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Conjugated linoleic acid-induced apoptosis in mouse mammary tumor cells is mediated by both G protein coupled receptor-dependent activation of the AMP-activated protein kinase pathway and by oxidative stress

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Abstract

Conjugated linoleic acid (CLA) has shown chemopreventive activity in several tumorigenesis models, in part through induction of apoptosis. We previously demonstrated that the t10,c12 isomer of CLA induced apoptosis of TM4t mouse mammary tumor cells through both mitochondrial and endoplasmic reticulum (ER) stress pathways, and that the AMP-activated protein kinase (AMPK) played a critical role in the apoptotic effect. In the current study, we focused on the upstream pathways by which AMPK was activated, and additionally evaluated the contributing role of oxidative stress to apoptosis. CLA-induced activation of AMPK and/or induction of apoptosis were inhibited by infection of TM4t cells with an adenovirus expressing a peptide which blocks the interaction between the G-protein-coupled receptor (GPCR) and G α_q , by the phospholipase C (PLC) inhibitor U73122, by the inositol trisphosphate (IP₃) receptor inhibitor 2-APB, by the calcium/calmodulin-dependent protein kinase kinase α (CaMKK) inhibitor STO-609 and by the intracellular Ca²⁺ chelator BAPTA-AM. This suggests that t10,c12-CLA may exert its apoptotic effect by stimulating GPCR through G α_q signaling, activation of phosphatidylinositol-PLC, followed by binding of the PLC-generated IP₃ to its receptor on the ER, triggering Ca²⁺ release from the ER and finally stimulating the CaMKK-AMPK pathway. t10,c12-CLA also increased oxidative stress and lipid peroxidation, and antioxidants blocked its apoptotic effect, as well as the CLA-induced activation of p38 MAPK, a downstream effector of AMPK. Together these data elucidate two major pathways by which t10,c12-CLA induces apoptosis, and suggest a point of intersection of the two pathways both upstream and downstream of AMPK.

Keywords

conjugated linoleic acid; AMP kinase; GPCR; ER stress; oxidative stress; CaMKK

1. Introduction

Conjugated linoleic acid (CLA) is a family of C18 polyunsaturated fatty acids with conjugated double bonds. The c9,t11-CLA isomer occurs naturally in dairy products and ruminant meat, and a mixture of c9,t11-CLA and t10,c12-CLA is available commercially and used for its putative health benefits, such as promoting fat loss and improving insulin sensitivity [1,2]. Although both isomers showed similar efficacy in mammary tumor growth inhibition [3,4], angiogenesis [5], and inducing apoptosis or inhibiting proliferation in some

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tumor cell lines [6,7], this is not always the case. For example, our laboratory showed that t10,c12-CLA, but not c9,t11-CLA, is the effective form in inducing apoptosis in the p53-mutant TM4t mouse mammary tumor cell line [8,9]. Similar observations of a greater *in vitro* efficacy of t10,c12-CLA have also been reported by others in different cell models [10-12].

The endoplasmic reticulum (ER) is the major organelle for protein synthesis and folding. It also serves as a Ca^{2+} reservoir regulated by two major Ca^{2+} release channels, the 1,4,5-trisphosphate receptor (IP_3R) [13,14] and the ryanodine receptor (RyR) [14,15], as well as by Ca^{2+} ATPases which regulate Ca^{2+} transport into the ER [16]. The lumen of the ER is an oxidative environment, and is critical for formation of disulfide bonds. Stresses that perturb the redox state or the ER Ca^{2+} concentration reduce the protein folding capacity of the ER, resulting in the accumulation and aggregation of unfolded or misfolded proteins; this is commonly referred to as ER stress [17]. ER stress has been associated with several pathophysiological conditions, including neurodegenerative diseases, cerebral ischemia and diabetes [18-20], and severe ER stress can initiate cell death in the form of apoptosis [21,22]. The mechanism underlying this process is not fully understood, however several key events have been proposed, including transcriptional induction of CHOP, activation of caspase 12, release of Ca^{2+} from the ER, oxidative stress, and perturbing the balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family [17,23,24].

