ATF3 Mediates Anti-Cancer Activity of Trans-10, cis-12-Conjugated Linoleic Acid in Human Colon Cancer Cells

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Abstract
Conjugated linoleic acids (CLA) are a family of isomers of linoleic acid. CLA increases growth arrest and apoptosis of human colorectal cancer cells through an isomer-specific manner. ATF3 belongs to the ATF/CREB family of transcription factors and is associated with apoptosis in colorectal cancer. The present study was performed to investigate the molecular mechanism by which t10, c12-CLA stimulates ATF3 expression and apoptosis in human colorectal cancer cells. t10, c12-CLA increased an apoptosis in human colorectal cancer cells in dose dependent manner. t10, c12-CLA induced ATF3 mRNA and luciferase activity of ATF3 promoter. mRNA stability of ATF3 was not affected by t10, c12-CLA treatment. t10, c12-CLA increases GSK3β expression and suppresses IGF-1-stimulated phosphorylation of Akt. The knockdown of ATF3 suppressed expression of GSK3β and NAG-1 and PARP cleavage. The results suggest that t10, c12-CLA induces apoptosis through ATF3-mediated pathway in human colorectal cancer cells.

Key Words: Conjugated linoleic acid, Activating transcription factor 3, Colon cancer

INTRODUCTION
Colorectal cancer is the third leading cause of cancer-related death in the United States (Jemal et al., 2011). There are many different and converging pathways for apoptosis or survival of cancer cells (Negrini et al., 2010). The PI3K/Akt pathway, a key regulator of cell growth and survival, is commonly dysregulated in human colon cancers (Lim and Counter, 2005). In particular, Akt has been implicated as an important prognostic factor in human cancer development and progression (West et al., 2003; West et al., 2004).

Aberrant activation of Akt leads to increase the inactive GSK3β, which is downstream target kinase of Akt in colorectal cancer (Cross et al., 1995; Agarwal et al., 2005). GSK3β is a multifunctional serine (Ser)/threonine (Thr) kinase and play an important role in a number of biological processes as a mechanistic link between the canonical Wnt signaling and survival of cancer cells (Jope and Bijur, 2002; Doble and Woodgett, 2003; Forde and Dale, 2007). Therefore, Akt/GSK3β pathway has emerged as an important and attractive therapeutic target for colon cancer therapy.

Recent studies have provided strong evidences that many dietary compounds including curcumin (Kumar et al., 2003), ellagic acid (Umesalma and Sudhandiran, 2011), resveratrol (Aziz et al., 2006; Shukla and Singh, 2011), and conjugated linoleic acid (CLA) (Lee et al., 2006) (Cho et al., 2009) target-ed Akt/GSK3β pathway in different types of cancer. CLAs are positional and geometric isomers of conjugated dienoic derivatives of linoleic acid, an n-6 polyunsaturated fatty acid. The naturally occurring dietary source of CLA is in the lipid fraction of meat of rumen origin, milk, and dairy products (Roche et al., 2003) because it is biosynthesized in the process of microbial fermentation in the rumen of ruminant animals (Chin et al., 1994). The most abundant isofrom of CLA in foods is cis-9, trans-11 (c9, t11) CLA, while synthetically prepared CLA contained the both cis-9, cis-11-CLA and trans-10, cis-12 (t10, c12) CLA in similar amounts. This preparation of CLA has been often used to determine its bioactivities previously (Dilzer and Park, 2012). In in vivo studies, the CLA has been shown to alter the carcinogen-induced tumorigenesis in rodent models (Ha et al., 1990; Ip et al., 1994; Belury, 2002; Lee et al., 2005; Dilzer and Park, 2012).
In terms of anticancer mechanisms of CLA, it has been shown that CLA induce apoptosis and suppress cell cycle progression due to suppress PI3K/Akt and ERK signaling cassette in cancer cell lines (Kim et al., 2003). Previously, our laboratory also demonstrated that t10, c12-CLA modulated colorectal cancer cells survival and proliferation (Lee et al., 2006). The proposed mechanisms include inhibition of Akt signaling and subsequent activation of proapoptotic proteins, non-steroidal anti-inflammatory drugs (NSAIDs)-activated gene-1 (NAG-1) (Lee et al., 2006).

