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Statins protect human endothelial cells from TNF-induced inflammation *via* ERK5 activation

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ABSTRACT

3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) exert pleiotropic effects on the cardiovascular system, in part through a decrease in reactive oxygen species (ROS) formation and reduction of vascular inflammation. To elucidate the molecular mechanisms involved in these effects, we investigated the effect of statins on TNF-α-induced ROS production, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression in human aortic endothelial cells (HAECs). Exposure of HAECs to TNF-α caused production of ROS *via* Rac-1 membrane translocation and activation. The Rac-1 activation and ROS liberation mediated TNF-stimulated NF-κB activation and the subsequent VCAM-1 and ICAM-1 expression. Extracellular-signal-regulated kinase 5 (ERK5) plays a central role in inhibiting endothelial inflammation. Immune complex kinase assay of protein extracts from HAECs treated with atorvastatin revealed increased ERK5 activity in a time- and dose-dependent manner. In addition, pretreatment with atorvastatin inhibited TNF-α-induced ROS production and VCAM-1 and ICAM-1 expression. Chemical or genetic inhibition of ERK5 ablated the statins inhibition of Rac-1 activation, ROS formation, NF-κB, VCAM-1 and ICAM-1 expression induced by TNF-α. Taken together, statins, *via* ERK5 activation, suppress TNF-stimulated Rac-1 activation, ROS generation, NF-κB activation and VCAM-1 and ICAM-1 expression in human ECs, which provides a novel explanation for the pleiotropic effects of statins that benefit the cardiovascular system.

1. Introduction

Inflammation plays a critical role in cardiovascular disease, and the inflammatory cascade is particularly important in the atherosclerotic process. The inflammatory mediator tumor necrosis factor (TNF, also known as TNF-α) has been implicated in the pathogenesis of a number of cardiovascular diseases, including atherosclerosis, myocardial infarction, heart failure, myocarditis and cardiac allograft rejection [1]. In response to TNF, vascular endothelial cells promote inflammation changes, which increase leukocyte adhesion, transendothelial migration and vascular leak and promote thrombosis, by displaying, in a distinct temporal, spatial and anatomical pattern [2–4], different combinations of adhesion molecules for leukocytes, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin [5,6]. In addition, TNF is vital for the ICAM-1-dependent recruitment of mononuclear cells and microvascular damage [7]. The central role of TNF in inflammation has also been demonstrated by the ability of TNF blocker to treat a range of cardiovascular disorders and inflammatory conditions, including acute myocardial infarction (AMI), heart failure, rheumatoid arthritis, diabetes and hyperlipidaemia [1,8]. Despite the important pathological role of TNF in cardiovascular diseases, the exact mechanisms underlying TNF-induced vascular inflammation and dysfunction remain unresolved and there is no current scientific consensus.

Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins) are potent inhibitors of cholesterol biosynthesis widely used to reduce serum cholesterol levels in hyperlipidemic patients [9]. In addition to lowering lipids, these drugs exhibit potent anti-inflammatory effects mediated by inhibition of macrophage function. Statins are the most effective agents available today for the reduction of vascular inflammation. However, the mechanisms by which statins may exert beneficial

Abbreviations: AMI, acute myocardial infarction; DCFDA, dihydrodichlorofluorescin diacetate; ECs, endothelial cells; ERK5, extracellular-signal-regulated kinase 5; HAECs, human aortic endothelial cells; HMG CoA, hydroxy-3-methylglutaryl coenzyme A; ICAM-1, intercellular adhesion molecule-1; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PMCA4, plasma membrane calcium ATPase 4; ROS, reactive oxygen species; TNF, tumour necrosis factor; VCAM-1, vascular cell adhesion molecule-1.

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anti-inflammatory effects independent of lipid-lowering have not been completely identified.

Extracellular-signal-regulated kinase 5 (ERK5) is the newest member of the mitogen-activated protein kinase (MAPK) family. Similar to other MAPK family members, ERK5 plays a significant role in cell growth and differentiation. Nevertheless, emerging evidence suggests ERK5's unique functional characteristics [10]. Recent studies have revealed distinctive features of the ERK5 pathway: ERK5 is a key factor to inhibit endothelial inflammation [10] and has a key role in cardiovascular development [11]. Intriguingly, ERK5 is strongly activated by steady laminar flow (s-flow) that generates a frictional dragging force on the endothelium surface (called fluid shear stress), which is known to possess anti-inflammatory and antiatherosclerotic effects and to protect endothelial cells (ECs) from becoming dysfunctional [12,13]. However, whether pharmaceutical stimulated ERK5 activation has a similar anti-inflammatory vasoprotective effect is not clear.

The accumulation of data implicating ERK5 as a key factor to inhibit endothelial inflammation prompted us to analyze the effect of statins on ERK5 activation and cross-talk between ERK5 and TNF inflammatory cascade in human endothelial cells. We found that TNF significantly increases NF-κB activity and VCAM-1 and ICAM-1 expression *via* Rac-1 activation and ROS generation. We provide evidence that statins can directly activate ERK5 and potently block TNF-induced Rac-1/ROS/NF-κB/VCAM-1 inflammation pathway. Therefore, the ERK5 activation and subsequent inhibition of TNF inflammation pathway in endothelial cells mediate statins-elicited anti-inflammatory vasoprotective effect.