ER stress can be induced by oxidative stress, in part via inactivation of ER-resident proteins [24,25]. Oxidative stress occurs as a result of an imbalance between the levels of reactive oxygen species (ROS) and the antioxidant defenses in the cell. Increased ROS production can occur in a physiological manner, for example as a response to bacterial infection, or from various pathological conditions including inflammation and cancer. ROS can also be generated in response to some chemotherapeutic drugs and radiation therapy, triggering oxidative stress and resulting in apoptosis [26]. Polyunsaturated fatty acids (PUFAs) are highly susceptible to ROS, which can non-enzymatically oxidize PUFAs into various biologically active metabolites, including 4-hydroxynonenal, 4-oxo-2-nonenal, and malondialdehyde [27]. These reactive aldehydes can exert both physiological, as signaling molecules, and pathological effects on cells, leading to activation of survival pathways, cell growth inhibition, ER stress, apoptosis, and/or cytotoxicity [28-32].

Recently we reported that the t10,c12 isomer of CLA induced an atypical ER stress response in TM4t mouse mammary tumor cells [8], and stimulated AMPK-mediated apoptosis in the same cell line [9]. We also demonstrated that the AMPK activation does not occur via an ATP-dependent LKB1 pathway, nor by nutrient deprivation. In the current study, we show that another AMPK upstream kinase, Ca^{2+} /calmodulin-dependent protein kinase kinase (CaMKK), plays a role in mediating CLA-induced AMPK activation. Our data further suggest that the activation of CaMKK results from Ca^{2+} release from the ER, a downstream event initiated by a plasma membrane G_q -coupled receptor. In addition, we demonstrate that CLA promotes oxidative stress and lipid peroxidation in TM4t cells. Finally, the finding that CLA-induced apoptosis was attenuated by several antioxidants confirms the contributing role of oxidative stress in the CLA apoptotic effect.

2. Materials and methods

2.1. Cell culture

The p53-mutant TM4t mouse mammary tumor cell line was obtained from Dr. Dan Medina at Baylor College of Medicine. Cells were cultured in DMEM-F12 supplemented with 2% adult bovine serum (ABS), 10 $\mu\text{g}/\text{ml}$ insulin, 5 ng/ml EGF and 5 $\mu\text{g}/\text{ml}$ gentamicin.

2.2. Materials

t10,c12-, and c9,t11-CLA were purchased from Larodan Fine Chemicals (Malmö, Sweden) and prepared as the sodium salt as described previously [33]. Anti-cleaved poly (ADP-ribose) polymerase (PARP), anti-cleaved caspase 9, anti-cleaved caspase 3, anti-phospho-AMPK (Thr172), anti-AMPK, anti-p38, anti-phospho p38 (Thr180/Tyr182), were purchased from Cell Signaling Technology (Danvers, MA). Anti-actin and Pertussis toxin were purchased from Calbiochem (La Jolla, CA). Anti-GADD 153 (CHOP) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). U-73122, SRB (sulforhodamine B), 2-aminoethyl diphenylborinate (2-APB), N-acetyl-L-cysteine (NAC), hypotaurine (HPT), and (+/-)- α -tocopherol were purchased from Sigma (St. Louis, MO). Dihydrorhodamine 123 and diphenyl-1-pyrenylphosphine (DPPP) were purchased from Invitrogen, Eugene, OR. The adenoviral vector containing the inhibitor of Gq signaling [34], as well as control vector, were generously provided by Dr. Andrea Eckhart at Thomas Jefferson University, Philadelphia, PA.

2.3. Western blot analysis

One $\times 10^5$ cells/ml were plated in dishes and cultured overnight. The next day, media were changed to fresh media +/- CLA. At the indicated times, cells were collected and lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS), supplemented with phosphatase inhibitor cocktail 1 and 2 (Sigma), and 1% (v/v) protease inhibitor cocktail 1 and 2 (Sigma) for western blot analysis. Protein concentration was determined by the Bio-Rad protein assay. Lysates were separated by SDS-PAGE and detected by chemiluminescence. Analysis of the western blots was performed using the Molecular Dynamics Personal Densitometer SI, followed by quantification with ImageQuant v5.2 software.

2.4. Viable cell density determination

The SRB assay was used to determine viable cell density as described [9]. Briefly, after 72 h CLA treatment with or without drug, culture media were aspirated followed by the addition of 400 μ l PBS plus 200 μ l ice cold 50% (w/v) trichloroacetic acid and incubation for 1 h at 4°C. Cells were then washed and dried before staining with SRB. The excess SRB dye was removed by 5 washes with 1% (v/v) acetic acid. The protein-bound SRB dye was extracted in 1 ml of 10 mM Tris base solution and the optical density measured at 570 nm in an Elx808 ultra microplate reader (BIO-TEK Instruments. Inc, Winooski, VT).

