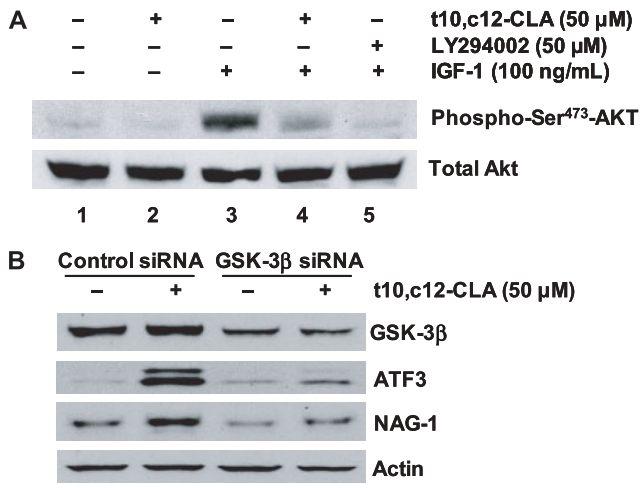

Fig. 8. Inhibition of AKT pathway by t10,c12-CLA. (**A**) Effect of t10,c12-CLA on IGF-1-induced AKT activation were measured. HCT-116 cells were treated with t10,c12-CLA (50 μM) or vehicle for 24 h followed by pretreatment with DMSO or LY294002 (50 μM) for 30 min. Subsequently, cells were stimulated with IGF-1 (100 ng/ml) for 5 h. Total proteins (100 μg) were loaded into 12% SDS-PAGE and western analysis was performed using antibody against anti-phospho-Ser^{473} AKT and total AKT. (**B**) HCT-116 cells were transfected with GSK-3 siRNA (100 nM) or control siRNA (100 nM) for 24 h and treated with t10,c12-CLA (50 μM) or vehicle for 24 h. Western analysis was performed for GSK-3β, ATF3, NAG-1 and actin.

t10,c12-CLA, suggesting that GSK-3β, as a downstream target of AKT, plays a pivotal role for t10,c12-CLA-induced ATF3 and NAG-1 expression.

Discussion

The anti-tumorigenic effects of CLA in a variety of experimental cancer models have raised an important question regarding the underlying molecular mechanisms. Here, we demonstrate direct evidence that t10,c12-CLA stimulates NAG-1 expression through inhibition of AKT/GSK-3β pathway and activation of ATF3-mediated pathway in human CRC cells.

Among two CLA isomers tested in this study, only t10,c12-CLA, but not c9,t11-CLA, is an effective inhibitor of cell growth and inducer of apoptosis. This is in agreement with observations that t10,c12-CLA is a specific inducer for both ATF3 and NAG-1. The positional isomer dependence of CLAs' effect on carcinogenesis has been previously demonstrated in several cancer models. Although both c9,t11- and t10,c12-CLA isomers were effective in animal model of breast cancer (36), only t10,c12-CLA, but not c9,t11-CLA induced apoptosis in colon (37) and prostate cancer cells (38). Thus, our study is consistent with previous reports, suggesting that t10,c12-CLA has a pro-apoptotic activity in an isomer specific manner in CRC cells. In contrast, Rajakangas *et al*. (39) showed that feeding *Min* mice with t10,c12-CLA, a mouse model with an activated β-catenin pathway increased the size of distal small intestinal adenomas by ~25%. The adenoma growth was accompanied by an increase in nuclear cyclin D1. In this report, we used HCT-116 cells in which the β-catenin degradation pathway is active and thus difference between the response to the CLA may be due to differences in the β-catenin/APC pathways. To examine this problem, we transiently transfected into HCT-116 cells an active β-catenin gene and confirmed an activated β-catenin pathway using cyclin D1 and TOP Flash reporter system (Figure 2C and D). With the activated β-catenin, HCT-116 cells are now similar to the *Min* mice in regard to the β-catenin pathway but this did not affect CLA-induced NAG-1 expression. Moreover, both APC wild (HCT-116) and mutant (HT-29) cell lines showed t10,c12-CLA-induced NAG-1 expression (Figure 6), further supporting our hypothesis. The contradictory results between *in vitro* with human cells and *in vivo* findings with mice were seen in other experiments. Several groups have shown that PPARγ ligands induce apoptosis with inhibition of human colonic cancer cell growth *in vitro* and in nude mouse models with human cell xenographs (40,41). Two independent groups have reported that treatment with PPARγ ligands may actually promote colorectal tumor growth in the genetic *Min* mouse model (42,43). These conflicting results have recently been partially explained by demonstrating that PPARγ has little effect on tumorigenesis in APC mutant mice but functions as a tumor suppressor gene in APC wild-type mice (44). In this regard, CLA/NAG-1 may have little effect on tumorigenesis in APC mutant model, compared to APC wild-type model. Therefore, we cannot completely exclude the possibility that APC/β-catenin pathway may play some roles in CLA-induced NAG-1 expression because several other factors may affect APC/β-catenin pathway including COX-2, PPARγ, PPARδ, E-cadherin, AKT, GSK-3β and Axin. NAG-1 is also regulated by GSK-3β, AKT, as well as PPARγ. Future studies are warranted to gain a better understanding of the molecular mechanism by which CLA affects NAG-1 expression.

