Nrf2 pathway modulates Substance P induced human mast cell activation and degranulation in the hair follicle

Capsule summary: Activation of Nrf2 in human mast cells exposed to Substance P induced oxidative stress suppresses pro-inflammatory gene transcription, activation and degranulation.

Key words: mast cells, Nrf2, oxidative stress, hair follicle, Substance P

Abbreviations: Nrf2 (nuclear factor (erythroid-derived 2)-like-2); SFN (Sulforaphane); MC (mast cell); CTS (connective tissue sheath); HO-1 (heme-oxygenase 1); NQO1 (NADP(H) dehydrogenase, quinone 1); PGD_2 (Prostaglandin D2); PGE_2 (Prostaglandin E2); SP (Substance-P); PBDMCs (Peripheral blood derived mast cells); HF (hair follicle); ROS (reactive oxygen species).

To the Editor:

Mast cells (MCs), are immune cells distributed throughout various tissues that respond to allergic and inflammatory reactions[1] . The neuropeptide Substance P (SP) is a stimulus that generates damaging reactive oxygen species (ROS) via activation of neurokinin-1 (NRK-1) and G-protein couple receptor MRGPRX2 and causes MC degranulation[2,3]. This SP-induced MC degranulation can result in the excessive accumulation of ROS causing inhibition of hair growth[3(S1-S3)].

Importantly, IgE-induced MCs degranulation in the RBL-2H3 cell line can be suppressed by activation of the Nrf2 (nuclear factor (erythroid-derived 2)-like 2) target gene, heme-oxygenase 1 (HO-1)[4], therefore we were interested in the role of Nrf2 in the SP-mediated activation of primary human MCs *in situ* and *in vitro*. Nrf2 controls cellular responses to oxidative stress by transcriptional regulation of antioxidant genes[(S4)]. The connective tissue sheath (CTS) of human HFs contains MCs, which allows investigation of human MC phenotype and activities under physiologically relevant conditions *ex vivo*[5(S5)]. We examined whether the Nrf2 transcriptional pathway can be activated within human MCs and whether this modulates SP-induced MC degranulation and secretory activity *in vitro* and *in situ.*

Nrf2/Mast Cell Tryptase (MCT) double immuno-staining was performed on human scalp HF sections. Intramesenchymal Nrf2 protein expression in the CTS was low (Figure 1A) and treatment with SFN did not increase Nrf2/MCT double+ cell number (Figure 1B). Recently we demonstrated Nrf2-mediated upregulation of HO-1 in human HF CTS by the Nrf2 activator, Sulforaphane (SFN)[6]. Therefore, isolated human HFs were pre-treated with SFN for 24 hrs followed by SP stimulation for 4 hrs to activate native CTS MCs *ex vivo*. This significantly increased the number of HO-1/MCT double-positive MCs in the CTS (Figure 1C-D). Importantly, stimulation with SP did not increase the number of MCs, as it has been shown in previous report[(S1)].

Next, Nrf2 activation by SFN, in primary human peripheral blood-derived cultured MCs (PBDMCs) was investigated. An increase in Nrf2 phosphorylation is observed following activation and is associated with increased nuclear translocation and transcriptional

activity[S4]. Using immunohistochemistry, Nrf2 displayed both cytoplasmic and nuclear localisations under basal conditions, whilst SFN treatment increasing nuclear Nrf2 accumulation (Figure 1E-F). However, in LAD2 Nrf2 localisation was only cytoplasmic in vehicle and SFN treated conditions (Supplementary Figure 1).

We asked whether the expression of Nrf2 target genes and pro-inflammatory MC genes is modulated by SFN 24 hrs or SP 6 hrs in PBDMCs. A significant increase in the mRNA expression of the Nrf2 target genes HO-1 and NADP(H) dehydrogenase-quinone 1 (NQO1) (Figure 1G-H) was observed following SFN treatment. SP induced significant mRNA transcription of the pro-inflammatory cytokine IL-1β (Figure 1I), followed by reduction prior SFN treatment. However, we did not observe the same transcriptional effect of the MC activation marker CD69[S8] or TNF-α (Supplementary Figure 2A-B). In LAD2 similar effect was detected (Supplementary Figure 3).

