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Activation of the AMP-activated protein kinase–p38 MAP kinase pathway mediates apoptosis induced by conjugated linoleic acid in p53-mutant mouse mammary tumor cells

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ABSTRACT

Conjugated linoleic acid (CLA) inhibits tumorigenesis and tumor growth in most model systems, an effect mediated in part by its pro-apoptotic activity. We previously showed that trans-10,cis-12 CLA induced apoptosis of p53-mutant TM4t mouse mammary tumor cells through both mitochondrial and endoplasmic reticulum stress pathways. In the current study, we investigated the role of AMP-activated protein kinase (AMPK), a key player in fatty acid metabolism, in CLA-induced apoptosis in TM4t cells. We found that t10,c12-CLA increased phosphorylation of AMPK, and that CLA-induced apoptosis was enhanced by the AMPK agonist 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and inhibited by the AMPK inhibitor compound C. The increased AMPK activity was not due to nutrient/energy depletion since ATP levels did not change in CLA-treated cells, and knockdown of the upstream kinase LKB1 did not affect its activity. Furthermore, our data do not demonstrate a role for the AMPK-modulated mTOR pathway in CLA-induced apoptosis. Although CLA decreased mTOR levels, activity was only modestly decreased. Moreover, rapamycin, which completely blocked the activity of mTORC1 and mTORC2, did not induce apoptosis, and attenuated rather than enhanced CLA-induced apoptosis. Instead, the data suggest that CLA-induced apoptosis is mediated by the AMPK–p38 MAPK–Bim pathway: CLA-induced phosphorylation of AMPK and p38 MAPK, and increased expression of Bim, occurred with a similar time course as apoptosis; phosphorylation of p38 MAPK was blocked by compound C; the increased Bim expression was blocked by p38 MAPK siRNA; CLA-induced apoptosis was attenuated by the p38 inhibitor SB-203580 and by siRNAs directed against p38 MAPK or Bim.

1. Introduction

A number of studies have demonstrated that the polyunsaturated fatty acid conjugated linoleic acid (CLA) exerts chemopreventive and therapeutic activities in a number of rodent and human tumor models, including mammary, gastric, skin, prostate and colon cancers (reviewed in [1,2]). CLA is a collective term for a group of geometrically-related linoleic acid isomers with conjugated double bonds. The c9,t11 isomer is the major form of CLA found in dairy products and ruminant meats, while t10,c12-CLA is found with the c9,t11 isomer in commercial supplements. Both CLA isomers inhibit angiogenesis [3], as well as carcinogenesis and metastasis in several, although not all, *in vivo* mammary model systems [4–7]. Contributing to the *in vivo* efficacy of CLA is the incorporation of both isomers into the triglyceride fraction of mammary adipocytes, where they serve as a highly concentrated local CLA reservoir within the MG [4,8]. *In vitro*, however, our previous studies showed that t10,c12 is the effective form of CLA in inducing apoptosis of p53-mutant TM4t mouse mammary tumor cells, an event that occurs through both Bcl-2 mediated-mitochondrial and ER stress pathways [9,10].

AMP-activated protein kinase (AMPK) is a heterotrimeric enzyme, which is comprised of a catalytic α subunit and two regulatory subunits (β and γ). Each subunit has multiple isoforms (α1, α2, β1, β2, γ1, γ2, and γ3) with different subcellular and tissue distributions [11,12]. AMPK was first recognized as a pivotal sensor of cellular

Abbreviations: ACC, acetyl coenzyme A carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (activator of AMPK); AMPK, AMP-activated protein kinase; CaMKKα, calcium/calmodulin-dependent protein kinase kinase α; CC or Comp C, compound C (inhibitor of AMPK); CLA, conjugated linoleic acid; t10,c12-CLA, trans-10, cis-12 CLA; c9,t11-CLA, cis-9, trans-11 CLA; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; p70S6K, p70S6 kinase; PPAR, peroxisome-proliferator activated protein; PKA, cyclic AMP-dependent protein kinase; PRAS40, proline-rich AKT substrate 40; SB, SB-203580 (inhibitor of p38 MAPK); SRB, sulforhodamine B; TAK1, TGFβ-activated kinase; TSC2, tuberous sclerosis complex 2; 4EBP1, eukaryotic initiation factor 4E-binding protein.

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energy status, and dysregulation of the AMPK pathway has been implicated in several cardiovascular-related diseases and metabolic disorders [13–15]. When activated by intracellular ATP depletion, AMPK can orchestrate numerous metabolic processes to minimize ATP consumption and promote ATP generation, including reduced protein synthesis, inhibition of lipogenesis, enhanced glucose transport and increased fatty acid oxidation [16,17]. In addition to the energy stress-dependent mechanism, AMPK can also be activated by several stress-independent mechanisms, including osmotic and oxidative stress, calcium signaling, and fatty acids [18–24]. Activation of AMPK occurs through phosphorylation of Thr172 on the α subunit by several upstream kinases (AMPKKs) including LKB1 [25,26], calcium/calmodulin-dependent protein kinase kinase α (CaMKKα) [27], CaMKKβ [28], and TGFβ-activated kinase-1 (TAK1) [29]. In contrast, both AKT and cyclic AMP-dependent protein kinase (PKA) have been shown to inhibit the activation of AMPK by phosphorylating Ser485/491 on its α1 and α2 subunits respectively [30–32].

