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Autophagy regulates trans fatty acid-mediated apoptosis in primary cardiac myofibroblasts

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ABSTRACT

Trans fats are not a homogeneous group of molecules and less is known about the cellular effects of individual members of the group. Vaccenic acid (VA) and elaidic acid (EA) are the predominant trans monoenes in ruminant fats and vegetable oil, respectively. Here, we investigated the mechanism of cell death induced by VA and EA on primary rat ventricular myofibroblasts (rVF). The MTT assay demonstrated that both VA and EA (200 μM, 0–72 h) reduced cell viability in rVF ($P<0.001$). The FACS assay confirmed that both VA and EA induced apoptosis in rVF, and this was concomitant with elevation in cleaved caspase-9, -3 and -7, but not caspase-8. VA and EA decreased the expression ratio of Bcl2:Bax, induced Bax translocation to mitochondria and decrease in mitochondrial membrane potential ($\Delta\psi$). *BAX* and *BAX*/*BAK* silencing in mouse embryonic fibroblasts (MEF) inhibited VA and EA-induced cell death compared to the corresponding wild type cells. Transmission electron microscopy revealed that VA and EA also induced macroautophagosome formation in rVF, and immunoblot analysis confirmed the induction of several autophagy markers: LC3-β lipidation, Atg5–12 accumulation, and increased beclin-1. Finally, deletion of autophagy genes, *ATG3* and *ATG5* significantly inhibited VA and EA-induced cell death ($P<0.001$). Our findings show for the first time that trans fat acid (TFA) induces simultaneous apoptosis and autophagy in rVF. Furthermore, TFA-induced autophagy is required for this pro-apoptotic effect. Further studies to address the effect of TFA on the heart may reveal significant translational value for prevention of TFA-linked heart disease.

1. Introduction

The impact of diet on the incidence of cardiovascular disease is well established, and nutrition may be responsible for ~40% of all cardiovascular disease (Canadian government report-Health Canada issued in 1995). Recent data indicate that the majority of myocardial infarctions (MI) are directly linked to modifiable environmental factors, including diet [1], in particular the relative intake of saturated fatty acids (SFA) and polyunsaturated fats (PUFAs) [2,3]. Trans fatty acids (TFA) are unsaturated fatty acids with at least one double bond in the trans configuration that renders them structurally/chemically unstable compared to saturated SFAs [4]. Elaidic acid (18:1 *trans*-9) is the main TFA isomer in hydrogenated vegetable oils and products containing hydrogenated margarines or vegetable shortening including fried foods, cookies, and crackers, and accumulates in atherosclerotic lesions and adipose tissue of obese patient [4–6]. Health professionals support removal or reduction of dietary TFAs for improved health [7,8]. This notwithstanding,

Abbreviations: VA, vaccenic acid; EA, elaidic acid; rVF, rat ventricular myofibroblast; MEF, mouse embryonic fibroblasts; TFA, trans fatty acid; MI, myocardial infarction; PUFA, poly unsaturated fat; SFA, saturated fatty acids; PI, propidium iodide; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; ATG5, autophagy protein 5; ATG3, autophagy protein 3; BAD, the Bcl2-associated death promoter protein; BAK, BCL-2-antagonist/killer; BAX, the Bcl2-associated X protein; Bcl2, B-cell lymphoma 2; BID, BH3 interacting domain death agonist; Caspases, cysteine-dependent aspartate-directed proteases; ER, endoplasmic reticulum; LC-3, microtubule-associated protein light chain 3; TEM, transmission electron microscopy

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some recent evidence indicates that specific isomeric configuration in different TFAs may exhibit different effect(s) on cardiovascular health [7]. Vaccenic acid (18:1 *trans*-11) is unique in structure and source, as it is enriched in dairy products and meats of ruminant species [9].

Several recent in vitro studies indicate that saturated fatty acids induce apoptosis (programmed cell death I) in somatic and cancer cells of many different origins, including cardiomyocytes and fibroblasts [10–21]. Though a recent report suggests a role for autophagy in TFA-induced cell death of hepatocytes [22], apoptosis is generally accepted as the principal mechanism driving cell demise in health and disease [23–26]. Apoptosis can be initiated via extrinsic and/or intrinsic pathways [24,27], driven by the activation of caspases. Macroautophagy is an evolutionarily conserved catabolic, homeostatic process that can support cell survival or, if excessive, can drive cell death. It consists of membrane isolation, autophagosome and autolysosome formation [28,29], the later driving breakdown of macromolecules and organelles by lysosomal enzymes [28,30,31]. Apoptosis and autophagy have many common regulators, and cross-talk regulates cell fate in response to cellular stress. The complex interaction of apoptotic and autophagic pathways necessitates the careful consideration of their integrated control and impact on cell fate to understand cell death phenomena [32,33].

Fibroblasts are a heterogeneous group of cells that exhibit distinct differentiated phenotypes in different organs [34]. In humans cardiac fibroblasts represent the most numerous non-myocytes in the myocardium, with these cells synthesizing and organizing collagens, fibronectins and other interstitial components to maintain the integrity of the cardiac extracellular matrix (matrix) [35]. In the current paper, we address the cell death effects of elaidic and vaccenic TFAs on rat ventricular myofibroblasts (rVF), dissecting the roles of apoptosis and autophagy and cross-talk between these pathways.