The transcriptional regulation of NAG-1 by chemopreventive compounds is mediated by several mechanisms (12,15, 16,34). One of the genes involved in the regulation of the NAG-1 expression is p53 tumor suppressor gene. In fact, some dietary compounds, including resveratrol and genistein, induce NAG-1 expression through p53 tumor suppressor protein (12,13). It has been reported that t10,c12-CLA induces p53 expression in human breast cancer cells, but only minimum induction was observed in colon cancer cells treated with 160 μM of CLA (6). However, in this study, we could not detect p53 induction at 100 μM of CLA (Figure 2). It is likely that t10,c12-CLA-induced anti-tumorigenic activity is p53 dependent or independent manner, depending on cell type and dosage. In addition, CLA-induced NAG-1 expression was observed in HT-29 cells (p53 mutant human CRC cells) (Figure 6C), further indicating that p53 expression does not mediate CLA-induced NAG-1 expression. Expression of cyclin D1 has been suppressed at the higher concentration of CLAs (6), however, we could not observe this effect at the 50 μM concentration. Another known biological activity of CLA is the PPAR activation. In particular, a number of human CRC cell lines express PPARγ and PPARγ agonists induce apoptosis and inhibit their clonal expansion both *in vitro* and *in vivo* (33). To observe whether the NAG-1 induction by t10,c12-CLA is associated with PPAR activation in human colon cancer cells, we measured the ability of LA and CLAs to bind different types of PPAR ligand binding domains (α, γ or δ) using a reporter gene containing ligand binding domains for PPARα, γ or δ. Our results indicate that both c9,t11-CLA and t10,c12-CLA are more potent ligands for PPARα than LA, which is consistent with other reports using a scintillation proximity assay (45). Both c9,t11-CLA and t10,c12-CLA showed small increase of PPARγ-dependent transactivation, but not for PPARδ (Figure 4). Although both c9,t11-CLA

and t10,c12-CLA induced PPAR-dependent luciferase activity, only t10,c12-CLA induces NAG-1/ATF3 expression, suggesting that the t10,c12-CLA-induced NAG-1 expression is PPARγ-independent activation. It has been reported that induction by other structural classes of PPARγ agonists is also PPARγ-independent (35).

The *NAG-1* promoter contains several binding sites for transcription factors previously identified to be important for its regulation, including p53, EGR-1, COUP-TF1 and Sp1 (28). In this report, we found that ATF3 plays an important role in the CLA-induced NAG-1 promoter activity. The observation that overexpression of ATF3 induces the NAG-1 promoter activity in a similar manner to CLA treatment, suggests that ATF3 is correlated with NAG-1 induction. We also showed that ATF3 is expressed earlier than NAG-1. The NAG-1 expression by t10,c12-CLA appears to be positively regulated by signals from ATF3 expression and NAG-1 may be one of the target genes for the ATF3 transcription factor. NAG-1 induction by t10,c12-CLA does not appear to be a general stress response since ATF3 was not up-regulated by other linoleic acids. The consensus binding site for ATF3 was defined as TGACGT(C/A)(G/A), a sequence identical to the CRE consensus (TGACGTCA). However, our results demonstrate that CREB is not involved in CLA-induced NAG-1 expression (Figure 6B). Two ATF3 bands were consistently detected on western analysis. Indeed, it has been reported that an alternatively spliced form of ATF3 (ATF3 delta Zip) encodes a truncated form of ATF3 protein lacking the bZIP protein-dimerization motif and does not bind to DNA (46). It is likely that alternative splicing of the *ATF3* gene is produced in HCT-116 cells and may be physiologically important in the regulation of CLA-induced NAG-1 expression. Competition for the same binding site by two transcription factors was seen on many genes including the NAG-1 promoter (16,19). We have previously reported that Sp1 and EGR-1 share the same binding site in the NAG-1 promoter, but in the presence of troglitazone or PPARγ-active DIM compounds, EGR-1 preferentially binds to the NAG-1 promoter, thereby inducing NAG-1 expression (35,47). Whether similar mechanisms between ATF3 and CREB are involved in cells treated with CLA is under investigation.

AKT is a downstream target of PI3K and the PI3K/AKT pathway has recently been recognized as one of the most important signals ensuring protection against apoptosis (48). Deregulation of PI3K/AKT pathways by specific inhibitor triggers a cascade of responses involved in apoptosis of a number of cancer cells (20,49). The present data indicate that inhibition of AKT phosphorylation may be a major mechanism by which t10,c12-CLA activates NAG-1 expression. This result is consistent with previous data that CLA induces apoptosis of HT-29 cells by inhibiting the AKT signaling pathways (5). GSK-3β is one of the primary target genes of AKT pathway and mediates apoptotic signals (50,51). In our previous study, transfection of specific GSK-3 siRNA blocked the induction of NAG-1 by PI3K inhibitor (20). The current study supports that GSK-3β acts as upstream gene of NAG-1 regulation and is responsible for NAG-1 induction by t10,c12-CLA. In addition, we found that *ATF3* is also a downstream target gene of GSK-3β, which is an important mediator to control t10,c12-CLA or other chemopreventive compounds. The results reported herein indicate that GSK-3β mediates NAG-1 expression through ATF3 activation in the presence of t10,c12-CLA.

In conclusion, the current study provides information on molecular events of anti-tumorigenic activity by t10, c12-CLA. AKT/GSK-3β pathway may influence t10,c12-CLA-induced NAG-1 expression and the resulting NAG-1 activation inhibits proliferation and induces apoptosis in colon cancer cells. The current study demonstrates the coordinated gene responses to t10,c12-CLA and expands our understanding of the nutrient regulation of gene transcription in chemoprevention.

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