2.5. Adenoviral infection

Five $\times 10^4$ cells/ml were plated in dishes and cultured overnight. The next day, cells were replenished with fresh medium and infected with an appropriate titer of adenovirus as determined previously [34]. Twelve hours after infection, the virus-containing medium was changed, fresh medium was added to the cells, and the cells incubated for another 12 hours. The 3rd day, the medium was changed to fresh media +/- CLA and the cells incubated for the indicated time.

2.6. Oxidative stress detection

Cells were cultured under the same conditions as described for western blot analysis. After 24 h, samples were washed once with pre-warmed PBS followed by incubation with 20 μ M dihydrorhodamine 123 at 37° C for 20 min. After incubation, samples were washed once with PBS then incubated with cell dissociation buffer (GIBCO, Grand Island, NY) for an additional 30 min. at 37° C before flow cytometry analysis using the BD FACScan.

2.7. Lipid peroxidation detection

Cells were cultured on coverslips under the same conditions as described above for western blot analysis. After 24 h, 5 μ M DPPP was added to the cells, followed by incubation at 37°C for 15 min. After incubation, samples were washed twice with PBS and analyzed by fluorescence microscopy using the 63X objective.

2.8. Statistics

Statistical analysis was performed using one-way analysis of variance, with the Holm-Sidak method used for pairwise multiple comparisons. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Role of CaMKK and Ca²⁺ in t10,c12-CLA - induced activation of AMPK

We previously demonstrated that AMPK activation played a central role in mediating t10,c12-CLA-induced apoptosis of TM4t mammary tumor cells, but that neither the upstream kinase LKB1 nor energy or nutrient depletion were responsible for this effect [9]. To further investigate the mechanism by which t10,c12-CLA induced AMPK activation, we examined the potential involvement of another AMPK upstream kinase, CaMKK, by treating TM4t cells with t10,c12-CLA in the presence or absence of the CaMKK inhibitor, STO-609. As shown in Fig. 1A, STO-609 effectively reduced the basal level of phosphorylated AMPK and abrogated CLA-induced phosphorylation of AMPK at 24 and 48 h. Since the activity of CaMKK is regulated by the cytosolic calcium level, we then tested whether the intracellular Ca²⁺ chelator BAPTA-AM could interfere with the activation of AMPK by t10,c12-CLA. Data in Fig. 1B show that in the presence of BAPTA-AM, the t10,c12-CLA-induced AMPK phosphorylation was largely inhibited, suggesting that intracellular calcium homeostasis was involved in stimulating CaMKK activity and mediating CLA-induced apoptosis.

3.2. Blocking Ca²⁺ release from the ER attenuates t10,c12-CLA- induced apoptosis

We recently demonstrated that t10,c12-CLA triggered an ER-stress-dependent apoptotic pathway through the induction of CHOP [8]. Since the ER is one of the major intracellular Ca²⁺ storage pools and Ca²⁺ depletion from the ER was shown to induce ER stress [35], we hypothesized that the ER stress induced by t10,c12-CLA may be due to the release of Ca²⁺ from the ER, with subsequent activation of the CaMKK-AMPK pathway and induction of apoptosis. To evaluate this notion, we used an inositol 1,4,5-trisphosphate (IP₃) receptor inhibitor, 2-APB, to block calcium efflux from the ER, since IP₃ receptors are the primary Ca²⁺ release channels on the ER [13]. The addition of 2-APB to CLA-treated cells increased viable cell number in a concentration-dependent manner as shown in the cell micrographs (Fig. 2A) and in the SRB assay (Fig. 2B). In addition, the cleavage of PARP and caspases 3 and 9 were each significantly reduced, and the ER stress marker CHOP was downregulated (Fig. 2C). Concurrently, the CLA effect on the enhancement of AMPK phosphorylation and the reduction in the non-phosphorylated form of AMPK were also attenuated (Fig. 2C).