Furthermore, we investigated whether Nrf2 activation could modulate SP-induced MC degranulation. In LAD2 cells, SP triggered high surface expression of MC degranulation markers CD107a and CD63 by nearly 70%, while SFN alone or prior SP 1 had no effect (Supplementary Figures 4A-B). We also measured well-established β-hexominidase release, yet no effect was detected (Supplementary Figure 4C). Therefore, PBDMCs were investigated next and while their surface expression of CD63 and CD107a after SP stimulation was approximately 30%, pre-treatment of PBMCs with SFN prior SP treatment significantly reduced SP-stimulated of both markers (Figure 2A-C). Furthermore, SP induced β-hexominidase release, whilst pre-treatment with SFN reduced β-hexominidase levels (Figure 2D). Pre-treatment with SFN for 24 hrs did not show this protective effect (Supplementary Figure 5A-B). Importantly, the inhibitory effect of SFN varies in intensity between PBDMCs cultures. Previously, Matsushima *et al.* (2009) determined that upregulation of HO-1 by the Nrf2 activator quercetin occurs rapidly, resulting in suppression of degranulation in RBL-2H3 cells. It may well be that the 1 hr stimulation with SFN used in this study stimulates a similarly rapid increase in HO-1 expression and enzymatic activity to suppress SP induced degranulation[4].

MCs are major source of Prostaglandin (PGE_2) and Prostaglandin D2 (PGD_2) production[7] and the release can occur very quickly upon stimulation[S9]. We investigated Nrf2 activation by SFN for 1 hr (Figure 2E-F) or 24 hrs (Supplementary Figure 6A-B) impact on the 1hr SP-induced secretion for these PGs into the PBDMCs supernatant by ELISA. A significant increase in PGD_2 release occurred following SP treatment, whereas pre-treatment with SFN prior to SP reduced this, though this did not reach the level of significance (Figure 2E). PGE_2 release was largely unaffected by either SFN pre-treatment or SP exposure (Figure 2F). However, we found that SFN and SP treatment does not disturb the levels of prostaglandin D2 synthase (PTGDs) and prostaglandin E2 synthase (PTGEs) at mRNA levels (Supplementary Figure 6C-D).

Finally, we asked whether the SP-induced degranulation of native human skin MCs can be suppressed by SFN *ex vivo*, using toluidine blue histochemistry. Results showed significant SP-stimulated MC degranulation within the CTS, whilst SFN pre-treatment reduced not only

SP-stimulated degranulation but also the SP-induced increase in the total number of toluidine blue$^+$ human skin MCs (Figure 2G-I).

Our results provide the first evidence that human MCs exhibit substantial Nrf2 activity, which can effectively supress MC activation and degranulation induced by the neuropeptide SP. Importantly, PGD_2 levels are increased in balding scalp HFs, whilst PGE_2 levels are decreased [S10-S11], therefore the intrafollicular balance between PGD_2 and PGE_2 has to be controlled for optimal hair growth[8][S12]. Increased MC numbers/degranulation have been reported in androgenic alopecia (AGA)[8], and in the most common inflammatory hair growth disorder, alopecia areata (AA), density, degranulation and proliferation of perifollicular MCs are also increased[9] [S13-14]. The therapeutic targeting of Nrf2 in perifollicular MCs may be of particular interest in the future management of both AGA and AA.

