**Deoxycholic Acid Is Involved in the Proliferation and Migration of Vascular Smooth Muscle Cells**

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**Summary** Obesity is increasingly becoming associated with increased risk of atherosclerosis. Serum levels of the bile acid deoxycholic acid (DCA) are elevated in mice with obesity induced by a high-fat (HF) diet. Therefore, we investigated the influence of DCA on the functions of vascular smooth muscle cells (VSMCs) because the initiation and progression of atherosclerosis are associated with VSMC proliferation and migration. DCA induced c-Jun N-terminal kinase (JNK) activation whereas a JNK inhibitor prevented DCA-induced VSMC proliferation and migration. Based on these findings, we examined whether DCA promotes the expression of platelet-derived growth factor \(\beta\)-receptor (PDGFR\(\beta\)) that has a c-Jun binding site in its promoter region. The mRNA and protein expression levels of PDGFR\(\beta\) were upregulated in VSMCs after a 24- and 48-h incubation with DCA, respectively. The effects of PDGF such as proliferation and migration of VSMCs were promoted after a 48-h incubation with DCA despite the absence of DCA during PDGF stimulation. These findings suggest that elevated serum concentrations of DCA are involved in the pathogenesis of atherosclerosis in HF-induced obesity.

**Key Words** high-fat diet, enterobacterium, bile acid, atherosclerosis

Atherosclerosis remains a major health problem throughout the western world, particularly in moderately to severely obese individuals because current therapeutic regimens prevent only 25% of all cardiovascular events (1). Obesity is an important established risk factor for the development of atherosclerosis and subsequent cardiovascular disease (CVD) (2). One of the main factors that contribute to the development of obesity is the consumption of high-fat (HF) diets that enhance bile secretion to facilitate lipid digestion (3). The gut microbiota in humans modifies the steroid nucleus of bile acids during transit to the large intestine to yield secondary bile acids (4). The most prevalent of these is deoxycholic acid (DCA) that is generated from cholic acid (CA), which is the most abundant bile acid in biliary bile (5). High-fat diets alter the gut microbiota of obese experimental animals, thus increasing serum DCA levels (6). Plasma and serum concentrations of DCA are increased in patients with insulin resistance (IR) and chronic renal failure (CRF), respectively (7, 8) and the risk of developing atherosclerosis is greater for these patients than healthy individuals. Furthermore, CVD is the leading cause of mortality in such patients (9, 10).

The initiation and progression of atherosclerosis are associated with the proliferation and migration of vascular smooth muscle cells (VSMCs), and with dysfunctional endothelial cells (ECs) (11). Although DCA directly promotes the adhesion of THP-1 cells, a human monocyctic cell line derived from a patient with acute monocytic leukemia, to human umbilical vein endothelial cells (HUVECs) by inducing the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (12), the functions of DCA in VSMCs remain unclear.

Platelet-derived growth factor (PDGF)-dependent signaling plays a key role in the development of atherosclerosis (11). Among the five isoforms of PDGF, PDGF-BB is involved in the proliferation and migration of VSMCs through binding its specific PDGF \(\beta\)-receptor (PDGFR\(\beta\)). Therefore, the present study aimed to determine the effects of DCA on VSMC proliferation and migration, and on the upregulation of PDGFR\(\beta\) expression in VSMCs. We also investigated whether DCA and PDGF-BB coordinately promote the VSMC functions.

