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The anti-inflammatory effect of LMWF5A and N-acetyl kynurenine on macrophages: Involvement of aryl hydrocarbon receptor in mechanism of action

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ABSTRACT

After a traumatic insult, macrophages can become activated leading to general inflammation at the site of injury. Activated macrophages are partially regulated by the aryl hydrocarbon receptor (AhR) which when activated suppresses inflammation by limiting the secretion of pro-inflammatory cytokines and promoting the over expression of immuno-modulatory mediators. This study aims to determine whether the low molecular weight fraction of 5% human serum albumin (LMWF5A) and N-acetyl kynurenine (NAK), an N-acetyl tryptophan (NAT) breakdown product in LMWF5A, can regulate inflammation by inhibiting macrophage activation through the AhR since kynurenine is a known AhR agonist. Using LCMS, we demonstrate that NAT is non-enzymatically degraded during accelerated aging of LMWF5A with high heat accelerating degradation. More importantly, NAK is a major degradation product found in LMWF5A. THP-1 monocytes were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA) and pre-treated with 2-fold dilutions of LMWF5A or synthetic NAK with or without an AhR antagonist (CH223191) prior to overnight stimulation with lipopolysaccharide (LPS). Treatment with LMWF5A caused a 50–70% decrease in IL-6 release throughout the dilution series. A dose-response inhibition of IL-6 release was observed for NAK with maximal inhibition (50%) seen at the highest NAK concentration. Finally, an AhR antagonist partially blocked the anti-inflammatory effect of LMWF5A while completely blocking the effect of NAK. A similar inhibitory effect was observed for CXCL-10, but the AhR antagonist was not effective suggesting additional mechanisms for CXCL-10 release. These preliminary findings suggest that LMWF5A and NAK partially promote the suppression of activated macrophages via the AhR receptor. Therefore, LMWF5A, which contains NAK, is potentially a useful therapeutic in medical conditions where inflammation is prevalent such as trauma, sepsis, and wound healing.

1. Introduction

Human serum albumin (HSA) is clinically used for the treatment of shock, acute restoration of blood volume, and hypoalbuminemia. In recently completed clinical studies, the low molecular weight fraction of 5% HSA (LMWF5A) has demonstrated efficacy in the treatment of knee osteoarthritis (OA) [1,2]. These improvements in knee OA symptoms include decreases in pain and increases in overall function.

Abbreviations: NAT, N-acetyl tryptophan; KYN, kynurenine; NAK, N-acetyl kynurenine; HSA, human serum albumin; LMWF5A, low molecular weight fraction of 5% albumin; ESI+, electrospray positive ionization; LCMS, liquid chromatography-mass spectrometry; AhR, aryl hydrocarbon receptor

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Recently published findings have documented biochemical and cellular events that assist in elucidating the anti-inflammatory effects of LMWF5A using various in vitro assays. In a cell culture model using bone marrow-derived mesenchymal stem cells, LMWF5A was shown to prime these cells for both mobilization and chondrogenic differentiation [3]. LMWF5A was also shown to significantly decrease TNFα release in LPS-stimulated human peripheral blood mononuclear cells (PBMC) [4]. Finally, the effect of LMWF5A on important mediators of pain and healing in knee OA such as prostaglandins has been suggested [5]. Indeed, LMWF5A has been shown to increase the release of prostaglandins involved in the resolution of inflammation in a PBMC model [6]. All of these anti-inflammatory and pro-healing observations occur far downstream from a possible membrane-initiating effect of LMWF5A. Therefore, the search for potential membrane targets of LMWF5A is warranted.

Various components of LMWF5A possess anti-inflammatory properties that might be relevant to nociception and initiation of healing. For example, the cyclic compound derived from the N-terminus of HSA, aspartate-alanine diketopiperazine (DA-DKP), is found in LMWF5A in micromolar concentrations that are high enough to decrease pro-inflammatory cytokine release from PBMC and T-cell lines following stimulation [7,8]. An excipient added to 5% HSA solutions, N-acetyl tryptophan (NAT), is known to have immuno-modulatory properties via inhibition of neurokinin 1 receptor (NK1R) thereby regulating important pro-inflammatory signals in immune cells [9,10]. NAT degradation products have also been identified in commercial HSA solutions with unknown biological activity [11]. Finally, various non-HSA derived peptides that co-elute with the HSA fraction have been characterized in commercial HSA solutions [12,13]. As a result, it is possible that any combination of known and unknown small molecular weight components of commercial HSA solutions contributes to the anti-inflammatory properties of LMWF5A.

Because it is present in large concentrations (4 mM) in 5% commercial HSA solutions, an obvious active ingredient candidate in LMWF5A is NAT. Besides the already discussed inhibitory effect on NK1R, it is possible that metabolites of NAT also have biological effects. This hypothesis is based on the documented biological activities of metabolites of the amino acid tryptophan. The oxidation of tryptophan is a complex metabolic pathway that results in the production of kynurenine (KYN) and associated biologically active molecules [14]. Additionally, tryptophan metabolites have certain redox properties making them important in aging and neurodegenerative processes [15]. Therefore, the purpose of this study is to characterize LMWF5A using LCMS-MS to identify non-enzymatic breakdown products of NAT. Additionally, the anti-inflammatory effect of LMWF5A and a synthetic NAT breakdown product, N-acetyl kynurenine (NAK), will be assessed using differentiated THP-1 macrophages stimulated with LPS with special emphasis on the involvement of the aryl hydrocarbon receptor (AhR) in the mode of action of LMWF5A.

