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Evidence for simvastatin anti-inflammatory actions based on quantitative analyses of NETosis and other inflammation/oxidation markers

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ABSTRACT

Simvastatin (SMV) has been shown to exhibit promising anti-inflammatory properties alongside its classic cholesterol lowering action. We tested these emerging effects in a major thermal injury mouse model (3rd degree scald, ~20% TBSA) with previously documented, inflammation-mediated intestinal defects. Neutrophil extracellular traps (NETs) inflammation measurement methods were used alongside classic gut mucosa inflammation and leakiness measurements with exogenous melatonin treatment as a positive control. Our hypothesis is that simvastatin has protective therapeutic effects against early postburn gut mucosa inflammation and leakiness. To test this hypothesis, we compared untreated thermal injury (TI) adult male mice with TI littermates treated with simvastatin (0.2 mg/kg i.p., TI+SMV) immediately following burn injury and two hours before being sacrificed the day after; melatonin-treated (Mel) (1.86 mg/kg i.p., TI+Mel) mice were compared as a positive control. Mice were assessed for the following: (1) tissue oxidation and neutrophil infiltration in terminal ileum mucosa using classic carbonyl, Gr-1, and myeloperoxidase immunohistochemical or biochemical assays, (2) NETosis in terminal ileum and colon mucosa homogenates and peritoneal and fluid blood samples utilizing flow cytometric analyses of the surrogate NETosis biomarkers, picogreen and Gr-1, and (3) transepithelial gut leakiness as measured in terminal ileum and colon with FITC-dextran and transepithelial electrical resistance (TEER). Our results reveal that simvastatin and melatonin exhibit consistently comparable therapeutic protective effects against the following: (1) gut mucosa oxidative stress as revealed in the terminal ileum by markers of protein carbonylation as well as myeloperoxidase (MPO) and Gr-1 infiltration, (2) NETosis as revealed in the gut milieu, peritoneal lavage and plasma utilizing picogreen and Gr-1 flow cytometry and microscopy, and (3) transepithelial gut leakiness as assessed in the ileum and colon by FITC-dextran leakiness and TEER. Thus, simvastatin exhibits strong acute anti-inflammatory actions associated with marked decreases in gut tissue and systemic NETosis and decreased gut mucosa leakiness.

1. Introduction

Accumulating evidence supports the emerging anti-inflammatory actions of simvastatin and melatonin alongside their classic roles in the treatment of hyperlipidemia and jetlag, respectively [1–12]. Recently, we have reported that melatonin suppresses neutrophil-mediated major postburn gut inflammation [1]. The similarity of our melatonin results to those of simvastatin actions reported in other systems triggered our interest in examining the effect of simvastatin treatment on postburn gut inflammation and leakiness.

The role of neutrophil hyperactivation in major postburn gut barrier pathogenesis has been assessed by a wide range of markers such as granulocyte-1 (Gr-1), myeloperoxidase (MPO), elastase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase proteins ($p47^{phox}$ and $p67^{phox}$), reactive oxygen species, and intracellular calcium, alongside immunological factors such as bactericidal/permeability-increasing protein (BPI), defensins, CD-11b, -11c, and -18, IL-18 and chemokines cytokine-induced neutrophil chemoattractant (CINC) [1,13–21]. Aside from these biomarkers, our search for tools to assess simvastatin's anti-inflammatory actions led us neutrophil extracellular traps (NETs) especially because they constitute a major aspect of neutrophil effector function that also encompasses many of the aforementioned markers [22–32]. Indeed, as neutrophil supramolecular fragments that are able to entrap and kill pathogens, NETs have been shown to

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contain nuclear DNA alongside cytoplasmic granules, proteases (MPO and elastase), and reactive oxygen species associated with neutrophil's oxidative burst.

Unfortunately, the powerful immune defense function of NETs, aimed at combating the spread of pathogens and limiting the spread of potentially harmful neutrophil byproducts, may occur at the cost of collateral tissue damage associated with excessive NETosis especially in cases of hyperinflammation encountered in the major postburn gut mucosa milieu [33–36]. Mechanistically, effects of NETosis may include immunomodulation, effector function, and intercellular signal transduction [34]. As such, NETs appear to activate adaptive immunity by priming T-cells thus resulting in a second wave of inflammation [36]. Such side effects may explain the role of NETs in autoimmune diseases, such as vasculitis, psoriasis, systemic lupus erythematosis (SLE), Felty's syndrome, and gout [24,25,33–36]. This is in addition to immunosuppressed individuals where several components of NETs (DNA, histones, and MPO) also act as autoantigens [34]. Similarly, NETosis has been linked to vascular pathogenesis (thrombosis, atherosclerosis, and preeclampsia) [34].

In recent works, the quantification of NETs has been proposed as diagnostic and prognostic inflammatory markers for sepsis [26,27,29]. NETs are ideal as a marker for inflammation due to their ability to traverse internal barriers and compartments as circulating free DNA (cf-DNA), as well as the rapid kinetics of NETosis [27,29–31]. Indeed, NETs have been found to be comparable and at times even better than classical inflammatory and septic markers, which include total leukocyte count, C-reactive protein (CRP), IL-6, and MPO [27,29].

