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Anacardic acid induces cell apoptosis associated with induction of ATF4-dependent endoplasmic reticulum stress

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HIGHLIGHTS

- Anacardic acid (AA) inhibits tumor growth *in vitro* and *in vivo*.
- AA induces endoplasmic reticulum (ER) stress *in vitro* and *in vivo*.
- Induction of ATF4-dependent ER stress contributes to AA-induced tumor cell death.
- Extrinsic and intrinsic apoptotic pathways are involved in AA-induced cell apoptosis.

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ABSTRACT

Anacardic acid (6-pentadecylsalicylic acid, AA), a natural compound isolated from the traditional medicine Amphipterygium adstringens, has been reported to possess antitumor activities. However, its molecular targets have not been thoroughly studied. Here, we report that AA is a potent inducer of endoplasmic reticulum (ER) stress, leading to apoptosis in hepatoma HepG2 and myeloma U266 cells. Induction of ER stress by AA was supported by a dose- and time-dependent increase in expression of the ER signaling downstream molecules, such as GRP78/BiP, phosphorylated eIF2α, ATF4 and CHOP in both HepG2 and U266 cell lines. Blockage of ATF4 expression by siRNA partially inhibited, while knock-down of CHOP expression by siRNA slightly increased AA-induced cell death in these cells. In addition, AA suppressed HepG2 xenograft tumor growth, associated with increased ER stress *in vivo*. These results suggest that AA induces tumor cell apoptosis associated with ATF4-dependent ER stress.

1. Introduction

Discovery of novel agents with anticancer activity from natural resources has gained a significant important position in cancer prevention and treatment. Anacardic acid (6-pentadecylsalicylic acid, AA) is a major active compound mainly isolated from Amphipterygium adstringens (Acevedo et al., 2006), Ozoroa insignis (Rea et al., 2003), *Anacardium occidentale* (Kubo et al., 1999) and Ginkgo biloba (Itokawa et al., 1987). The bark of Amphipterygium adstringens has been used for the treatment of gastric ulcers, gastritis, and stomach cancers in Mexico (Acevedo et al., 2006).

Several targets of AA have been reported. AA exerted anti-inflammatory effect by inhibiting IL-8 expression which is important for initiation and persistence of inflammation. AA inhibited decreased acetylation of histone 4 at the IL-8 promoter, resulting in inhibition of LPS-induced IL-8 expression (Trevisan et al., 2006). AA also possessed anti-microbial (Kubo et al., 1999; Muroi and Kubo, 1996) and antioxidant effects (Trevisan et al., 2006). Recent studies have reported that AA exhibited antitumor activities. AA induced caspase-independent apoptosis in pituitary adenoma and lung adenocarcinoma cells (Seong et al., 2013a; Sukumari-Ramesh et al., 2011). AA inhibited activities of histone

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acetyltransferase and NF-κB, both of which are potential targets for chemotherapy (Balasubramanyam et al., 2003; Sun et al., 2006; Sung et al., 2008). AA could also inhibit angiogenesis *in vitro* and *in vivo* through suppressing vascular endothelial growth factor receptor-2 (VEGFR-2) signaling (Wu et al., 2011). Treatment with AA inhibited the estrogen receptor alpha-DNA binding and reduced target gene transcription, leading to inhibition of breast cancer cell proliferation (Schultz et al., 2010). AA also induced apoptosis through down-regulation of androgen receptor (AR) by suppressing p300 and upregulation of p53 in prostate cancer cells (Tan et al., 2012).

Although several cellular proteins or activities could be affected by AA treatment as mentioned before, its specific molecular target(s) remain to be discovered. Protein folding in the endoplasmic reticulum (ER) could be impaired under various physical and pathological conditions, termed ER stress. The ER has evolved highly specific signaling pathways to ensure that its protein folding capacity is not overwhelmed, all of which are collectively termed the unfolded protein response (UPR). However, severe or prolonged ER stress can induce apoptosis when the UPR fails to compensate for the stimulus (Huang et al., 2009; Jiang et al., 2012). Multiple pathways may be involved in ER stress-initiated apoptosis. Three distinct branches of the UPR have been identified based on distinct sensors: IRE1, PKR-like endoplasmic reticulum kinase (PERK), and ATF6 (Ron and Walter, 2007; Sano and Reed, 2013; Xu et al., 2005). Among them, the PERK pathway leads to phosphorylation of eukaryotic initiation factor 2α (eIF2α) and enhances translation of mRNAs such as ATF4. The eIF2α-ATF4 signaling has a preapoptotic function that involves the activation of binding protein (BiP), phosphorylated eIF2α, ATF4 and C/EBP homologous protein (CHOP) (Armstrong et al., 2010; Jiang and Wek, 2005; Qing et al., 2012).

Here we report that when applied in HepG2 and U266 cell lines and HepG2 xenografts, AA could induce ATF4-dependent ER stress *via* a mechanism distinct to previously reported (Tan et al., 2012), associated with its antitumor activity.