3.3. Involvement of G α_q signaling and phospholipase C activation in stimulation of AMPK phosphorylation and apoptosis by t10,c12-CLA

The observation that an IP₃ receptor inhibitor blocked CLA-induced apoptosis suggests that CLA may act at the cell membrane to activate the enzyme, phosphatidylinositol - phospholipase C (PI-PLC), which generates IP₃ via hydrolysis of phosphatidylinositol 4,5-bisphosphate [36]. This hypothesis was tested using a PLC inhibitor, U73122, to investigate whether blocking PLC activity would hinder the CLA effect. As seen in Fig. 3A, U73122

not only attenuated CLA-induced AMPK activation but also reduced CHOP upregulation by CLA at 48 and 72h. These findings suggested that the PLC-IP₃ pathway may play a role in mediating the CLA effect via mobilization of Ca²⁺ from the ER. Upstream, PLC is known to be activated by ligand binding to a plasma membrane G-protein coupled receptor (GPCR) followed by the interaction of GPCR with the alpha subunit of Gq (Gα_q) [37]. To determine the role of the GPCR- Gα_q interaction, we next inhibited Gα_q signaling by infecting cells with adenovirus expressing a carboxyl-terminal peptide of the α subunit Gα_q (GqI) to block the interaction between agonist-occupied receptor and Gα_q. As shown in Fig. 3B, cells expressing GqI had a reduced CLA-induced AMPK activation and PARP cleavage compared to cells with mock infection.

3.4. t10,c12-CLA induces oxidative stress and antioxidants inhibit apoptosis induced by t10,c12-CLA

An interplay among oxidative stress, ER stress and apoptosis has been well documented in many studies [25,38-41]. We thus asked whether oxidative stress might play a role in CLA-induced apoptosis of TM4t cells. First, the oxidative stress level was measured using the reactive oxygen species (ROS) detection reagent dihydrorhodamine 123 (DHR-123) in cells treated with t10,c12- or c9,t11-CLA; the latter does not induce apoptosis in these cells [8,9]. The flow cytometry data in Fig. 4A and 4B show that CLA indeed enhanced the oxidative stress level after only 24 h of treatment, with a 1.67 and 1.25 fold increase in signal intensity in ROS-positive cells with t10,c12-CLA and c9,t11-CLA treatment, respectively. Next, we used the lipid peroxidation detection reagent diphenyl-1-pyrenylphosphine (DPPP) to see whether t10,c12-CLA could also induce lipid peroxidation in these cells. Data in Fig. 4C show that cells treated with t10,c12-CLA had a much stronger signal under the immunofluorescence microscope than cells without treatment, indicating that lipid peroxidation is a form of oxidative stress generated by t10,c12-CLA. To further validate that oxidative stress induction by t10,c12-CLA could actually induce apoptosis, we treated cells with several antioxidants, including N-acetyl-L-cysteine (NAC), hypotaurine, α-tocopherol, and Tiron. As shown in Fig. 5, all antioxidants tested effectively attenuated CLA-induced PARP cleavage and CHOP upregulation. In addition, NAC significantly increased the viable cell numbers as indicated in the microscope images (Fig. 6A) and the SRB assay (Fig. 6B). Finally, we asked whether the oxidative stress induced by t10,c12-CLA used p38 MAPK as its downstream effector to initiate apoptosis, since other groups have suggested a link between oxidative stress, p38 MAPK and apoptosis [42,43]. Furthermore, we previously demonstrated the role of p38 MAPK in mediating CLA-induced apoptosis [9]. Data in Fig. 6C show that in the presence of NAC, CLA-induced activation of p38 was attenuated at 48 h. At the 72h time point, however, NAC by itself unexpectedly enhanced p38 phosphorylation, and concurrent treatment of NAC and CLA modestly further enhanced p38 phosphorylation at this time. These data suggest that p38 MAPK may play a partial role in mediating the effect of CLA-induced oxidative stress, at least at 48 h.

4. Discussion

We previously demonstrated that t10,c12-CLA, but not c9,t11-CLA, induced ER stress and activated AMPK, leading to apoptosis of TM4t mammary tumor cells via the p38 MAPK/ Bim pathway [8,9]. Furthermore, activation of AMPK was key to the apoptotic effect of t10,c12-CLA, since CLA-induced apoptosis was enhanced by the AMPK agonist AICAR, and inhibited by the AMPK inhibitor compound C [9]. Data presented herein demonstrate that CaMKK is the kinase responsible for activating AMPK in response to treatment with t10,c12-CLA.

