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Short communication

Attenuation of TNF-induced neutrophil adhesion by simvastatin is associated with the inhibition of Rho-GTPase activity, p50 activity and morphological changes

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ABSTRACT

Neutrophil adhesion to the vasculature in response to potent inflammatory stimuli, such as TNF-α (TNF), can contribute to atheroprogression amongst other pathophysiological mechanisms. Previous studies have shown that simvastatin, a statin with known pleiotropic anti-inflammatory properties, can partially abrogate the effects of TNF-induced neutrophil adhesion, in association with the modulation of β_2-integrin expression. We aimed to further characterize the effects of this statin on neutrophil and leukocyte adhesive mechanisms *in vitro* and *in vivo*. A microfluidic assay confirmed the ability of simvastatin to inhibit TNF-induced human neutrophil adhesion to fibronectin ligand under conditions of shear stress, while intravital imaging microscopy demonstrated an abrogation of leukocyte recruitment by simvastatin in the microvasculature of mice that had received a TNF stimulus. This inhibition of neutrophil adhesion was accompanied by the inhibition of TNF-induced RhoA activity in human neutrophils, and alterations in cell morphology and β_2-integrin activity. Additionally, TNF augmented the activity of the p50 NFκB subunit in human neutrophils and TNF-induced neutrophil adhesion and β_2-integrin activity could be abolished using pharmacological inhibitors of NFκB translocation, BAY11-7082 and SC514. Accordingly, the TNF-induced elevation of neutrophil p50 activity was abolished by simvastatin. In conclusion, our data provide further evidence of the ability of simvastatin to inhibit neutrophil adhesive interactions in response to inflammatory stimuli, both *in vivo* and *in vitro*. Simvastatin appears to inhibit neutrophil adhesion by interfering in TNF-induced cytoskeletal rearrangements, in association with the inhibition of Rho A activity, NFκB translocation and, consequently, β_2-integrin activity.

1. Introduction

As the most abundant leukocytes in the peripheral blood, neutrophils play a key role in the host defense against infection and are often the first leukocytes to be recruited to sites of inflammation, where they employ multiple mechanisms to eliminate pathogens, including degranulation, phagocytosis, reactive oxygen species generation and the release of neutrophil extracellular traps [1]. However, if inflammatory mechanisms are not resolved, the continued recruitment of activated leukocytes to the vascular wall, *via* selectin and integrin mediated ligand–binding interactions, can induce endothelial activation and inflammatory molecule release, culminating in the chronic inflammation and tissue damage that is characteristic of a number of inflammatory diseases [2].

Neutrophil adhesion to the vasculature in response to potent inflammatory stimuli, such as TNF-α (TNF), can contribute to atheroprogression [3] and trigger vaso-occlusive processes in sickle cell disease [4], amongst other pathophysiological mechanisms. TNF can prime neutrophils, enhancing their oxidative burst in response to stimuli, increasing surface β_2-integrin expression [5,6], where the Src kinases, PI3K/Akt, p38 MAPK and ERK 1/2, are reported to mediate Mac-1 (CD11b/CD18) integrin up-regulation [7]. Neutrophil leukocyte

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recruitment and chemotaxis is regulated by various intracellular molecules and cytoskeletal components, including the small Rho GTPases, where the small GTPases, Rac, Cdc42 and RhoA, coordinate polarization of the cell's leading edge and uropod formation during cell migration [8]. Recent data indicate a role for RhoA GTPase-mediated signaling and actin-filament rearrangement in TNF-induced neutrophil adhesive mechanisms [9–11] and cell surface TNF receptors can also mediate the effects of TNF *via* the nuclear factor κB (NFκB) transcription factor [12,13] in neutrophils and other cell types.

Statins, a class of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors that are generally used to treat hyperlipidemia, are known to have pleiotropic anti-inflammatory properties, altering endothelial cell activity and adhesion molecule expression, and also leukocyte β2 integrin expression and cytokine production [9,14–16], amongst other effects, although concentrations of statins employed to achieve such anti-inflammatory effects differ depending on the assay system and target cell. Indeed, use of statins has demonstrated beneficial anti-inflammatory effects in patients with conditions associated with inflammatory processes, such as rheumatoid arthritis, acute ischemic stroke and sickle cell disease [17–19].

Our previous studies have shown that simvastatin is able to partially abrogate the effects of TNF-induced neutrophil adhesion under static conditions, *in vitro* [9]. Interestingly, inhibition of the Rho GTPase effector, Rho kinase (ROCK), reversed the effects of simvastatin on neutrophil adhesion and β2-integrin expression, suggesting that simvastatin may modulate neutrophil Rho GTPase-dependent signaling pathways [9]. We, herein, aim to further characterize the effects of simvastatin on neutrophil and leukocyte adhesive mechanisms *in vitro* and *in vivo* using microfluidic and intravital imaging and investigate the effects of this statin on the activities of RhoA and NFκB transcription factor and on cell morphology.