**Materials and Methods**

**Materials and reagents.** Antibodies were obtained from the following suppliers: anti-PDGF-\(\beta\) receptor antibody, Santa Cruz Biotechnology (Santa Cruz, CA); anti-a-tubulin, Calbiochem (La Jolla, CA); anti-phospho-c-Jun N-terminal kinase (JNK), anti-rabbit IgG horse-radish peroxidase (HRP)-linked antibody, anti-mouse 1

**Note**

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IgG, HRP-linked antibodies and PDGF-BB, Cell Signaling Technology (Beverly, MA); the JNK inhibitor SP600125 and Dulbecco’s modified Eagle’s medium (DMEM), Wako Pure Chemical Industries, Ltd. (Osaka, Japan); trypsin-EDTA and fetal bovine serum (FBS), GIBCO (Grand Island, NY); DCA and penicillin/streptomycin, Nacalai Tesque, Inc. (Kyoto, Japan). DCA and SP600125 were dissolved in dimethyl sulfoxide. PDGF-BB was dissolved in distilled deionized water.

Cell culture. We isolated VSMCs from the thoracic aorta of adult Sprague-Dawley rats as described (13). The Institutional Animal Care and Use Committees of the University of Tsukuba approved all experimental protocols (approval number: 12-088). We maintained VSMCs in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. VSMCs were analyzed between passages 8 and 10 after a 48-h incubation in DMEM containing 0.1% FBS.

Immunoblotting. Serum-starved VSMCs (2×10^5 cells/3.5 cm dish) were incubated with or without SP600125 (5 μM) for 30 min, then with or without DCA (5 μM) for 10 min in Fig. 1A and 48 h in Fig. 2C. Proteins from cell lysates were immunoblotted as described (13). In brief, cell lysates were fractionated by SDS-PAGE and proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA). Levels of phospho-JNK and PDGFRβ detected using specific antibodies were normalized to those of α-tubulin. Protein bands were visualized using the enhanced Chemiluminescent system (Nacalai Tesque), and densitometrically analyzed and calculated using Bio-Rad Quantity One software.

Evaluation of cell proliferation. Serum-starved VSMCs (2×10^5 cells/6 cm dish) were incubated with or without SP600125 (5 μM) for 30 min, then with or without DCA (5 μM) for 48 h in Fig. 1B. In Fig. 3A, serum-starved VSMCs (2×10^5 cells/6 cm dish) were incubated with or without DCA (5 μM) for 48 h. After removing DCA, cells were stimulated with or without PDGF-BB (20 ng/mL) for another 48 h. After culture under various conditions, VSMCs were harvested by trypsin digestion, stained with Trypan blue and counted.

Cell migration assay. Cell migration was assayed as described (13). In brief, it was determined using a modified Boyden chamber assay (Corning Costar, Acton, MA). Polyvinylpyrrolidone-free polycarbonate filters with an 8 μm-pore size were coated with fibronectin. In Fig. 1C, serum-starved VSMCs (2×10^5 cells/well) were incubated with or without SP600125 (5 μM) for 30 min. In Fig. 3B, serum-starved VSMCs (2×10^5 cells/well) were incubated with or without DCA (5 μM) for 48 h. Reagent-treated cells added to the upper Boyden chambers. These were inserted into the bottom chamber containing medium and incubated with or without DCA (5 μM) or PDGF-BB (20 ng/mL) for 5 h. Cells that migrated to the bottom surface of the membrane were fixed in methanol, stained with hematoxylin and eosin, and counted in 10 representative fields.

Quantitative real-time PCR. Serum-starved VSMCs (2×10^5 cells/3.5 cm dish) were incubated with or with-
out SP600125 (5 μM) for 30 min, then with or without DCA (5 μM) for 48 h. After removal of DCA, cells were incubated with or without PDGF-BB (20 ng/mL). (A) Cell proliferation assessed after stimulation with PDGF-BB for 48 h. (B) Cells migration assessed after stimulation with PDGF-BB for 5 h. Data are expressed as means±SE of four independent experiments for (A) and (B).

Results

The JNK activation leads to neointimal formation through VSMC proliferation and migration (15, 16). Therefore, we initially investigated whether DCA induces
JNK activation in VSMCs. Figure 1A shows that DCA stimulation activated JNK, and Figs. 1B and 1C show that the JNK inhibitor SP600125 suppressed the DCA-induced proliferation and migration of VSMCs. These results indicate that DCA triggered VSMC proliferation and migration through JNK activation.