CaMKK is one of the upstream kinases responsible for phosphorylating and activating AMPK [44-46]. We demonstrated its role in mediating the CLA-induced activation of

AMPK using the CaMKK inhibitor STO-609 as well as the cell permeable Ca^{2+} chelator BAPTA-AM, both of which inhibited CLA-induced phosphorylation of AMPK, with the latter suggesting that CLA was acting by elevating the cytosolic Ca^{2+} concentration and subsequently activating CaMKK. The endoplasmic reticulum appears to be the source of the increased Ca^{2+} , at least in part, since 2-APB, an inhibitor of the IP_3 receptor which is a major Ca^{2+} channel of the ER, blocked AMPK activation and downregulated CHOP expression, an ER stress marker whose expression was enhanced by t10,c12-CLA, but not by c9,t11-CLA [8]. We previously demonstrated that CHOP contributes to the apoptosis induced by t10,c12-CLA, since CHOP knockdown with siRNA reduced the apoptotic effect [47]. Consistent with our findings is the recent report of Kennedy *et al* [48], demonstrating that t10,c12-CLA was more potent than c9,t11-CLA in elevating intracellular Ca^{2+} levels in human adipocyte primary culture.

Several orphan G protein-coupled receptors (GPCR) have been identified which mediate free fatty acid signaling and modulate cytosolic Ca^{2+} levels, with GPR40 specifically shown to bind and mediate the physiological effects of medium and long chain fatty acids [49-54]. Kotarsky *et al* [51] demonstrated that t10,c12-CLA bound to a free fatty acid receptor (FFAR), later identified as GPR40 [55], and Schmidt *et al.* recently reported that the enhancement of glucose-stimulated insulin secretion by CLA was significantly reduced in pancreatic islets from FFA1/GPR40 – deficient mice [56]. At the time we initiated our studies, no one had investigated the G protein pathway through which the t10,c12-CLA-activated GPR40 mediated its effects. A number of studies had shown that GPR40 signals through both the $\text{G}\alpha_q$ and $\text{G}\alpha_{i/o}$ pathways [49,50,54,55,57], with the former leading to activation of PLC and elevation of cytosolic Ca^{2+} [37]. Our observation that t10,c12-CLA treatment led to activation of the CaMKK/AMPK pathway, which was blocked by an intercellular Ca^{2+} chelator, led us to hypothesize that $\text{G}\alpha_q$ mediated the effect of this CLA isomer, at least in part. This notion was confirmed when we found a significant decrease in t10,c12-CLA activation of AMPK and induction of apoptosis when we infected TM4t mammary tumor cells with an adenovirus expressing a polypeptide that blocks the interaction between the $\text{G}\alpha_q$ subunit and its G protein-coupled receptor, and is supported by the recent studies of Schmidt *et al.* [56] who showed a CLA-induced increase in intracellular calcium and inositol phosphates. Additionally, we found that the PLC inhibitor U73122 reduced CLA-induced activation of AMPK. Together, these findings suggest that t10,c12-CLA triggers a $\text{G}\alpha_q$ -coupled receptor pathway to activate phosphatidylinositol-specific PLC (PI-PLC) and hydrolyze phosphatidylinositol 4,5-bisphosphate into IP_3 and diacylglycerol. IP_3 then binds to its receptor on the ER membrane to mobilize Ca^{2+} from the ER and subsequently stimulate the CaMKK-AMPK signaling pathway.

Interestingly, it has been demonstrated that the activity of the IP_3R is controlled by Bcl-2 or Bcl-xL, which bind to the receptor and inhibit Ca^{2+} release [58]. We previously demonstrated that t10,c12-CLA markedly depletes both mitochondrial and total cellular Bcl-2 in TM4t mammary tumor cells [47], suggesting another mechanism by which this isomer can stimulate Ca^{2+} release and activate signaling through the CaMKK-AMPK pathway. Furthermore, the IP_3R is also sensitive to redox state, with oxidative stress increasing the affinity of the receptor for IP_3 [14], and potentially disrupting Ca^{2+} homeostasis by enhancing Ca^{2+} release from the ER.

It is unlikely that $\text{G}\alpha_{i/o}$ plays a role in mediating the apoptotic effect of CLA since in a preliminary experiment, pertussis toxin, which inhibits $\text{G}\alpha_{i/o}$ signaling, did not block CLA-induced cleavage of PARP, caspase 3 or caspase 9 (Supplementary Figure 1) and further reduced viable cell number (Supplementary Table 1). However, the possibility remains that the $\text{G}\alpha_s$ pathway, whose effectors include cyclic AMP - dependent protein kinase (PKA) and adenylate cyclase [37], contributes to the action of t10,c12-CLA since this isomer robustly

enhanced phosphorylation of the PKA substrate cyclic AMP response element binding protein (CREB), and inhibition of PKA activity with H-89 attenuated the apoptotic effect of t10,c12-CLA [59].

