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4-phenylbutyrate and valproate treatment attenuates the progression of atherosclerosis and stabilizes existing plaques

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ABSTRACT

Background and aims: Recent evidence suggests that endoplasmic reticulum (ER) stress signaling through glycogen synthase kinase (GSK)-3α/β is involved in the activation of pro-atherosclerotic processes. In this study, we examined the effects of small molecules that interfere with ER stress-GSK3α/β signaling on the progression and regression of atherosclerosis in a mouse model.

Methods: To examine atherosclerotic progression, low-density lipoprotein receptor deficient ($Ldlr^{-/-}$) mice were placed on a high-fat diet (HFD) and treated with the chemical chaperone, 4-phenylbutyrate (4PBA, 3.8 g/L drinking water), or the GSK3α/β inhibitor, valproate (VPA, 625 mg VPA/kg diet), for 10 weeks. To examine potential effects on atherosclerotic regression, 4 week old $Ldlr^{-/-}$ mice were placed on a HFD for 16 weeks. Subsets of mice were harvested at this time or switched to a chow (low fat) diet, or a chow diet with 4PBA or VPA treatment for 4 weeks.

Results: In the progression model, the 4PBA- and VPA-treated mice had significantly reduced lesion and necrotic core size. Treatments had no effect on metabolic parameters, including plasma and hepatic lipid levels, or plaque composition. In the regression model, mice with 4PBA or VPA treatment showed no alterations in lesion size, but the lesions had significantly smaller necrotic cores, increased vascular smooth muscle cell content, and increased collagen content. These features are consistent with more stable plaques.

Conclusions: The pharmacological attenuation of ER stress or inhibition of GSK3α/β impedes the development of atherosclerosis in $Ldlr^{-/-}$ mice and appears to promote the stabilization of existing lesions.

1. Introduction

Atherosclerosis is an inflammatory disease characterized by the accumulation of fatty plaques within the arterial wall. It is the major underlying cause of cardiovascular disease, which is the leading cause of mortality worldwide, accounting for approximately 30% of all deaths [1,2]. Risk factors that contribute to the progression of atherosclerosis include diabetes mellitus, dyslipidemia, hypertension, obesity, and smoking [3]. The major complications of atherosclerosis can be attributed to thrombus formation following plaque rupture, which may occlude the artery and result in myocardial ischemia or infarction [4,5]. Despite many advances, our understanding of the molecular mechanisms that link cardiovascular risk factors to the development of atherosclerosis is incomplete. Furthermore, the potential to reverse the atherogenic process, through the resolution or stabilization of existing plaques, has only just begun to be investigated.

Recently, our group and others have established a causative role for endoplasmic reticulum (ER) stress in the development and progression of atherosclerosis [6–8]. The ER is a eukaryotic organelle responsible for protein modification, protein folding, and protein trafficking. When the processing capacity of the ER is exceeded, there is an accumulation of misfolded and unfolded proteins in the ER, a condition known as ER stress. The proximal intracellular response to ER stress is the activation of the unfolded

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protein response, which acts to reduce the protein load and increase ER folding capacity [9]. Chronic, unresolved ER stress can lead to activation of intracellular pathways that, depending on the magnitude and cell type, can include increased expression of inflammatory cytokines [10], increased biosynthesis and accumulation of cholesterol and triglycerides [11], and activation of pro-apoptotic processes [12]. There is a growing body of evidence that implicates ER stress in the development of several metabolic diseases and disorders including cancer, neurodegeneration, type 2 diabetes, liver disease, and atherosclerosis [13].

The mitigation of ER stress using a chemical chaperone, 4-phenylbutyrate (4PBA), has been shown to be protective against atherosclerosis in apolipoprotein E deficient (*Apoe*$^{-/-}$) mice [14]. However, the underlying molecular mechanisms by which ER stress affects downstream pro-atherogenic pathways are not well understood. Results from our lab and others have suggested that ER stress may signal through the activation of glycogen synthase kinase (GSK)-3α/β to promote the development of atherosclerosis [15–17]. GSK3α/β is a serine/threonine kinase that is involved in the regulation of several different metabolic pathways and implicated in the development of a number of diseases [18]. We have shown that atherosclerosis is attenuated in *Apoe*$^{-/-}$ mice treated with valproate (VPA), an anti-epileptic drug and a known GSK3α/β inhibitor, as well as in low density lipoprotein receptor deficient (*Ldlr*$^{-/-}$) mice with either whole body or myeloid GSK3α deficiency [17,19,20]. Together, these studies suggest that the ER stress-GSK3α/β pathway is involved in the activation of pro-atherosclerotic processes, and therefore may be a target for anti-atherosclerotic therapies. The effect of pharmacological mitigation of ER stress and the inhibition of GSK3α/β on established atherosclerotic lesions have not been investigated. Therefore, in this study we examined the impact of the chemical chaperone 4PBA, and the GSK3α/β inhibitor VPA, on the development, progression and regression of atherosclerosis in *Ldlr*$^{-/-}$ mice.

