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Simvastatin reduces the TLR4-induced inflammatory response in human aortic valve interstitial cells

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ABSTRACT

Background: Calcific aortic stenosis is a chronic inflammatory disease. Proinflammatory stimulation via toll-like receptor 4 (TLR4) causes the aortic valve interstitial cell (AVIC) to undergo phenotypic change. The AVIC first assumes an inflammatory phenotype characterized by the production of inflammatory mediators such as intercellular adhesion molecule-1 (ICAM-1), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1). This change has been linked with an osteogenic phenotypic response. Statins have recently been shown to have anti-inflammatory properties. We therefore hypothesized that statins may have an anti-inflammatory effect on human AVICs by down-regulation of TLR4-stimulated inflammatory responses. Our purposes were (1) to determine the effect of simvastatin on TLR4-induced expression of inflammatory mediators in human AVICs and (2) to determine the mechanism(s) through which simvastatin exert this effect.
Materials and methods: Human AVICs were isolated from the explanted hearts of four patients undergoing cardiac transplantation. Cells were treated with simvastatin (50 μM) for 1 h before stimulation with TLR4 agonist lipopolysaccharide (LPS, 0.2 μg/mL). Immunoblotting (IB) was used to analyze cell lysates for ICAM-1 expression, and enzyme-linked immunosorbent assay was used to detect IL-8 and MCP-1 in cell culture media. Likewise, lysates were analyzed for TLR4 and nuclear factor-kappa B activation (IB). After simvastatin treatment, lysates were analyzed for TLR4 levels (IB). Statistics were by analysis of variance ($P < 0.05$).
Results: Simvastatin reduced TLR4-induced ICAM-1, IL-8, and MCP-1 expression in AVICs. Simvastatin down-regulated TLR4 levels and suppressed TLR4-induced phosphorylation of nuclear factor-kappa B.
Conclusions: These data demonstrate the potential of a medical therapy (simvastatin) to impact the pathogenesis of aortic stenosis.

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Introduction

Calcific aortic stenosis is diagnosed in at least 2% of the US population over the age of 75 y.[1] It is the third most common cardiovascular disease, behind only coronary artery disease and hypertension, and it is the most common indication for aortic valve replacement.[2] Heretofore, no pharmacologic therapy has been identified for the prevention or treatment of aortic stenosis.

The pathogenesis of calcific aortic stenosis is not well defined. Although traditionally considered a degenerative disease in which calcium passively accumulates on the aortic valve leaflets, recent data suggest that aortic stenosis may be a chronic inflammatory disease. The aortic valve interstitial cell (AVIC) has been implicated in the pathogenesis of aortic stenosis.[3,4] In response to toll-like receptor 4 (TLR4) stimulation, AVICs adopt an inflammatory phenotype, characterized by the production of inflammatory proteins like intercellular adhesion molecule-1 (ICAM-1), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1).[5,6] This inflammatory phenotypic change is linked to an osteogenic phenotypic change, which is thought to be involved in valve calcification.[5,7] Prior studies have yet to identify a pharmacologic therapy that could interrupt these pathologic changes. Such a discovery could have an impact on the prevention or treatment of aortic stenosis.

Commonly used for the treatment of hypercholesterolemia, statins have been shown to possess important anti-inflammatory properties.[8] Given the role of inflammation in the pathogenesis of aortic stenosis, other investigators have explored a possible therapeutic role for statins in aortic stenosis with mixed results. While some clinical investigators have demonstrated no effect of statin therapy on the progression of aortic stenosis,[9,10] others have suggested a benefit.[11] However, at the cellular level, some recent data suggest that statins may reverse procalcific pathways in human AVICs.[12,13] Furthermore, simvastatin has been shown to inhibit calcium nodule formation in porcine AVICs.[14] These data led us to hypothesize that statins may have anti-inflammatory actions in human AVICs. Given the important role of TLR4 in mediating proinflammatory responses in AVICs, we further hypothesized that the anti-inflammatory effects of statins are mediated via the downregulation of TLR4-stimulated responses in human AVICs.

The purposes of this study were to (1) evaluate the effect of simvastatin therapy on TLR4-induced inflammatory protein expression in human AVICs, and (2) determine the mechanism(s) through which this effect is mediated. The results of this study demonstrate that simvastatin can reduce TLR4-induced inflammatory responses in human AVICs, and this effect may be mediated through both downregulation of total TLR4 expression and modulation of nuclear factor-kappa B (NF-κB) activation.

Materials and methods

This work was approved by the Colorado Multiple Institutional Review Board at the University of Colorado Health Sciences Center.

Reagents

Earle's balanced salt solution, culture medium 199, penicillin G, streptomycin, and amphotericin B were all purchased from Lonza (Walkersville, MD). Fetal bovine serum (FBS) was obtained from Aleken chemicals (Nash, TX). Laemmli sample buffer, nitrocellulose membranes, and mini-protean gels (4%-20% gradient) were bought from Bio-rad (Hercules, CA). Chemiluminescent substrate and lactate dehydrogenase (LDH) assay kits were purchased from Thermo Scientific (Rockford, IL). Rabbit-derived antibodies against human ICAM-1 and TLR4 were obtained from Santa Cruz (Dallas, TX). Rabbit-derived antibodies against human phospho–NF-κB and total NF-κB, and rabbit-derived antibody against human beta-actin were purchased from Cell Signaling (Danvers, MA). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN). All other reagents, including simvastatin, were purchased from Sigma Aldrich (St. Louis, MO).

Cell isolation and culture

Human AVICs were isolated and cultured as described previously.[5] Preoperative consent was obtained according to institutional review board protocol. At the time of heart transplantation, the recipient's native heart was removed, and the aortic valves were inspected to ensure that no calcium nodules or fibrotic areas were identified. H&E staining is performed on our donors to ensure normal cellular architecture within the valve as described previously.[5] The aortic valve leaflets were carefully excised and placed into a sterile saline container. This container was transported on ice from the operating room to the laboratory. The leaflets were washed five times with Earle's balanced salt solution and then sectioned into small pieces. The pieces were placed into a 15 mL conical tube and digested using collagenase diluted in full strength cell culture medium (medium 199 with penicillin G, streptomycin, amphotericin B, and 10% FBS) to a concentration of 2.5 mg/mL. After 30 min of digestion with gentle agitation at a temperature of 37°C, the mixture was centrifuged at 500 revolutions per minute for 2 min. Supernatant containing the stripped endothelial cells was then discarded. The tissue was again mixed with a lower concentration of collagenase (0.8 mg/mL) for 3 h. After this time, the cells were again centrifuged at 500 revolutions per minute for 2 min. The supernatant was then centrifuged again for 8 min at 1100 revolutions per minute. The pellet was resuspended with culture medium and then placed into a small (25 cm^2) flask along with 5 mL of culture media. This flask was placed into an incubator maintained at 37°C and 5% CO_2. AVIC phenotype was verified using immunofluorescent staining as described previously.[5] Cells are stained for actin and vimentin to identify them as myofibroblasts (the most common phenotype for valve interstitial cells). Baseline expression of inflammatory proteins such as ICAM-1 is almost negligible in isolated human AVICs as demonstrated by immunofluorescence imaging in a prior study.[15,16] In addition, normal human AVICs express low baseline expression of other inflammatory proteins like transforming growth factor beta-1 as previously demonstrated by Western blotting.[17] Cells were grown to passages 2-6 prior to use for experiments.