The role of oxidative stress in mediating the activity of CLA has been a somewhat controversial topic. Ip *et al.* [60,61] reported that a diet containing both c9,t11-CLA and t10,c12-CLA reduced lipid peroxidation in the rat mammary gland, although not in the liver. However the decrease in the mammary gland was modest in comparison to the more robust chemopreventive efficacy of CLA, and moreover, oxidative damage to mammary gland DNA was unchanged. In another *in vivo* model, Tsuzuki *et al.* [62] reported that mixed isomers of dietary CLA inhibited growth of transplanted DLD-1 colon tumor cells in mice, without affecting lipid peroxidation within the tumor. In contrast, in humans fed a mixture of the t10,c12 and c9,t11 isomers, there was an increase in urinary excretion of 8-iso-PGF2 α and 15-keto-dihydro-PGF2 α , suggesting that lipid peroxidation had been stimulated [63]; this effect was attributed mainly to the t10,c12-CLA isomer [63-65]. A number of *in vitro* studies also reported an increase in lipid peroxidation or ROS levels in human breast and colon cancer cells and in Jurkat human lymphoblastic T cells cultured with mixed isomers of CLA [66-68]. In human adipocytes, t10,c12-CLA was found to be solely responsible for increasing ROS levels [48]; however in differentiated THP-1 cells, both t10,c12- and c9,t11-CLA isomers increased ROS formation, although t10,c12-CLA was more efficacious in this regard [69]. Using DHR-123 as a ROS detection probe, we also found that both CLA isomers increased ROS levels in TM4t cells, and confirmed that the increase was greater with t10,c12-CLA. Extending the results of Stachowska *et al.* [69], we showed that lipid peroxidation was increased to a much greater extent with t10,c12-CLA than with c9,t11-CLA, suggesting the importance of lipid peroxides for the apoptotic effect of t10,c12-CLA *in vitro*. It is tempting to speculate that an increase in lipid peroxidation in an *in vivo* microenvironment in which t10,c12-CLA is inducing apoptosis of lipid-filled adipocytes in the mammary gland [5], is a major contributor to the stimulation of mammary tumorigenesis that we have observed in the transgenic erbB2 mouse mammary tumor model [70]. In this regard, Kumar *et al.* [71] have demonstrated a correlation between ROS generation and aggressiveness of prostate cancer cells.

Several mechanisms can be proposed by which CLA may increase ROS production. *First*, based on experiments in which we found a very rapid decrease (> 40% within 1 h) in succinate dehydrogenase (SDH) activity, as measured by the MTT assay, prior to any change in cell number (Hsu and Ip, data not shown), we speculate that CLA inhibits mitochondrial respiratory chain activity. A number of studies have shown an increase in ROS accompanying an inhibition of mitochondrial complexes I, II (SDH) and/or III of the respiratory chain, and that this can lead to cell death [72-77]. Interestingly Soller *et al.* [75] found that thiazolidinedione-induced cell death in Jurkat T cells was mainly due to its inhibition of complex II, and moreover, that maintenance of ATP levels was required for death to be apoptotic, rather than necrotic. We previously demonstrated that ATP levels are maintained in TM4t cells treated with t10,c12-CLA [9], supporting the notion that the t10,c12-CLA - induced ROS results from an inhibition of complex II, and as a result, apoptosis is stimulated. *Second*, disruption of lysosomes, as occurs in dRLh-84 rat hepatoma cells treated with t10,c12-CLA [78], with subsequent release of lysosomal proteases, has been inferred to cause mitochondrial membrane permeabilization and increase ROS generation [79]. Phosphatidylcholine-specific PLC (PC-PLC), which is activated by t10,c12-CLA in adipocytes [48], has been shown to cause lysosomal destabilization [80]. This enzyme can also activate sphingomyelinase to promote the processing of sphingolipid into ceramide [81,82], a lipid messenger which has been shown to promote oxidative stress, induce ER stress and cause apoptosis [83-85]. *Third*, release of Ca²⁺ from the ER promotes ER stress [86,87], and there is increasing evidence suggesting that ER stress can induce

ROS formation [38,41,88], thus creating a vicious cycle since oxidative stress can induce ER stress [24,25].

