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Cinnamic aldehyde inhibits vascular smooth muscle cell proliferation and neointimal hyperplasia in Zucker Diabetic Fatty rats

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ABSTRACT

Atherosclerosis remains the number one cause of death and disability worldwide. Atherosclerosis is treated by revascularization procedures to restore blood flow to distal tissue, but these procedures often fail due to restenosis secondary to neointimal hyperplasia. Diabetes mellitus is a metabolic disorder that accelerates both atherosclerosis development and onset of restenosis. Strategies to inhibit restenosis aim at reducing neointimal hyperplasia by inhibiting vascular smooth muscle cell (VSMC) proliferation and migration. Since increased production of reactive oxygen species promotes VSMC proliferation and migration, redox intervention to maintain vascular wall redox homeostasis holds the potential to inhibit arterial restenosis. Cinnamic aldehyde (CA) is an electrophilic Nrf2 activator that has shown therapeutic promise in diabetic rodent models. Nrf2 is a transcription factor that regulates the antioxidant response. Therefore, we hypothesized that CA would activate Nrf2 and would inhibit neointimal hyperplasia after carotid artery balloon injury in the Zucker Diabetic Fatty (ZDF) rat. In primary ZDF VSMC, CA inhibited cell growth by MTT with an EC_{50} of 118 ± 7 μM. At a therapeutic dose of 100 μM, CA inhibited proliferation of ZDF VSMC *in vitro* and reduced the proliferative index within the injured artery *in vivo*, as well as migration of ZDF VSMC *in vitro*. CA activated the Nrf2 pathway in both ZDF VSMC and injured carotid arteries while also increasing antioxidant defenses and reducing markers of redox dysfunction. Additionally, we noted a significant reduction of neutrophils (69%) and macrophages (78%) within the injured carotid arteries after CA treatment. Lastly, CA inhibited neointimal hyperplasia evidenced by a 53% reduction in the intima:media ratio and a 61% reduction in vessel occlusion compared to arteries treated with vehicle alone. Overall CA was capable of activating Nrf2, and inhibiting neointimal hyperplasia after balloon injury in a rat model of diabetic restenosis.

1. Introduction

Cardiovascular disease (CVD) remains the number one cause of death and disability worldwide [1]. Atherosclerosis is the major underlying cause for most coronary and peripheral artery diseases. Diabetes mellitus (DM) is a series of metabolic disorders associated with a chronic state of hyperglycemia, systemic redox imbalance, inflammation, advanced glycation end products, dyslipidemia, and endothelial dysfunction. DM patients are two to three times more likely to have CVD than non-diabetics [2] and exhibit an increased rate of atherosclerotic plaque development [3]. Revascularization procedures for occluded arteries include balloon angioplasty with or without stent placement. The arterial injury response after revascularization often results in vessel re-occlusion, or restenosis, which in turn requires

Abbreviations: ARE/EpRE, antioxidant/electrophile response element; BrdU, bromodeoxyuridine; CA, cinnamic aldehyde; CVD, cardiovascular disease; DHE, dihydroethidium; DM, diabetes mellitus; GCLC, glutamate-cysteine ligase catalytic subunit; GSH, glutathione; HO-1, heme oxygenase-1; I:M, intima:media; MPO, myeloperoxidase; NOX1, NADPH oxidase 1; Nrf2, nuclear factor erythroid 2-related factor 2; PDGF, platelet derived growth factor; Prx, peroxiredoxin; ROS, reactive oxygen species; SFN, sulforaphane; SOD, superoxide dismutase; VSMC, vascular smooth muscle cells; XO, xanthine oxidase; ZDF, Zucker Diabetic Fatty rat

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further intervention. DM also accelerates restenosis as evidenced by lower patency rates after revascularization in diabetic patients [4–6].