NAG-1 is a member of the TGF-β superfamily (Baek et al., 2001) and NAG-1 expression leads to an inhibition of primary tumor growth in carcinogen-driven colorectal cancer (Baek et al., 2006) and lung cancer in vivo (Cekanova et al., 2009). ATF3 is a member of the activating transcription factor/cAMP-responsive element binding protein (ATF/CREB) family of transcription factors (Hai and Curran, 1991). ATF3 gene expression is elevated by a variety of stress signals, including carcinogens as well as chemotherapy (Chen et al., 1996; Ameri et al., 2007). These data suggest that ATF3 could be regarded as selective and a promising molecular target for chemoprevention and chemotherapy. However, the molecular mechanisms that facilitate these effects are still unclear. Here, we report that the presence of t10, c12-CLA results in an increase of ATF3 expression through transcriptional upregulation in the promoter of ATF3 gene and subsequently activates GSK3β and NAG-1 in human colon cancer cell lines.

MATERIALS AND METHODS

Materials and DNA

Human colorectal adenocarcinoma cells, HT-29, Lovo, and HCT-116 were purchased from American Type Culture Collection (Manassas, VA, USA). Antibodies for ATF3 were purchased from Santa Cruz (Santa Cruz, CA, USA). Antibodies for GSK3β, PARP, actin, phospho-AKT (Ser473) and total AKT were purchased from Cell Signaling (Beverly, MA, USA). The trans-10, cis-12 CLA (t10, c12-CLA) was purchased from Natural Lipids (Hovdebygda, Norway). The t10, c12-CLA preparation was 94% pure, with 2% cis-9, trans-11 isomer (c9, t11-CLA) and 3% other conjugated linoleic acid isomers. IGF-1 was purchased from BD Biosciences (Bedford, MA, USA). Human ATF3 promoter constructs were previously reported (Lee et al., 2010a). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

Cell culture and treatment

HT-29, Lovo, and HCT-116 cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin antibiotics mixture at 37°C in 5% CO2 Incubator. The cells were plated in 6 well culture dishes (for Western blot and RT-PCR) or 12 well culture dish (for luciferase assay) and grown until cells were 80-90% confluent. Then, the cells were incubated with t10, c12-CLA in serum-free media at indicated doses and times in Figure legends. t10, c12-CLA was prepared and added to serum-free media as described previously (Lee et al., 2006). Bovine serum albumin (BSA) without CLA was used as vehicle.

Transient transfection and assay of luciferase activity

The cells were plated in 12-well plates at the concentration of 2×104 cells/well and ATF3 promoter construct were transfected using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instruction. DNA mixtures containing 0.5 μg of ATF3 promoter and 0.05 μg of pRL-null vector were transfected in serum-free media for 5 h and the cells were incubated with serum-containing media overnight. Then, the transfected cells were exposed to different doses of t10, c12-CLA for 24 h. The media was removed and cell lysates were obtained using 1X luciferase lysis buffer, and luciferase activity was measured using a dual luciferase assay kit (Promega, Madison, WI, USA).

RNA interference

HCT-116 cells were transfected with control or ATF3 small interference RNA (siRNA; Santa Cruz; Cat# sc-29757) at a concentration of 100 nM using TransIT-TKO transfection reagent (Mirus, Madison, WI, USA), as described previously (Yamaguchi et al., 2004). After 24 h transfection, the cells were treated with vehicle or t10, c12-CLA (50 μM) for 24 h for Western blot.

Measurement of apoptosis

Apoptosis was assayed using Cell Death Detection ELISA-PLUS Kit (Roche Diagnostics, Indianapolis, IN, USA) as we previously described (Jeong et al., 2013). This kit detects qualitatively and quantitatively the amount of cleaved DNA/histone complexes (nucleosomes). Briefly, the cells were treated with t10, c12-CLA for 24 h and cytosolic extracts and immunoreagent were mixed and incubated for 2 h at room temperature. After washing with the incubation buffer, 100 μl of ABTS solution was added and incubated for 20 min. The absorbance was recorded at 405 nm and 490 nm in an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

Western analysis

Cell lysates were extracted by incubating the cells with radiolunmunoprecipitation assay (RIPA) buffer for 30 min on ice. To prevent protein degradation, the RIPA buffer was supplemented with protease inhibitors (1 mM PMSF, 5 μg/ml apro tinin and 5 μg/ml leupeptin) and phosphatase inhibitors (1 mM Na3VO4, and 1 mM NaF) and centrifuged at 12,000 rpm for 5 min at 4°C. The protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL, USA) using BSA as the standard. The proteins (30 μg) were separated on SDS-PAGE and transferred to nitrocellulose membranes (Osmonics, Minnetonka, MN, USA). The membranes were incubated with a specific primary antisrum 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 for 30 min, the blots were incubated with horse radish peroxidase (HRP)- conjugated IgG for 1 h at room temperature, visualized using ECL (Amersham Biosciences, Piscataway, NJ, USA) and quantified by Gel Doc 2000 system and Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA).

