Cellular Signalling 23 (2011) 2013–2020

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

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

Yung-Chung Hsu, Margot M. Ip *

Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

ARTICLE INFO

Article history:
Received 4 May 2011
Received in revised form 30 June 2011
Accepted 13 July 2011
Available online 23 July 2011

Keywords:
Conjugated linoleic acid
AMP kinase
GPCR
ER stress
Oxidative stress
CaMKK

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 $G\alpha_q$, by the phospholipase C (PLC) inhibitor U73122, by the inositol trisphosphate (IP_3) receptor inhibitor 2-APB, by the calcium/calmodulin-dependent protein kinase kinase α (CaMKK) inhibitor STO-609 and by the intracellular Ca^{2+} chelator BAPTA-AM. This suggests that t10,c12-CLA may exert its apoptotic effect by stimulating GPCR through $G\alpha_q$ signaling, activation of phosphatidylinositol-PLC, followed by binding of the PLC-generated IP_3 to its receptor on the ER, triggering Ca^{2+} 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.

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 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,

Abbreviations: 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 tetraacetoxy-methyl 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; $G\alpha_q$, alpha subunit of G_q; GPCR, G protein coupled receptor; Gql, a carboxy-terminal peptide of the α subunit $G\alpha_q$; HPT, hypotaurine; IP_3, 1,4,5-inositol trisphosphate; IP_3R, 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.

* Corresponding author at: Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA. Tel.: +1 716 845 2356; fax: +1 716 845 5865.
E-mail address: margot.ip@roswellpark.org (M.M. Ip).

0898-6568/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellsig.2011.07.015

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 $G\alpha_q$-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 μg/ml insulin, 5 ng/ml EGF and 5 μg/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 μl PBS plus 200 μl 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 h. The 3rd day, the medium was changed to fresh media +/− CLA and the cells incubated for the indicated time.

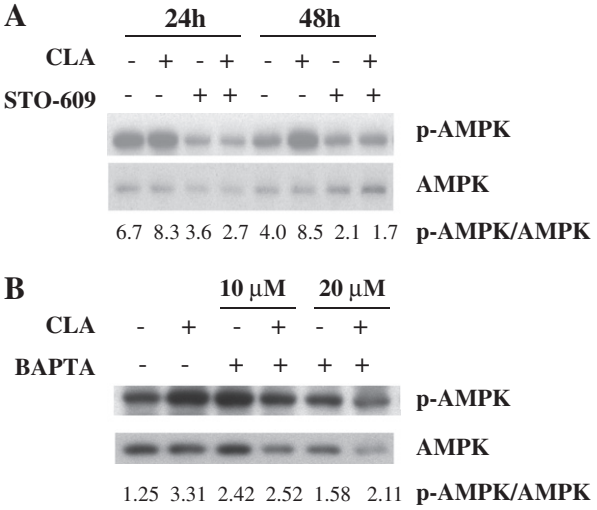

Fig. 1. The CaMKK inhibitor STO-609 and the cell membrane permeable Ca^{2+} chelator BAPTA-AM attenuate CLA-induced AMPK activation. (A) TM4t cells were treated with or without 40 μM t10,c12-CLA and/or 5 μM STO-609 for 24 or 48 h. (B) TM4t cells were treated with or without 40 μM t10,c12-CLA and/or 10 or 20 μM 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.