Restenosis is secondary to vessel constrictive remodeling and neointimal hyperplasia, or the inward migration and proliferation of both vascular smooth muscle cells (VSMC) [7] from the media and fibroblasts from the adventitia [8] into the intima. Drug-eluting stents are currently the most effective therapy for restenosis as they inhibit all cellular proliferation and migration within the vessel, but their use increases the risk of thrombosis by preventing vessel reendothelialization [9]. Thus, emerging therapies aim to prevent the proliferation and migration of VSMC and fibroblasts after injury without inhibiting endothelialization.

Local redox imbalance has been described as a major contributing factor for this VSMC pathology [10,11]. Specifically, reactive oxygen species downstream of NADPH oxidase 1 (NOX1), superoxide and subsequently hydrogen peroxide, are overproduced after injury and promote VSMC growth and migration [12–15]. Additionally, the family of platelet derived growth factors (PDGF) have been implicated as one of the major growth signals upstream of NOX1 [16]. Small molecules that target the redox imbalance have shown promise as potential therapeutics for preventing the VSMC phenotypic changes [17–19]. A novel therapeutic approach is to restore the redox balance by activating nuclear factor erythroid 2-related factor 2 (Nrf2), the main antioxidant defense pathway present in our cells [20,21]. Indeed, treatment with sulforaphane, a Nrf2 activating small molecule, has been shown to inhibit restenosis in a non-diabetic Sprague Dawley rat balloon injury model [22] as well as wire injured mouse models [23,24]. To date, however, no study has evaluated the efficacy of these small molecules in a diabetic model of restenosis.

Cinnamic aldehyde (CA) is an α,β-unsaturated aldehyde extracted from cinnamon that activates Nrf2 [25,26]. CA has been shown to prevent hyperglycemia-induced endothelial dysfunction [27], be protective against diabetes-induced hypertension in rats [28], and exhibit overall anti-diabetic effects [29]. We hypothesized that CA would inhibit neointimal hyperplasia in a diabetic restenosis model. Accordingly, we used Zucker Diabetic Fatty (ZDF) rats, a well characterized type 2 DM model, for our *in vitro* and *in vivo* evaluation. Primary aortic ZDF VSMC were treated in the presence of high glucose (25 mM) and PDGF-BB (25 ng/mL) as an *in vitro* model of injury in diabetes. We used the carotid artery balloon injury in the ZDF rats after onset of diabetes to evaluate the therapeutic potential of CA at inhibiting neointimal hyperplasia formation.

2. Materials and methods

2.1. Materials

Cinnamic aldehyde (CA) (C80687; Sigma-Aldrich, St. Louis, MO). DMEM (11885-084; Gibco, Grand Island, NY). DMSO (BP231; Thermo-Fisher Scientific, Waltham, MA). F-12 (11765-054; Gibco). Glucose (50-99-7, Sigma-Aldrich). Heat-inactivated fetal bovine serum (FBS) (16140071; Gibco). Paraformaldehyde (158127; Sigma-Aldrich). PBS (20–134; Apex Bioresearch Products, San Diego, CA). Sulforaphane (SFN) (S6317; Sigma-Aldrich). Trypsin-EDTA (0.05%) (25300054; Gibco).

2.2. Animals

All animal handling and experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of North Carolina – Chapel Hill. (Ref no. IACUC16-254 (10-2016-09-2019)). Male Zucker Diabetic Fatty (ZDF) rats were obtained from Charles River Laboratories (Wilmington, MA). Animals were fed Irradiated Purina 5008 (Granville Milling, Granville, OH) chow. Blood glucose was measured by tail nick using a Freestyle Precision Neo Blood Glucose Monitoring System (Abbott Laboratories, Abbott Park, Illinois). Glycemia > 400 mg/dL after two consecutive random weekly measurements was used to determine type 2 diabetes onset.