Isolation and analysis of RNA

Total RNA was isolated from cells using Trizol Reagent (Invitrogen), according to the manufacturer’s instructions. RNA samples with an OD260/OD280 ratio greater than 1.8 were used.
Fig. 1. t10, c12-CLA increases ATF3 expression and apoptosis in different human colorectal cancer cells. (A, C) HCT116, LoVo and HT-29 cells were incubated with media containing indicated concentrations of t10, c12-CLA for 24 h. Western analysis was performed for ATF3, cleaved PARP, caspase-3, and actin as described in Methods. Data represent one experiment. (B) Apoptosis of LoVo and HT-29 cells were analyzed with Cell Death Detection ELISAPLUS Kit (Roche Diagnostics). *p<0.05 versus vehicle-treated cells.

for semi-quantitative RT-PCR. One μg of total RNA was used to produce cDNA using the Verso cDNA synthesis Kit (Thermo Scientific, Pittsburgh, PA, USA). PCR was performed using one μL of cDNA mixture and the PCR Master Mix solution (Promega, Madison, WI, USA) according to the manufacturer’s instruction. The sequence of the oligonucleotide primers were as follows; ATF3 sense (5'-gtttgaggattttgctaacctgac-3') and antisense (5'-agctgcaatcttatttctttcgt-3'); GAPDH sense (5'-accagaagactgtggatgg-3') and antisense (5'-ttctagacggcagcagtggt-3'). The PCR products were run on 1.5% agarose gels containing ethidium bromide dye and photographed. A densitometric analysis was performed using Image J software (National Institute of Health, Bethesda, MD, USA).

Statistical analysis
Statistical analysis of ATF3 promoter assay was performed with Student’s unpaired t-test, with statistical significance set at *p<0.05.

RESULTS

The t10, c12-CLA stimulates expression of ATF3 and apoptosis
Our previous study demonstrated that CLA increased growth arrest and apoptosis of human colorectal cancer cells through isomer-specific manner (Lee et al., 2006). We also found that t10, c12-CLA increased expression of tumor suppressor gene, NAG-1 through ATF3-mediated pathway. To further extend our understanding on anti-cancer mechanisms of t10, c12-CLA, we tested effect of t10, c12-CLA on expression of ATF3 in a well-established human colorectal cancer cell lines including HT-29, Lovo and HCT-116. As shown in Fig. 1A, t10, c12-CLA stimulated expression of ATF3 protein in dose-dependent manner in all cell lines we tested. Since we observed that t10, c12-CLA increased apoptosis of HCT-116 cells (Lee et al., 2006), we measured apoptosis in HT-29 and Lovo cells. As shown in Fig. 1B, treatment of t10, c12-CLA for 24 h increased apoptosis in dose-dependent manner in LoVo and HT-29 cells. Next, we also measured the cleavage of PARP and caspase3, a hallmark of apoptosis (Wang et al., 1997). t10, c12-CLA resulted in an increase of cleaved PARP and caspase 3 which is positively correlated with ATF3 expression (Fig. 1), although there is no change in caspase-3 cleavage in HT-29 cells.

The t10, c12-CLA increase ATF3 expression through transcriptional upregulation of ATF3 gene
To test whether t10, c12-CLA affects the transcriptional activity of the ATF3 gene, we measured mRNA of ATF3 gene in the cells treated with different doses of t10, c12-CLA. As shown in Fig. 2A, t10, c12-CLA treatment increase ATF3 mRNA in dose-dependent manner. This result is consistent with Western blot analysis, indicating that increased protein expression is caused by increased transcription of ATF3 gene in HCT-116 cells treated with t10, c12-CLA.

To observe if increased mRNA is associated with transcriptional upregulation, we analyzed the promoter activity using luciferase construct containing ATF3 promoter (pATF3-1850/+34).
ATF3 promoter construct were transfected into HCT-116 cells and treated with 0, 25, 50, and 100 μM of t10, c12-CLA for 24 h. The luciferase activities were measured to assess the transactivation for ATF3 promoter. The t10, c12-CLA treatment resulted in the significant induction of luciferase activity in the cells treated with t10, c12-CLA (Fig. 2B). Next, we performed the promoter assay using different size of the ATF3 promoter clones to identify the promoter region responsible for the transcriptional upregulation of the ATF3 gene by t10, c12-CLA. We transfected seven different size of the ATF3 promoter (pATF3-84/+34, pATF3-147/+34, pATF3-318/+34, pATF3-514/+34, pATF3-718/+34, pATF3-1420/+34, and pATF3-1850/+34) into the HCT-116 cells and incubated for 24 h with and without 50 μM of t10, c12-CLA. As shown in Fig. 2C, t10, c12-CLA showed 1.2, 1.4, 1.5, 1.3, and 1.8 fold increase of transactivation of ATF3 in pATF3-318/+34, pATF3-514/+34, pATF3-718/+34, and pATF3-1420/+34 and no change in pATF3-1850/+34 transfected cells, respectively. However, t10, c12-CLA did not change luciferase activity of pATF3-147/+34 and pATF3-318/+34 transfected cells. These data indicate that t10, c12-CLA probably affects the promoter region between -147 and -1850 bp for ATF3 gene transcription.