2. Materials and methods

2.1. Materials and reagents

Cell culture plasticware was obtained from the Corning Costar Company (Thermo Fisher Canada). Cell culture media, propidium iodide (PI), rabbit anti-LC3β, rabbit anti-beta actin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), vaccenic acid, elaidic acid, and vitamin C were obtained from Sigma (Sigma-Aldrich, Oakville, Canada). Rabbit anti-cleaved caspase-7, -8, rabbit anti-Bak, Bax, Bcl2, Atg12, Atg3, Atg5, cleaved caspase-3, and cleaved caspase-9 were purchased from Cell Signaling (MA, USA). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), Mitotracker Red, was obtained from Life Technologies Inc. (Burlington, Canada). Caspase-Glo®-3/7, Caspase-Glo®-8 and Caspase-Glo®-9 assay were purchased from Promega (WI, USA).

2.2. Primary cardiac fibroblast preparation

Cardiac fibroblasts were isolated as previously described [36,37]. Briefly, hearts from adult male Sprague–Dawley rats (150–200 g) were subjected to Langendorff perfusion with DMEM-F-12 (GIBCO) followed by serum-free MEM (SMEM; Life Technologies, Inc., Burlington, Canada). Perfused hearts were digested with 0.1% wt/vol collagenase type 2 (Worthington) in SMEM for 20 min. Hearts were minced in dilute collagenase solution (0.05% wt/vol collagenase type 2 in SMEM) for a further 15 min before addition of growth media DMEM-F-12 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin (GIBCO-BRL), 100 μg/ml streptomycin (GIBCO BRL), and 1 μM ascorbic acid (Sigma-Aldrich). Upon settling of large tissue pieces to the bottom of a 50-ml tube, supernatant was centrifuged at 2000 rpm for 5 min. Cell pellets were re-suspended in growth media and plated on 75-cm^2 culture flasks. Cells were allowed to adhere for 2–3 h in a 5% CO_2 37 °C incubator, then washed twice with phospho-buffered saline (PBS) followed by the addition of fresh growth media. Media were changed the following day, and cells were allowed to grow for 3–4 days before passaging into first passage (P1) myofibroblasts. P1 myofibroblasts were transferred to DMEM/F12 media and after 24 h all cultures have been done in DMEM medium. For all experiments, *passages* 3–7 of rat cardiac myofibroblast were used in DMEM/F12 complete media.

2.3. Cell viability assay

We measured the viability of cardiac myofibroblasts under various treatment conditions, as described previously using MTT [38,39]. Briefly, primary cardiac myfibroblasts, wild type murine embryonic fibroblasts (Wt MEF), MEF *Bax* knock out (MEF *Bax*$^{-/-}$), and MEF *Bax*/*Bak* double knock out (MEF *Bax*/*Bak*$^{-/-}$) cells were treated with vaccenic or elaidic acid (0–400 μM, 0–72 h). Relative cell viability (percent of control) was calculated using the equation: (mean OD of treated cells/mean OD of control cells)×100. For each time point, the treated cells were compared with control cells that had been treated with vehicle only (DMSO, 0.1% V/V). In experiments investigating if vitamin C can modulate the cytotoxic effects of vaccenic and elaidic acids, vitamin C (2.5 and 5 mM) was added to culture media 4 h before the treatment and later the cells were co-treated with vaccenic and elaidic acids (200 μM, 72 h).

2.4. Measurement of apoptosis by flow cytometry

Apoptosis in our cell preparations was measured using the Nicoletti method [40,41]. Briefly, cells grown in 12-well plates were treated with 200 and 400 μM vaccenic and elaidic acids for the indicated time intervals. After scraping, the cells were harvested by centrifugation at 1500×*g* for 5 min, washed once with phosphate-buffered saline, and resuspended in hypotonic propidium iodide lysis buffer (1% sodium citrate, 0.1% Triton X-100, 0.5 mg/ml RNase A, 40 μg/ml propidium iodide). Cellular nuclei were incubated for 30 min at 30 °C and subsequently analyzed by flow cytometry. Nuclei to the left of the G1 peak containing hypo-diploid DNA were considered apoptotic.

2.5. Luminescence caspase activity assays

Luminometric assays Caspase-Glo® 8, 9 and 3/7 (Promega, Canada, Nepean, ON) were used to measure the proteolytic activity of caspases-3/7 (DEVD-ase), 8 (IETD-ase), and 9 (LEHD-ase) as we have previously done [42].

2.6. Measuring mitochondrial membrane potential

Mitochondrial membrane potential was measured employing the mitochondria-specific cationic ratiometric dye JC-1 that undergoes $\Delta\Psi_m$-dependent aggregation in the mitochondria. Normally, JC-1 exists as a green fluorescent (540 nm, excitation 490 nm) monomer at $\Delta\Psi_m$<140 mV, but when $\Delta\Psi_m$>140 mV, then JC-1 aggregates and emits red spectra fluorescence (590 nm, excitation 540 nm). We measured the $\Delta\Psi_m$ of cardiac myofibroblasts under various treatment conditions, as described previously [43].

2.7. Analysis of cellular morphology

To assess cell viability based on gross cellular appearance (chromatin condensation and cell shrinkage) rat cardiac myofibroblast cells were grown on 12-well plates and morphology was assessed by phase contrast microscopy (Olympus CKX41) using a Olympus Infinity 1 CCD digital camera to capture images.