2. Material and methods

2.1. Reagents

Recombinant human and murine TNF-α were purchased from R&D Systems (Minneapolis, USA). The TransAM™ NF-κB p50 assay was purchased from Active Motif (Carlsbad, CA, USA), and the G-LISA® RhoA Activation Assay Biochem Kit™ was from Cytoskeleton, Inc. (Denver, CO, EUA). BAY11-7082 ((E)3-[(4-Methylphenyl)sulfonyl]-2-propenenitrile) and SC514 ((thienothienyl)amino-acetamide) were from Calbiochem (La Jolla, CA, USA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA), unless stated.

2.2. Animals and human subjects

Male C57BL/6 mice, obtained from the animal facility of the University of Campinas (SP, Brazil), were used in the study. Mice were maintained under controlled humidity and temperature and were exposed to 12 h light-dark cycles. Animals were fed on an irradiated 22% protein diet (NUVILAB - CR1) and water *ad libitum*. All procedures using animals were carried out in accordance with the 'Principles of Laboratory Animal Care' (http://grants.nih.gov/grants/guide/notice-files/not96-208.html), and following Brazilian laws for the protection of animals; this study was approved by the Commission for Ethics in Animal Experimentation of the University of Campinas (CEUA/Unicamp, protocol 3470-1).

With regard to human samples, peripheral blood from healthy individuals (aged 18–60 years) was collected in heparin and processed within 1 h. Informed written consent was obtained from all participants in the study, which was approved by the Ethics Committee of the University of Campinas, Brazil (CAAE: 10550012.3.0000.5404) and conducted in accordance with national guidelines for human research. None of the study participants had taken anti-inflammatory drugs or statins during the 2 weeks preceding the study.

2.3. Human neutrophil isolation and treatment

Neutrophil suspensions were obtained by centrifuging peripheral blood samples over Ficoll-Paque of densities of 1.077 and 1.119 g/mL. Contaminating erythrocytes were then lysed (155 mM NH_4Cl, 10 mM $KHCO_3$) and cells were washed in phosphate-buffered saline (PBS) before resuspension in RPMI 1640 medium for immediate use in assays. Morphological and viability analyses of isolated neutrophil populations indicated > 97% purity and > 98% viability.

Isolated human neutrophils were first pretreated, or not, with 10 μM simvastatin, 10 μM BAY11-7082 (irreversible inhibitor of phosphorylation of IκBα) or 100 μM SC514 (IKK-2 Inhibitor) for 15 min (37 °C, 5% CO_2) before co-incubating with human TNF-α (TNF; 200 ng/mL, 30 min, 37 °C, 5% CO_2; the stimulating TNF concentration used was based on previous reports [6,9,20]). The effects of all vehicles used for compound solubilization were also tested and demonstrated no significant effects on the parameters assayed (data not shown). MTT viability assays demonstrated that none of the agents used, or their vehicles, affected neutrophil viability (data not shown).

2.4. Microfluidic adhesion assays

Neutrophil adhesion under a physiologically-relevant shear rate was determined using the Venaflux microfluidic system (Cellix, Dublin, Ireland), employing a Mirus nanopump 2.0 Evo. Biochip microchannels (400-μm width) were coated with human fibronectin (FN; 20 μg/mL) for 2 h and blocked with 1% (w/v) BSA for 30 min at room temperature to reduce non-specific binding sites. After washing channels with phosphate-buffered saline (PBS), cells (5×10^6 cells/mL in RPMI) were infused over channels at a shear stress of 0.5 dyn/cm^2 (1 min, 37 °C). The number of cells adhered after this time was observed in three fields of 0.09 mm^2 each using an inverted microscope (Carl Zeiss Axiovert 40 CFL, Göttingen, Germany) and camera (DeltaPix Camera, Smorum, Denmark). Adhesion is presented as % (± SEM) adhesion relative to basal (non TNF-treated) neutrophil adhesion, which was considered as 100%.

2.5. Flow cytometry

Human neutrophils in suspension were pretreated with drugs of choice and then ressuspended in PBS (1×10^6 cells). For detection of activation-specific epitopes on the CD11b molecule, cells were incubated with PE-conjugated mouse anti-human CD11b antibody (clone CBRM1/5; eBioscience-ThermoFischer Scientific, San Jose, USA) before analyzing at 488 nm on a FACScalibur (BD Biosciences, San Jose, USA). CellQuest Software was used for acquisition and data are expressed as mean fluorescence intensities (MFI) compared to a negative isotype control.

2.6. RhoA activity assay

RhoA activity was determined in neutrophil suspensions (minimum of 5×10^6 cells resuspended in ice-cold assay lysis buffer with a protease inhibitor provided in the kit) using the G-LISA RhoA Activation Assay, according to the manufacturer's instructions. Fifty micrograms of protein/sample were used in the G-LISA assay (protein was quantified using the Bradford assay [21]). Bound active RhoA was detected in the microplate at 490 nm and quantified using a VersaMax micro plate reader (Molecular Device, Sunnyvale, CA); RhoA activity is expressed as a percentage compared to basal RhoA activity in unstimulated neutrophils.

2.7. Rho kinase (ROCK) activity assay

Neutrophils (minimum of 5×10^6 cells) were resuspended in ice-cold lysis buffer (10 mM EDTA, 100 mM Tris base, 10 mM $Na_4P_2O_7$,