The t10, c12-CLA does not regulate ATF3 RNA stability

Since the increase of ATF3 mRNA (Fig. 2A) is more prominent than the increase of the promoter activity (Fig. 2B, C), we hypothesized that t10, c12-CLA may affect the post-transcriptional regulation. To determine whether the treatment of t10, c12-CLA influence the mRNA stability, HCT-116 cells were treated with either vehicle or 50 μM of t10, c12-CLA for 24 h and then exposed to actinomycin D (inhibitor of RNA synthesis) for 0, 1, 2, 4 or 8 h. mRNA of ATF3 was compared using semi-quantitative RT-PCR to compare the half-lives of the ATF3 mRNA. The results indicate that half-life of ATF3 mRNA is the same between vehicle- and t10, c12-CLA-treated cells.
Fig. 4. Dependency of ATF3 in t10, c12-CLA-induced GSK3β and NAG-1 expression, and apoptosis (A) HCT116 cells were exposed to different doses of t10, c12-CLA for 24 h and Western blot was performed GSK3β and actin. (B) The HCT116 (upper) and HT-29 (lower) cells were treated with vehicle or t10, c12-CLA (50 μM) for 24 h and stimulated with IGF-1 (100 ng/mL). Western analysis was performed using antibodies for phospho-Akt (Ser473), total Akt and actin. (C, D) HCT-116 cells were transfected with control or ATF3 siRNA (100 nm) for 24 h and treated with t10, c12-CLA (50 μM) for 24 h. Western analysis was performed for ATF3, NAG-1, GSK3β, PARP, and actin. Data represent one experiment.

(Fig. 3A, B). These results suggest that t10, c12-CLA does not affect ATF3 mRNA stability.

The t10, c12-CLA modulates Akt phosphorylation and GSK3β expression, and ATF3 mediates t10, c12-CLA-stimulated expression of GSK3β and NAG-1, and apoptosis

GSK3β is an important mediator of PI3K/Akt pathway and modulator of apoptosis. Akt is also an apoptotic regulator that is elevated in cancer. In previous study, we found that Akt/GSK3β axis mediates t10, c12-CLA-stimulated apoptosis (Lee et al., 2006). ATF3 directly upregulated the transactivation of NAG-1 gene and both are the downstream target of GSK3β (Yamaguchi et al., 2004; Lee et al., 2006). Here, we found that treatment of t10, c12-CLA increased expression of GSK3β in dose-dependent manner (Fig. 4A) while it did not affect phosphorylation of GSKβ (data not shown).

IGF-I is one of the most potent natural activators of the Akt signaling. The receptor for IGF-I (IGF-IR) is expressed in both normal and colon cancer cells, including HCT116 (Donovan and Kummar, 2008). We tested if t10, c12-CLA inhibited IGF-I-stimulated Akt phosphorylation. We observed repression of IGF-1-stimulated Akt phosphorylation in the cells treated with t10, c12-CLA (Fig. 4B).

Next we tested the effect of the ATF3 status on the expression of NAG-1 and apoptosis. HCT116 cells were transiently transfected with control siRNA and ATF3 siRNA and then treated with 50 μM of t10, c12-CLA. As shown in Fig. 4C, the transfection of ATF3 siRNA dramatically repressed t10, c12-CLA-stimulated expression of NAG-1, confirming our previous claim that ATF3 is an upstream molecule of NAG-1 transactivation. In addition, the knockdown of ATF3 ameliorated t10, c12-CLA-stimulated cleavage of PARP, implying that ATF3 mediates t10, c12-CLA-induced apoptosis (Fig. 4D). Interestingly, we found that the ATF3 knockdown led to the inhibition of t10, c12-CLA-stimulated GSK3β expression (Fig. 4D). These data suggest that ATF3 might act as GSK3β regulator in human colorectal cancer cells.