2. Materials and methods

2.1. Materials

5% human serum albumin (HSA) (Octapharma, Hoboken, NJ) was used for the production of LMWF5A. LCMS solvents were purchased from Fisher Scientific (Pittsburgh, PA). 0.9% (w/v) sodium chloride (10 ml saline injection syringe flush, USP) was obtained from Excelsior Medical (Neptune, NJ, USA). All cell culture reagents were obtained from Thermo Fisher Scientific (Waltham, MA). A 1 mg/ml PMA stock was made in DMSO, and a 1 mg/ml LPS (O55:B5) stock was made in non-supplemented RPMI 1640 media. N-acetyl kynurenine (NAK) was synthesized by IsoSciences (King of Prussia, PA). All other reagents were obtained from Sigma (St. Louis, MO) unless otherwise stated.

2.2. Collection of LMWF5A

LMWF5A was isolated by Ampio Pharmaceuticals, Inc. (Englewood, CO, USA) using a tangential flow filtration (TFF) process with a 5 kDa MWCO PVDF filter membrane (Sartorius Stedim Biotech GmbH, Germany). In accordance with cGMP guidelines, the isolation process involved the removal of the > 5 kDa component (primarily HSA) and the aseptic filling of sterile glass vials with 4.2 ml LMWF5A. Each vial was sealed with a rubber stopper and a proper metal closure. The vials were stored in the dark at ambient temperature.

2.3. LCMS Analysis of LMWF5A

LMWF5A was injected on an Acquity UPLC BEH C18 column (Waters, Milford, MA, USA) connected to an Acquity H-Class liquid chromatography system (Waters, Milford, MA, USA) and Xevo G2S tandem mass spectrometer (Waters, Milford, MA, USA). Starting mobile phase conditions consisted of 99% HPLC-grade water with 0.1% TFA (Solvent A) and 1% acetonitrile with 0.1% TFA (Solvent B) at a flow rate of 0.5 ml/min. The gradient was adjusted to 40% Solvent A and 60% Solvent B during the 25 min run. A 5 min equilibration was included to return to starting conditions. MS survey conditions consisted of capillary (2.5 kV), sampling cone (30 V), source temperature (110 °C), desolvation temperature (500 °C), cone gas (150 L/h), desolvation gas (850 L/h) and collision energy (6 V). Accurate mass determination was accomplished using leucine enkephalin. MS-MS was performed using the same conditions as the LCMS settings above except the collision energy was set at 15 V.

2.4. THP-1 cell differentiation and dosing

All cell cultures were incubated at 37 °C and 5% CO_2. Suspensions of human THP-1 monocytes (ATCC, Manassas, VA) were initially cultured in 75 cm^2 flasks at 2–3 × 10^5/ml in RPMI 1640 media supplemented with 10% fetal calf serum (FCS) and 1% penicillin/ streptomycin (Pen/Strep). Cells were counted with trypan blue, and 1 × 10^5cells were added to each well of a 96-well flat bottom plate. 50 ng/ml (final) phorbol 12-myristate 13 acetate (PMA) was added to all wells.

Dosing solutions consisted of 2 × dilutions of LMWF5A or 200 μM NAK in saline. Since the starting solutions are saline-based, the dosing solutions were diluted 1:1 with RPMI 1640 supplemented with 20% FCS, 2% Pen/Strep, 1% L-glutamine, 1% sodium bicarbonate, 1% sodium pyruvate, and 1% non-essential amino acids (NEAA). After 3 days of differentiation with PMA, the media was aspirated from each well. To triplicate wells, 200 μl of the appropriate dosing solution was added with or without the AhR antagonist (CH223191, final concentration of 10 μM) and incubated for 1 h. 20 μl of 1.1 μg/ml LPS (final = 100 ng/ml) was added to all wells and incubated overnight.

2.5. qPCR Toll-like receptor signaling pathway array

In 25 cm^2 flasks, 3 × 10^5/cm^2 THP-1 cells were plated and differentiated as described above in Section 2.4 scaling up to 10 ml total volume. Flasks were dosed and stimulated scaling up to 8.8 ml total volume (8 ml 2 × diluted LMWF5A + 0.8 ml 1.1 μg/ml LPS). After the overnight incubation, media was aspirated from all flasks. RNA was isolated from each flask using RNeasy Plus Mini Kit and QIAshredder spin columns (Qiagen, Hilden, Germany). 0.5 μg of total RNA was then reverse transcribed into cDNA with QuantiTect kit (Qiagen, Hilden, Germany). 4.25 ng cDNA was added to each well of a 96-well RT^2 Profiler PCR Array for the Human Toll-Like Receptor Signaling Pathway (Qiagen, Hilden, Germany). Real time (RT) PCR was performed using a 480 Lightcycler (Roche Diagnostics, Indianapolis, IN). Relative gene expression was calculated using the comparative threshold cycle ($\Delta\Delta C_T$) method versus LPS only with normalization to internal controls (actin, β − 2-microglobulin, and GAPDH).