5. Conclusions

The current study provides evidence that t10,c12-CLA induces apoptosis of TM4t mammary tumor cells by activation of a GPCR/phospholipase C/IP₃/CaMKK/AMPK pathway, as well as by inducing oxidative stress and lipid peroxidation. Apoptosis and/or activation of AMPK could be inhibited by antioxidants, as well as by agents that disrupted the interaction between GPCR and its intracellular binding partner Gα_q, inhibited phospholipase C, binding of IP₃ to the IP₃ receptor on the ER, or activity of CaMKK, or which chelated intracellular Ca²⁺. Our previous studies demonstrated that AMPK plays a central role in CLA-induced apoptosis, and our current data suggest that the apoptotic signals from AMPK and oxidative stress converge downstream of AMPK on p38 MAPK, the effector of AMPK activity in this model [9] (see schematic in Figure 7).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

The abbreviations used are as follows:

AMPK	AMP-activated protein kinase
2-APB or APB	2-aminoethyl diphenylborinate
BAPTA-AM	1,2-bis(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester
CaMKKα	calcium/calmodulin-dependent protein kinase kinase α
CLA	conjugated linoleic acid
t10,c12-CLA	trans-10, cis-12 CLA

c9,t11-CLA	cis-9, trans-11 CLA
DPPP	diphenyl-1-pyrenylphosphine
ER	endoplasmic reticulum
Gα_q	alpha subunit of G $_q$
GPCR	G-protein coupled receptor
GqI	a carboxy-terminal peptide of the α subunit G α_q
HPT	hypotaurine
IP$_3$	1,4,5-inositol trisphosphate
IP$_3$R	IP $_3$ receptor
MAPK	mitogen-activated protein kinase
NAC	N-acetyl-L-cysteine
PARP	poly ADP ribose polymerase
PLC	phospholipase C
PC-PLC	phosphatidylcholine-specific PLC
PI-PLC	phosphatidylinositol-specific PLC
PKA	protein kinase A
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
SDH	succinate dehydrogenase
SRB	sulforhodamine B

Fig. 1.

The CaMKK inhibitor STO-609 and the cell membrane permeable Ca^{2+} chelator BAPTA-AM attenuate CLA-induced AMPK activation. **(A)** TM4t cells were treated with or without 40 μ M t10,c12-CLA and/or 5 μ M STO-609 for 24 or 48 h. **(B)** TM4t cells were treated with or without 40 μ M t10,c12-CLA and/or 10 or 20 μ M BAPTA-AM for 48 h. In both cases, total cell lysates were analyzed for p-AMPK and AMPK by western blot. The numbers indicate the ratio of phospho-AMPK (p-AMPK) to the total AMPK. The experiments shown in A and B are each representative of 2 independent experiments.

Fig. 2.

The IP₃ receptor inhibitor 2-APB, which blocks Ca²⁺ efflux from the ER, inhibits CLA-induced apoptosis and activation of AMPK. (**A,B**) Cells were treated 72 h with or without 40 μ M t10,c12-CLA and/or various concentrations of 2-APB. Photographs were taken under the 10X microscope objective (**A**). Viable cell numbers, presented as optical density (OD), were estimated by the SRB assay, and are shown as mean $\pm$ SD (n=3) from a representative experiment (**B**). Letters without a common superscript denote a statistical difference. (**C**) Cells were treated 72 h with or without 40 μ M t10,c12-CLA and/or 20 μ M 2-APB. Cleaved PARP, caspase 9, caspase 3, CHOP, p-AMPK and AMPK were evaluated by western blot. The numbers indicate the ratio of p-AMPK to the total AMPK. The addition of 2-ABP with CLA reduced cleavage of PARP, caspase 9, caspase 3, and CHOP expression, relative to actin, respectively, to 42.1, 27.1, 66.7, and 23.5% of the CLA group at 48 h, and to 81.2, 46.5, 23.8, and 18.2% of the CLA group at 72 h. The experiment shown is representative of 2 experiments using the 20 μ M concentration of 2-ABP, and was confirmed using 2-ABP at 50 μ M.

Fig. 3.