DISCUSSION

In our previous study, we proposed a novel molecular mechanism of anti-cancer activity of t10, c12-CLA. It includes increased expression of a novel proapoptotic protein, NAG-1 through AKT/GSK3β pathway (Lee et al., 2006). Another interesting finding is that ATF3 may mediate t10, c12-CLA-induced NAG-1 expression. However, the effect of t10, c12-CLA on ATF3 expression and potential link of ATF3 to other signaling components such as Akt and GSK3β and subsequent apoptosis remains unanswered. Therefore, we raise an important question regarding the underlying molecular mechanisms of ATF3 activation by t10, c12-CLA. Here, we demonstrate direct evidence that t10, c12-CLA increase ATF3 expression through transcriptional upregulation of ATF3 gene and ATF3 mediate t10, c12-CLA-stimulated NAG-1 expression and apoptosis in human colorectal cancer cells.

The transcriptional regulation of ATF3 gene is mediated by several mechanisms involving other CREB/ATF transcription factors and C/EBPs (Lee et al., 2010a, 2013). In fact, some dietary compounds, including tolfenamic acid and diindolylmethane, induce ATF3 transcription through ATF2 or ATF4-dependent mode. Unlike tolfenamic acid and diindolylmethane, responsible promoter region for ATF3 transcription by t10, c12-CLA is localized between -147 and -1859 region, imply-
ing that another transcriptional mechanism might be involved in transactivation of ATF3 gene by r10, c12-CLA. However, the increase of ATF3 mRNA (Fig. 2A) is more prominent than the increase of the promoter activity (Fig. 2B, C), suggesting involvement of post-transcriptional regulation. So we compared mRNA stability. The results indicate that half-life of ATF3 mRNA is the same between vehicle- and r10, c12-CLA-treated cells. For this inconsistency, we speculate that the promoter region we used in this study may not include responsible cis-acting elements responsible for transcriptional activity of ATF3 gene. And this also suggests that ATF3 gene regulation by r10, c12-CLA is different from that by other compounds we reported previously.

Deregulation of PI3K/AKT pathways by specific inhibitors triggers a cascade of cellular responses of apoptosis in numerous cancer cells. The current results suggest that inhibition of AKT phosphorylation may be a responsible mechanism by which r10, c12-CLA activates ATF3 expression.

Interesting finding is that knockdown of ATF3 gene ameliorates r10, c12-CLA-stimulated NAG-1 expression. This result is in agreement with the previous result that overexpression of ATF3 increased NAG-1 promoter activity (Lee et al., 2006), confirming our previous results that ATF3 is an upstream regulator of NAG-1 gene.

Another interesting finding of our current study is a dose-dependent increase of GSK3β expression (Fig. 4A). GSK3β is one of the primary target genes of AKT pathway and mediates apoptotic signals (Jope and Bijur, 2002; Doble and Woodgett, 2003; Forde and Dale, 2007). Increased GSK3β expression was also observed in caspasein treated human colorectal cancer cells (Lee et al., 2010b), suggesting that increased expression of GSK3β could be a common phenomenon in cancer cells exposed to anti-cancer compounds. In fact, cellular level of GSK3β is highly associated with process of apoptosis and by modulation of expression of numerous target transcription factors (Bijur and Jope, 2001).

In our previous study, the knockdown of GSK3β inhibited r10, c12-CLA-mediated ATF3 induction (Lee et al., 2006). However, current study supports that ATF3 might be one of upstream gene of GSK3β. Another study indicated that GSK3β directly associate with ATF3 and form big complex proteins (Lee et al., 2010b). Further study is required to explain the inconsistency and obtain more detailed information on how ATF3 interact with GSK3β in response to dietary compounds including r10, c12-CLA and caspasein.

Among the two major CLA isomers, the cis-9, trans-11 CLA (c9, t11-CLA) is the primary isomer found in meat and dairy foods. The r10, c12 CLA is present in small quantity in foods but synthetically prepared CLA contains significant amounts of this isomer, approximately 35-45% along with about the same quantity of the c9, t11-CLA. Anti-cancer activity of CLA shows isomer specific in human colorectal cancer. In our previous study, we observed that r10, c12-CLA, but not c9, r11-CLA, increased apoptosis and expression of ATF3 and GSK3β in human colorectal cancer cells (Lee et al., 2006). Further studies will be required to address how CLAs show the positional isomer dependence in terms of carcinogenesis.

The current data demonstrate that r10, c12-CLA increases transcription of ATF3 gene which might be associated with multiple signaling components including Akt, GSK3β and NAG-1 in human colorectal cancer cells.

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