Because JNK activation induces PDGFR expression in hepatic stellate cells (17), we postulated that DCA would also promote PDGFRβ in these cells. Figure 2A shows that DCA time-dependently increased the amount of PDGFRβ mRNA expression in VSMCs. Figures 2B and 2C show that SP600125 partially suppressed the mRNA and protein expression of PDGFRβ induced by DCA, respectively. Thus, DCA evokes PDGFRβ expression via JNK activation in VSMCs.

We investigated the relationship between DCA-induced upregulation of PDGFRβ and the PDGF-BB-elicited proliferation and migration of VSMCs. Figures 3A and 3B show augmented VSMC proliferation and migration after a 48-h incubation with DCA even when the cells were not exposed to DCA during the incubation with PDGF-BB. These results suggest that DCA promotes PDGF-induced VSMC proliferation and migration through the upregulation of PDGFRβ expression promoted by JNK.

Discussion

The novel findings of the present study are that DCA elicited VSMC proliferation and migration through JNK activation and that DCA accelerates PDGF-BB-induced VSMC proliferation and migration via the upregulation of PDGFRβ expression promoted by JNK. Together with a report indicating that DCA evokes EC dysfunction (18), the present findings support the notion that DCA is critically involved in the development of atherosclerosis and that DCA acts in concert with PDGFRβ to stimulate the pathogenesis of atherosclerosis.

We found that 5 μM DCA can induce VSMC functions, whereas most previous studies have examined cellular functions using ≥100 μM. Our results suggest that even a very low concentration of DCA can lead to VSMC dysfunction. The effects of low DCA concentrations should also be investigated in other types of cells.

In healthy WKAH rats, the maximum plasma concentration of DCA is about 1 μM (18). However, we consider 5 μM DCA to be a physiological concentration because the plasma concentration of bile acids is about 20 μM in rats with CRF (19) and DCA comprises about 25% of serum bile acids in patients with CRF (8).

Bile acids are ligands for farnesoid X receptors (FXR) that are expressed in VSMCs and prevent neointima formation (20, 21). However, our results suggested that DCA is involved in VSMC proliferation and migration. Although chenodeoxycholic acid (CDCA) is reportedly the best activator of FXR (22), DCA interferes with the ability of CDCA to cause the recruitment of steroid receptor coactivator-1 (SRC-1) to FXR and thus might result in a decline in the activation of some FXR pathways (23). Thus, we speculate that DCA does not activate FXR and that it induces the activation of an unknown signaling pathway through JNK activation for VSMC functions.

The present results showed that DCA promotes PDGFRβ expression in VSMCs. The expression of PDGFRβ might be upregulated in VSMCs during the process of atherosclerosis induced by diabetes or CRF because increased concentrations of serum glucose in diabetic rats and of indoxyl sulfate, a tryptophan metabolite, in CRF rats promote such expression (13, 24). Therefore, analysis of crosstalk between DCA and high glucose or indoxyl sulfate might identify novel upregulatory mechanisms of PDGFRβ expression in atherosclerosis induced by diabetes and CRF.

To study diet-induced atherosclerosis, atherogenic diets are used and CA is supplemented in the diets (25). Because a part of CA is converted to DCA by intestinal bacteria (5), on the basis of the present results, DCA might influence diet-induced atherosclerosis.

We propose that the intestinal microbiota plays a role in these cardiometabolic diseases because DCA generated by enterobacteria induces the initiation and development of atherosclerosis through VSMC proliferation and migration. Older literature supports our notion that germ-free animals are less susceptible to atherosclerotic plaque (26). In addition, prebiotics administered to ApoE−/− mice for 16 wk obviously altered the composition of the gut microbiota and reduced the size of atherosclerotic lesions by 35% (27). Therefore, controlling intestinal microbiota using prebiotics might attenuate the initiation and progression of CVD.

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