Inhibition of PLC activity or G_{α_q} signaling attenuates CLA-induced AMPK activation and apoptosis. **(A)** Cells were treated with or without 40 μ M t10,c12-CLA and/or 2.5 μ M of the PLC inhibitor U73122 for 24, 48 or 72 h. **(B)** Cells were infected with the adenovirus encoding the inhibitor of Gq or control vector, then treated with or without 40 μ M t10,c12-CLA for 24, 48 or 72 h. CHOP or cleaved PARP, p-AMPK and AMPK were evaluated by western blot. The numbers indicate the ratio of p-AMPK to the total AMPK. The addition of U-73122 with CLA reduced CHOP expression, relative to actin, to 63.1% and 29.9% of the CLA controls at 48 and 72 h, respectively (A). The addition of Gqi reduced PARP cleavage, relative to actin, to 50.1% and 81.6% of the CLA controls at 48 and 72 h, respectively (B). The experiments shown in A and B are each representative of two independent experiments.

Fig. 4.

t10,c12-CLA induced both oxidative stress and lipid peroxidation in TM4t cells. Cells were treated with or without 40 μ M CLA for 24 h. (A,B) After incubation with CLA, cells were stained with 20 μ M DHR-123 for 20 min and analyzed by flow cytometry. Fig. 4A shows representative flow cytometry data; Fig. 4B shows the mean number of ROS-positive cells from a representative flow cytometry experiment. Each bar represents the mean $\pm$ SD from a triplicate experiment. Letters without a common superscript denote a statistical difference. (C) After incubation with t10,c12-CLA, cells were stained with 5 μ M DPPP for 15 min and examined under an immunofluorescence microscope using a 63x objective. Control cells were exposed for 14 seconds, and CLA-treated cells for 4 seconds.

Fig. 5.

The antioxidants N-acetyl-cysteine (NAC), hypotaurine (HPT), α -tocopherol (vitamin E) and Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid) attenuated CLA-induced CHOP expression and cleavage of PARP. Cells were treated with or without 40 μ M t10,c12-CLA and/or 5 mM NAC (**A**), and/or 2 mM HPT (**B**), and/or 100 μ M α -tocopherol (**C**), and/or 100 μ M Tiron (**D**) for the indicated times. For cells treated with NAC, fresh NAC was supplemented at 24 and 48 h. For cells treated with Tiron, fresh Tiron at a 50 μ M concentration was added at 24 and 48 h. The levels of CHOP and cleaved PARP were evaluated by western blot. The addition of NAC with CLA reduced cleavage of PARP and CHOP expression, relative to actin, respectively, to 34.5 and 4.6% of the CLA group at 48 h,

and to 14.2 and 22.2% of the CLA group at 72 h. (A). The addition of HPT with CLA reduced cleavage of PARP and CHOP expression, relative to actin, respectively, to 69.5 and 71.4% of the CLA group at 48 h, and to 45.5 and 17.6% of the CLA group at 72 h. (B). The addition of α -tocopherol with CLA reduced cleavage of PARP, relative to actin, to 32.1% of the CLA group at 48 h, and to 18.4% of the CLA group at 72 h. CHOP could not be quantified (C). The addition of Tiron with CLA reduced cleavage of PARP and CHOP expression, relative to actin, respectively, to 58.8 and 12.8% of the CLA group at 48 h, and CHOP to 23.8% of the CLA group at 72 h. Relative to actin, PARP cleavage in the Tiron + CLA group at 72 h was 101% of the CLA alone group at this time point (D). Experiments shown in A, B and C are representative of two independent experiments for each treatment. The experiment shown in D was done once, but a similar result was obtained when cells were treated at time zero with 200 μ M Tiron, then fresh 100 μ M added at 24 and 48 h.

Fig. 6. NAC inhibited CLA-induced apoptosis and attenuated CLA-induced p38 MAPK activation. Cells were treated with or without 40 μ M CLA and/or 5 mM NAC for 72 h (A,B), or for the indicated times (C). Fresh NAC was supplemented every 24h. (A) Cell images were photographed under the 10X microscope objective. (B) Viable cell numbers were estimated by the SRB assay. The y-axis shows the ratio of the optical density for the CLA-treated cells to the respective control (bar 1, CLA/control; bars 2-4, [CLA+NAC]/NAC). Each bar represents the mean $\pm$ SD from a triplicate experiment. Each bar is significantly different from the others with two exceptions: bar 1 vs. bar 2; bar 1 vs. bar 3. (C) Whole cell lysates

were evaluated for phospho-p38 (p-p38) and p38 by western blot. This experiment is representative of two independent experiments.

Fig. 7. Schematic representation of pathways involved in CLA-induced apoptosis. This figure is compiled from data presented in the current paper, as well as that published in reference 9.