2. Materials and method

2.1. Materials

Antibodies used in the present study and their commercial sources were as follows: anti-Rac-1, anti-IκB and anti-ERK5 (EMD Millipore Corporation, Billerica, MA, USA); anti-PMCA4 (plasma membrane calcium ATPase 4) (Sigma–Aldrich, St. Louis, MO, USA); anti-VCAM-1, ICAM-1 (OriGene Technologies, Rockville, MD, USA). Rac-1 inhibitor was purchased from Calbiochem (San Diego, CA, USA); PEG-catalase were purchased from Sigma; BAY 11-7085 and XMD 8-92 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); human recombinant tumor necrosis factor-α (TNF) were obtained from R&D Systems (Minneapolis, MN, USA) and used at a concentration of 100 U/ml. Atorvastatin was from Toronto Research Chemicals, Inc (North York, Canada). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless stated otherwise. Rac1 activation was measured by GTP-Rac1 pull-down assays using a Rac1 Activation Assay Kit from Upstate Biotechnology Inc. (Lake Placid, NY, USA).

2.2. Cell culture

Human aortic endothelial cells (HAEC) were growth in EGM-2 (Lonza Walkersville, MD, USA) at 37 °C in an atmosphere of 95% air–5% CO_2. HAEC from passages 2–5 were cultured for 0–12 h in presence of 1 μM atorvastatin with or without 100 U/ml TNF, 50 μM Rac-1 inhibitor, 500 U/ml PEG-catalase, 5 μM BAY 11-7085 or 5 μM XMD 8-92. The cells were harvested at the time points indicated.

2.3. Small interfering RNA (siRNA)

A commercial siRNA to human ERK5 was obtained from Ambion (Austin, TX, USA). A nonspecific control siRNA from Invitrogen was used as a negative control. siRNA was transiently transfected using the Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA, USA) following the protocols provided by the manufacturer. The cells were harvested 36 h after siRNA transfection, and protein expression was measured using immunoblotting with antibodies against ERK5 (Cell Signaling Technology, Beverly, MA, USA) or actin (Sigma–Aldrich, St. Louis, MO, USA).

2.4. Western blotting

HAEC were lysed with a buffer that contained 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X 100, 10% glycerol, 10 μg/ml leupeptin, 60 μg/ml aprotinin, and 1 mmol/L phenylmethanesulfonyl fluoride. EC lysates were resolved on SDS-PAGE according to standard protocols. After being transferred to membranes, the samples were immunoblotted with primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase. Bands were revealed by use of an enzyme-linked chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ, USA), and density was quantified by use of Scion Image software (Scion Corp, Frederick, MD, USA).

2.5. Membrane preparation

The plasma membrane preparation was carried out as described previously [14]. In brief, HAEC were washed in ice-cold phosphate-buffered saline three times, drained, and incubated with 1 ml of ice-cold hypotonic lysis buffer (10 mM Tris–HCl at pH 7.4, 1.5 mM $MgCl_2$, 5 mM potassium chloride, 1 mM dithiothreitol, 1.0 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 5 min. Cells were scraped and homogenized with 15 strokes of a Dounce homogenizer. Homogenates were centrifuged at 700 × *g* for 3 min to pellet nuclei and intact cells. The supernatants were spun at 100,000 × *g* for 30 min at 4 °C to sediment particulates before being removed. The crude membrane was gently washed with a hypotonic lysis buffer. Membrane-bound Rac-1 was measured by western analysis using antibodies specific to Rac-1. The plasma membrane calcium ATPase (PMCA4) was used as a plasma membrane loading control [15].

2.6. Measurement of reactive oxygen species

To detect the generation of intracellular reactive oxygen species (ROS), the ROS-sensitive fluorescent indicator 2′,7′-dihydrodichlorofluorescin diacetate (DCFDA, Molecular Probes, Eugene, OR, USA) was used in HAEC as described previously [16]. Confluent ECs in 96-well plates were preincubated with the fluorescence probe DCFDA (10 μM) for 30 min. After removal of medium from wells, cells were washed three times in PBS, followed by measurement of fluorescence intensity at 485-nm excitation and 538-nm emission spectra with a fluorescence microplate reader. The ROS formation in mouse aorta was determined as described previously [17]. Data are presented as the fold increase in DCF fluorescence compared with that in unstimulated cells.

2.7. ERK5 kinase assay

Endogenous ERK5 kinase assays were performed as described previously with minor modifications [18]. After treatment, the cells were washed once in ice-cold HEPES-buffered saline, scraped in a lysis buffer consisting of 20 mM Tris–Cl (pH 7.5), 5 mM EGTA, 25 mM β-glycerophosphate, 1% Triton X-100, 2 mM dithiothreitol, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, and 1% aprotinin (400 μl of buffer/100-mm dish),