2. Material and methods

2.1. Materials, reagents, and antibodies

AA and Cremophor EL were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), RPMI 1640, penicillin and streptomycin were purchased from Invitrogen Life Technology (Carlsbad, CA USA). Rabbit polyclonal antibody against GAPDH (FL-335) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against nuclear poly (ADP-ribose) polymerase (PARP), eIF2α, Phospho-eIF2α (Ser51), rabbit monoclonal antibodies against BiP (C50B12), Bcl-2 (50E3), Bim (C34C5), ATF-4 (D4B8), caspase-3 (8G10), caspase-8 (D35G2) and mouse monoclonal antibodies against caspase-9 (C9), CHOP (L63F7) were obtained from Cell Signaling (Beverly, MA, USA). Rabbit polyclonal antibodies against actived-caspase-3, -8, -9 and Bid were purchased from Bioworld Technology Inc. Enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences (Piscataway, NJ, USA). Propidium idodide (PI) and Annexin V-FITC Apoptosis Detection Kit were from Keygene Company (Nanjing, China).

2.2. Cell lines and cell culture

Human hepatoma cell line HepG2, myeloma cell line U266 and non-transformed bronchial epithelial 16HBE cell line were purchased from American Type Culture Collection (Manassas, VA, USA) and grown in RPMI 1640 supplemented with 10% FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin. Cell cultures were maintained at 37 °C and 5% CO_2.

2.3. MTS assay

The effects of compounds on cell viability were determined by the MTS assay (Cell Titer 96® AQueous One Solution Cell Proliferation assay, Promega Corporation, Madison, WI, USA) as described

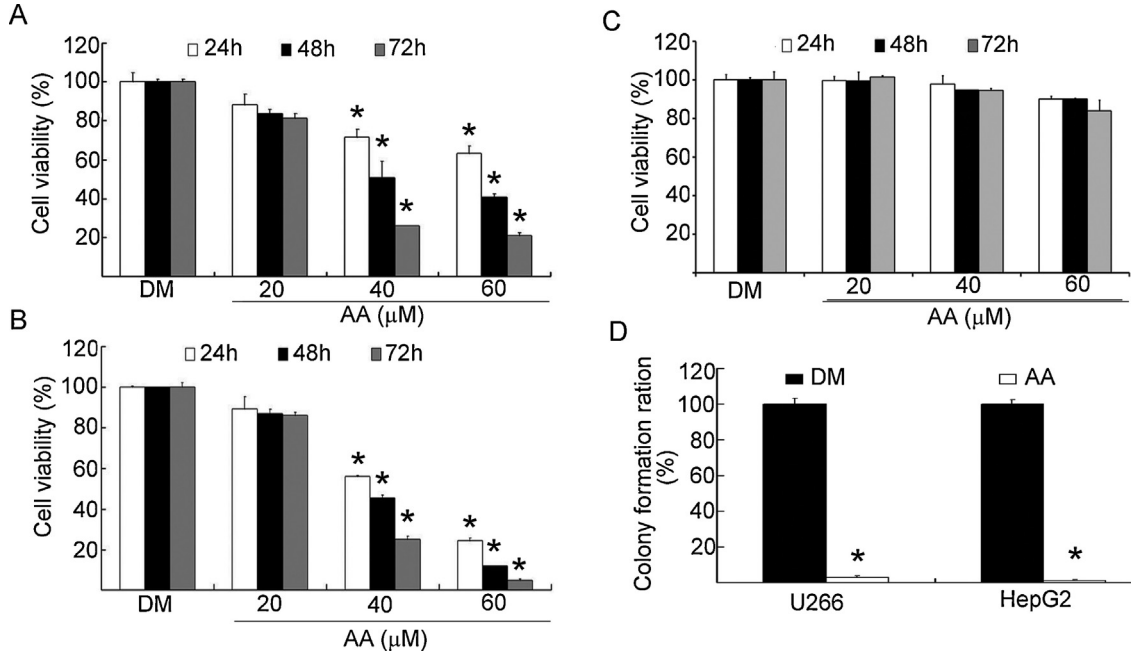

Fig. 1. The growth inhibitory effect of anacardiac acid (AA) on cancer and mom-transformed cells. ((A)–(C)) HepG2, U266 and 16HBE cells were treated with increasing concentration of AA (20 to 60 μM) for 24 h, 48 h, and 72 h, and cell viability was detected by MTS. Cell viability in HepG2, U266 and 16HBE cells were shown in (A)–(C), respectively. Mean ± SD ($n = 3$). * $P < 0.05$, *versus* each DMSO control. DM:DMSO. (D) AA inhibits colony formation in HepG2 and U266 cells. HepG2 and U266 cells were treated with AA for 12 h, and then suspended in soft agar. The number of colonies >60 μm were counted after 7 days' culture under light microscope. The experiments were tested in triplicate. Quantification of cells as a percentage of solvent DMSO treated cells was shown. Mean ± SD ($n = 3$). * $P < 0.05$, *versus* DMSO control.