2.3. Rat carotid artery injury model

Adult male ZDF rats weighing 350–500 g were used for surgery after onset of diabetes. Rats were anesthetized with inhaled isoflurane (0.5–2%). Atropine (0.1 mg/kg) was administered subcutaneously (SC) to reduce airway secretions and Carprofen (5 mg/Kg) was administered SC for pain management. After a sterile prep and midline neck incision, the left common, internal, and external carotid arteries were dissected and the internal and common carotid arteries were occluded. A No. 2 French Fogarty balloon catheter (Edwards Lifesciences, Irvine, CA) was inserted through an arteriotomy in the external carotid artery and advanced into the common carotid artery. The balloon was inflated to 5 atm of pressure for 5 min to create a uniform injury. After removal of the balloon, the external carotid artery was ligated and blood flow was restored. 100 μM CA was applied in 100 μL of Pluronic-127 (P2443; Sigma-Aldrich) gel periadventitially to the external surface of the injured common carotid artery and then the neck incision was closed. Two separate cohorts of rats, one from each of the two surgeons that performed the model, were sacrificed 2 weeks (total n = 6/group) after surgery for morphometric analysis of neointimal hyperplasia and macrophage presence. Rats were sacrificed 3 days after surgery for analyzing proliferation, redox biomarker levels, and inflammatory cell invasion. To analyze cell proliferation, bromodeoxyuridine (BrdU) (B5002; Sigma-Aldrich) was administered via intraperitoneal injection 1 day and 1 h prior to euthanasia. One vehicle-treated, 3-day injury rat was not included in analysis due to thrombus development in the common carotid artery.

2.4. Tissue processing

Carotid arteries were harvested after in situ perfusion-fixation with 200 mL of PBS and 200 mL of cold 2% paraformaldehyde. Arteries were placed in 2% paraformaldehyde for 1 h at 4 °C followed by 30% sucrose overnight at 4 °C. Arteries were quick-frozen in O.C.T. (4583; Tissue-Tek, Torrance, CA) and stored at − 80 °C. 5 μm sections were cut throughout the entire common carotid artery for staining.

2.5. Histological and Immunofluorescence analysis

Immunofluorescent (IF) staining for 3-day injured arteries (n = 5 vehicle alone; n = 6 CA-treatment): 10 μM dihydroethidium (DHE) (D23107, Thermo-Fisher Scientific) diluted in DMSO for 10 min in the dark; 1:1000 anti-3-nitrotyrosine antibody (ab61392; Abcam, Cambridge, UK) in IHC-Tek diluent (1W-1000; IHC World, Woodstock, MD) for 1 h followed by 1:1000 Alexa Fluor 555 goat anti-mouse IgG (A-21236, Invitrogen, Carlsbad, CA) in PBS for 1 h; 1:100 anti-myeloperoxidase (ab9535; Abcam) in IHC-Tek diluent for 1 h after permeabilization with 0.3% Triton X-100 (X100; Sigma-Aldrich) followed by 1:100 Alexa Fluor 647 goat anti-rabbit IgG (A21245; Invitrogen) in PBS for 1 h; 1:1000 anti-CD68 (MCA341R; Bio-Rad, Hercules, CA) in IHC-Tek diluent for 1 h followed by 1:500 Alexa Fluor 647 goat anti-mouse IgG (A-21236; Invitrogen) in PBS for 1 h; counterstain with 0.6 μM DAPI (D3571; Invitrogen) diluted 1:500 in PBS for 5 min was performed with all immunofluorescent staining. ProLong Gold Antifade Reagent (P36930, Thermo-Fisher Scientific) was used for mounting coverslips. 3-day injured arteries were stained for BrdU uptake according to the manufacturer's instructions (ab125306; Abcam). 2-week injured arteries were H&E stained for measuring neointimal hyperplasia and IF stained for CD68. For *in vitro* IF, after 24 h treatment with 100 μM CA or 4 μM SFN, VSMC were stained with 10 μM DHE for 1 h followed by 0.6 μM DAPI diluted 1:500 in PBS for 5 min. Slides were imaged on the day after staining using a Zeiss Axio Imager. A2 microscope (Oberkochen, Germany). Images were taken on a 5x objective for