2. Materials and methods

2.1. Mouse models

All animal experiments were pre-approved by the McMaster University Animal Research Ethics Board. Female *Ldlr*$^{-/-}$ mice (B6.129S7-*Ldlr*^{tm1Her}/J) were purchased from Jackson Labs. Mice were fed a high-fat diet (HFD) containing 21% fat and 0.2% cholesterol, with 42% calories from fat (Harlan Teklad, TD97363) or a standard chow diet containing 18% protein and 5% fat, with 18% calories from fat (Envigo, 2918). Treatment of the chemical chaperone, sodium 4-phenylbutyate (4PBA), consisted of 3.8 g/L drinking water, which corresponds to approximately 1 g 4PBA/kg body weight/day when water intake/mouse is taken into account. Treatment of the GSK3α/β inhibitor, sodium valproate (VPA) consisted of 625 mg VPA/kg diet. All mice had unrestricted access to food and water, and were maintained on a 12-h light/dark cycle. Mice were fasted for 6 h prior to sacrifice. Fasting blood glucose was measured using an UltraMini blood glucose meter (OneTouch). Mice were anesthetized with 3% isoflurane, blood was collected via cardiac puncture and livers and perigonadal fat pads were weighed and flash frozen. Perigonadal fat pads were collected as they are large, easily identified, and readily accessible. The vasculature was flushed with 1× PBS buffer and perfusion fixed with 10% neutral buffer formalin. Hearts and aortas were collected and formalin fixed.

2.2. Plasma PBA and VPA quantification

Sample preparation, liquid chromatographic system and mass spectrometer parameters were as previously described [21]. L-phenylalanine-d8 and L-tryptophan-d5 were used as recovery and internal standard, respectively. Gradient elution was modified to allow for a faster method for targeted quantification. Mobile phase A was 100% acetonitrile (LCMS grade, Sigma Aldrich) and mobile phase B was 100% water (LCMS grade, Sigma Aldrich). Gradient elution started with 95% A for 0.5 min and then linearly decreased to 30% A at 10 min, held at 30% A for 1 min followed by a ramping up to 95% A for 1 min. The columns were then re-equilibrated for 12 min at 95% A prior to subsequent sample analysis.

The acquired mass spectra were calibrated internally using endogenous sodium formate clusters (Bruker Daltonics DataAnalysis 4.0). Peak areas integrated were normalized with both recovery and internal standards.

2.3. Determination of lipid content

Plasma was fractioned using fast performance liquid chromatography with the FRAC-950 FPLC (Amersham Pharmacia Biotech) as previously described [22], and the cholesterol concentration was measured using the Infinity cholesterol reagent (Thermo Scientific). Frozen livers were homogenized and lipids were extracted with chloroform, followed by resuspension in isopropanol. Total plasma and tissue cholesterol and triglyceride levels were determined using the Infinity Reagent (Thermo Scientific).

2.4. Characterization of aortic lesions

Hearts and aortas were embedded in paraffin and 5 μm sections of the aortic root were collected onto slides as previously described [23]. Sections were deparaffinized and stained with Harris hematoxylin and eosin (Sigma) or Masson's Trichrome (Sigma) for atherosclerotic lesion, necrotic core, and lesional collagen quantification. After removing surrounding fat and connective tissue, aortic arch and descending aorta were opened longitudinally and stained with Sudan IV solution.

For immunofluorescent and immunohistochemical staining, sections were deparaffinized and antigen retrieval performed, where necessary. After blocking with 10% normal serum, sections were immunostained with primary antibodies against phospho-GSK3β-Ser9 (Cell Signaling), phospho-GSK3β-Tyr216 (BD transductions), the macrophage marker CD107b (Mac3, BD Transductions), vascular smooth muscle cell (VSMC) marker α-actin (Santa Cruz), and cell proliferation marker Ki67 [SP2] (Abcam). Secondary antibodies (BD transductions) conjugated to a fluorophore were used for detection. Separate sections were stained with pre-immune IgG instead of primary antibodies to control for non-specific staining (Supplemental Fig. 1). The DeadEnd™ Fluorometric TUNEL System kit (Promega) was used according to manufacturer's instructions to measure apoptotic cell death. 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen) was used for immunofluorescent counterstaining. On a separate subset of mice, hearts were imbedded in optical cutting temperature (OCT) compound (Tissue-Tek) and frozen. OCT imbedded frozen tissue was serial sectioned at 10 μm and stained with Oil Red O (Sigma). Images of the stained sections were collected using Leitz LABORLUX S microscope connected to a DP71 Olympus camera. Lesion area and immunofluorescent staining was quantified using Image J 1.48v software. Regions of colocalization were visualized using the Colocalization plug-in by Pierre Bourdoncle (Institut Jacques Monod, Service Imagerie, Paris) (Supplemental Fig. 2).

2.5. Statistical analysis

GraphPad Prism 7 was used to perform all statistical analysis.