In addition to its role in the homeostatic regulation of ATP, AMPK was found to mediate apoptosis in response to energy stress and chemotherapeutic agents [33–36], and its activation was shown to inhibit cell proliferation and tumorigenesis [34,37–41]. The anti-tumor activity of AMPK has been attributed to its inhibitory action on the mTOR survival pathway, which occurs through AMPK-catalyzed phosphorylation and subsequent activation of tuberous sclerosis complex 2 (TSC2), a negative regulator of the mammalian target of rapamycin (mTOR) [42].

mTOR is a key regulator of cell growth and proliferation, and its dysregulation has been linked with many human diseases, including cancer, diabetes, and cardiovascular disease [43–46]. Recent studies revealed that mTOR is integrated within two structurally and functionally distinct multi-protein complexes, mTORC1 and mTORC2. mTORC1, which consists of mTOR, mLST8, Raptor, and proline-rich AKT substrate 40 (PRAS40, a negative regulator), is a nutrient/energy sensor [43,44,47,48]. On the other hand, mTORC2, which consists of mTOR, mLST8, mSIN1, Protor 1 and Rictor, responds to growth factors but is nutrient-insensitive. mTORC1 activates protein synthesis and cell growth through regulating p70S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein (4E-BP1) activity, while mTORC2 phosphorylates AKT on Ser473 [49], activating cell growth, proliferation, and survival [43,49]. A number of studies have suggested that the ATP-sensing capability of mTORC1 is regulated by the LKB1/AMPK/TSC1/2 pathway [44,48].

Because of the central role played by AMPK in modulating fatty acid metabolism and cell viability, the major objective of this study was to determine whether AMPK might be involved in mediating the apoptotic effect of t10,c12-CLA in TM4t mammary tumor cells. Our previous studies had shown that apoptosis induced by t10,c12-CLA is via the caspase 9 and 3 pathway [9]. In this study, we examined the upstream events responsible for mediating the caspase-dependent apoptosis. Here, we demonstrate that the AMPK–p38–Bim pathway is activated by t10,c12-CLA, but not by c9,t11-CLA. Blocking this pathway with an AMPK inhibitor or siRNAs against p38 or Bim reduced cleavage of PARP, caspase 9 and caspase 3. Inhibiting mTOR activity by rapamycin was found to attenuate t10,c12-CLA-induced PARP cleavage, suggesting that the AMPK-mediated t10,c12-CLA apoptotic effect cannot be via the suppression of the AKT/mTOR/p70S6K survival pathway. Moreover, results from ATP assay and LKB1 knockdown suggest that the activation of AMPK is independent of the LKB1-dependent energy depletion pathway.

2. Materials and methods

2.1. Cell culture

The 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 μg/ml insulin, 5 ng/ml EGF and 5 μg/ml gentamicin.

2.2. Materials

The CLA isomers, each ~98% pure, were purchased from Larodan Fine Chemicals (Malmö, Sweden) and prepared as the sodium salt as described previously [50]. Anti-p38, anti-phospho AKT-Thr308, anti-phospho AKT-Ser473, anti-phospho ACC, anti-ACC, anti-phospho mTOR, anti-mTOR, anti-LKB1, anti-cleaved poly (ADP-ribose) polymerase (PARP), anti-cleaved caspase 9, anti-cleaved caspase 3, anti-phospho-AMPK-Thr172, anti-AMPK, anti-phospho p70S6K, anti-p70S6K, and AICAR were purchased from Cell Signaling Technology (Danvers, MA). Anti-Bim was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin and compound C were purchased from Calbiochem (La Jolla, CA). Rapamycin was purchased from LC laboratories. SRB (sulforhodamine B) and p38 inhibitor SB-203580 were purchased from Sigma (St. Louis, MO).

2.3. Western blot

1×10^5/ml cells were plated in dishes and cultured overnight before CLA treatment. To generate the cell lysates for western blot, 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 (Sigma). Protein concentration was determined by Bio-Rad protein assay. Lysates were separated by SDS-PAGE and proteins detected by chemiluminescence. All data reported are representative of at least two independent experiments.

2.4. Cell number determination

The SRB assay modified from the original protocol [51] was used to estimate cell number. Briefly, cells were plated in a 24-well plate and cultured overnight before CLA treatment. After 72 h CLA treatment with or without drug, culture media were aspirated followed by the addition of 400 μl PBS plus 200 μl ice cold 50% (w/v) TCA and incubation for 1 h at 4 °C to fix the viable cell monolayer. After fixation, cells were washed 5 times with distilled water and the plates inverted to drain. The cells were then stained with 300 μl of 0.4% SRB solution for 5 min at room temperature. The excess dye was removed by washing 5 times with 1% (v/v) acetic acid. The protein-bound dye was dissolved in 1 ml of 10 mM Tris base solution for OD determination at 570 nm using a microplate reader.

2.5. ATP assay

Cellular ATP concentration was determined by the luminescence ATP detection assay system from PerkinElmer (Boston, MA), using a modification of the company's protocol. Briefly, at each time point, cells were harvested and lysed in 100 μl lysis buffer for 10 min at room temperature. After incubation, mixtures were passed through a 21G syringe 5 times. Supernates were collected by centrifugation at $16{,}000\times g$ for 10 min at 4 °C. Lysates were used directly for luciferase and protein assays. Protein concentrations were determined by the DC protein assay kit from Bio-Rad (Hercules, CA) and used to normalize luciferase activity.

2.6. siRNA transfection

Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) was used for siRNA transfection. Negative control siRNA (4611) was purchased from Ambion (Austin, TX). LKB1, p38α and p38β siRNAs were purchased from Santa Cruz. Before transfection, TM4t cells were plated in 60 mm dishes, cultured overnight, then transfected with 1 μg of siRNA. Cells were transfected for 6 h, followed by replacement