As such, we hypothesize that NETs are the quintessential choice as a diagnostic marker for postburn gut inflammation that may be tested in multiple body fluids and compartments to assess the anti-inflammatory effects of drugs such as simvastatin. In this investigation, we provide evidence for beneficial effects of simvastatin (0.2 mg/kg) treatment comparable to those of melatonin (1.86 mg/kg) on the postburn inflammatory state of multiple body compartments as well as gut leakiness. These simvastatin and melatonin doses were developed and tested in our lab based on several MSc thesis projects as well as clear anti-inflammatory effects seen in previous published works using mouse models [1,37].

2. Materials and methods

2.1. Thermal injury and treatment protocols

Male BALB/c mice weighing 25–30 g (Harlan laboratories, Indianapolis, IN) were used in this work. All animal handling, housing, feeding, and experimentation were in accordance with Chicago State University's Institutional Animal Care and Use Committee (IACUC) approved protocols and with NIH guidelines and based on our previously published protocols [1,17–21]. All mice were acclimatized in the animal facility for at least one week and were continually maintained under a 12-h light: 12-h dark cycle (LD 12:12) with free access to water and standard mice chow *ad libitum*. Mice were separated into four groups: control (CT), thermal injury (TI), and TI with post treatment of either melatonin (TI+Mel) or simvastatin (TI+SMV). Thermal injury and sacrifice were around Zeitgeber Time (ZT) 4 with ZT 0 being the onset of the light period. Thermal injury was performed as described previously [1,17–21]. Briefly, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, IP, and unresponsiveness to a hind limb pinch) before shaving their dorsum then placing them in a bottomless plastic mold to expose only ~20% of their total body surface area (TBSA) to scalding water (90–95 °C) for 10 s. Mice were immediately blotted dry then resuscitated with 0.5 mL normal saline intraperitoneal injection. Treatment groups received intraperitoneal melatonin or simvastatin (Sigma-Aldrich, St. Louis, MO) at doses of 1.86 mg/kg (TI+Mel) and 0.2 mg/kg (TI+SMV) immediately following injury and around 2 h before being sacrificed based on published doses and protocols [1,10,12].

2.2. Specimen collection and preparation

Peritoneal lavage was induced by 4% thioglycolate (1 mL, IP) injection 2 h before sacrifice [13]. Circulating blood was collected transcardially upon chest opening under deep anesthesia (sodium pentobarbital, 50 mg/kg, IP, and unresponsiveness to a hind limb pinch) followed by immediate fresh collection of peritoneal lavage and dissection of the terminal ileum. Blood and peritoneal lavage samples were collected in heparinized syringes and tubes and set on ice then immediately used for flow cytometry and fluorescent microscopy. The terminal ileum and proximal colon segments were gently flushed with cold sterile phosphate buffered saline (PBS) then cut in 1–3 cm segments that were either immediately used fresh for transepithelial diffusion or electrical resistance, prepared for flow-cytometric and microscopic NETosis analyses, carbonyl slot blotting, MPO colorimetric assay (see below), or preserved in 4% paraformaldehyde fixative at 4 °C for cryostat-sectioning.

2.3. Gr-1 immunohistochemistry

Fixed ileum and colon segments were mounted in Neg-50 embedding solution (Richard-Allan Scientific, Kalamazoo, MI) on a cryostat chuck for cut into 30 μm cross sections (HM 550 Cryostat, Richard-Allan Scientific) and thaw-mounted on Superfrost-Plus slides (Fisher Scientific, Hampton, NH) and stored at −20 °C until use. Gr-1 immunohistochemistry was based on previously published protocols [19]. Briefly, cryostat sections were thawed for 10 min and sequentially passed through the following solutions (pH 7.4 and at room temperature, unless specified): PBS washing buffer (3 × 3 min), blocking solution containing 0.1% normal goat serum and 0.1% Triton-X 100 (1 h), primary antibody (rabbit anti-Gr1, Sigma-Aldrich) diluted in blocking buffer at 1:1000 primary antibody (overnight at 4 °C), PBS washing buffer (3 × 3 min), secondary antibody (goat anti-rabbit IgG (for Gr-1) diluted 1:1500 in blocking buffer (45 min), then PBS washing buffer (3 × 3 min)). Immunostaining was processed for microscopic imaging with an Eclipse TE2000-S Inverted Fluorescent Microscope (Nikon), camera (Cool Snap ES, Photometric, Tucson, AZ), and Metamorph software (Universal Imaging, Downingtown, PA) or EVOS fluorescent digital microscopy.

2.4. Carbonyl slot blotting

Total protein was isolated from freshly homogenized terminal ileum and colon mucosa scrapings processed with Ready Prep Protein Extraction Kit (Bio-Rad, Hercules, CA) and then blotted as 5 μg samples onto a polyvinylidene difluoride (PVDF) membrane using Bio-Dot SF Microfiltration Apparatus (Bio-Rad) in accordance with previously published protocols [38]. The PVDF membrane was processed for chemiluminescence by being sequentially passed through the following solutions (pH 7.4 and at room temperature, unless specified): 100 μg/mL 2.4-dinitrophenylhydrazine (DNPH) in 2 N hydrochloric acid (HCl) (5 min), 2 N HCl washing solution (3 × 5 min), 100% methanol (7 × 5 min), blocking solution that contained 5% non-fat dry milk in Tris-buffered saline (1 h), monoclonal rabbit anti-carbonyl primary antibody diluted 1:25,000 in blocking buffer (overnight at 4 °C), washing solution that contained 1% non-fat dry milk in 0.1% TBS