Figure 1: Nrf2 activity within human MCs. (A) Nrf2 and MCT immunofluorescent staining in isolated human HFs. MCT$^+$ cells (green) showed low expression of Nrf2

immunofluorescence (red) with infrequent Nrf2/MCT double positive immunofluorescence. (B) The number of Nrf2/MCT double+ cells did not increase after 2 hr and 4hrs (the graph presents combined 2hrs and 4hrs treatment of SFN) (C-D) Treatment with SFN and SP significantly increased expression of MCT/HO-1 double+ cells. MCT (purple) detected in the CTS and HO-1 (red) activity was found in the HF epithelium and vasculature of CTS. (n=3; data are mean ± SEM; One-Way ANOVA; Significance indicated by *P<0.05;**P<0.01; ***P<0.001). (E-F) Expression of phoshoprylated nuclear Nrf2 (red) in human primary MCs is increased after treatment with SFN (n=3; ±SD). (G) HO-1, (H) NQO1, (I) IL-1β, (J) CD69 in PBDMCs were measured by qRT-PCR and data reported as fold changes in normalized expression (n=3; data are mean ±SEM; RM-One Way ANOVA; Significance indicated by *P<0.05; **P<0.01; ***P<0.001).

Figure 2: Stimulation with SP induces MC degranulation and upregulates secretion of mediators. (A) Gating strategy identifying expression of CD107a and CD63 in PBDMCs. (B-C) Decrease of CD63 and CD107a PBDMCs after pre-treatment with SFN (D) Pre-treatment with SP suppressed SP induced β-hexominidase release (n=7; data are mean ±SEM; paired t-test; Significance indicated by *P<0.05) (E-F) Secretion of lipid-derivatives after treatment with SFN and SP (n=4-5; data are mean ±SEM; One-Way ANOVA; Significance indicated by *P<0.05) (G-I) Toluidine blue staining indicated MCs degranulation and number within CTS of human HFs (n=3; data are mean ±SEM; One-Way ANOVA; Significance indicated by *P<0.05;**P<0.01; ***P<0.001).

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Methods

Human HF collection

Occipital scalp hair follicles (HFs) from male hair transplant surgeries were delivered from the Farjo Medical Centre (Manchester, UK). Male patients that undergone hair transplant surgery were suffering from AGA, but the occipital scalp HFs are androgen-insensitive and not affected by AGA, therefore suitable for the experimental procedures. The tissue was collected after informed patient consent according to the 'Declaration of Helsinki Principles' together with institutional approval ethics from the University of Manchester.

Human HF organ culture

Isolated full-length scalp HFs in anagen VI were cultured in William's E media (Gibco ®, Leicestershire, UK) supplemented with 2 mM of L-Glutamine (Invitrogen, Paisley, UK), 10ng/mL hydrocortisone (Sigma-Aldrich ®, UK), 1 µg penicilin/streptomycin antibiotic mixture (Gibco®, Leicestershire, UK), insulin-transferin-selenium (Life technologies, Paisley, UK) at 37^0C 5 % CO_2 incubator[S15-S16]. Nrf2 activation was performed using 20 µM of SFN (Sigma-Aldrich ®, UK) for 2 hours, 4 hours and 24 hours and 2 µM of SP (Milipore, Nottingham, UK) for 4 hours.

Immunofluorescence microscopy and immunohistochemistry

Double staining of Nrf2/MCT was performed using PerkinElmer TSA plus Kit (PerkinElmer, UK). Cryosections (6 µm) were fixed in 4 % PFA followed by permeabelisation in 0.1 % Triton X. The blocking was performed using 3 % H_2O_2 (Sigma-Aldrich ®, UK) followed by washes with TNT buffer. Additional blocking was done using TNB and primary Nrf2 antibody (Abcam 31163, Cambridge, UK) was left overnight at 4^0C before incubation with HRP goat-anti-rabbit (Life Technologies, Paisley, UK). Additional blocking was done using Bloxall (Vector, Peterborough, UK) before addition of secondary MCT antibody (Abcam 2378, Cambridge, UK).

PBDMCs were dried at room temperature after stimulation with SFN (5 µM) for 2 hrs and stained for Nrf2 phospho-S40 (Abcam76026, Cambridge, UK). Washes were done using TBS + 0.1 % Tween 20. The blocking was performed using normal goat serum (NGS) 10 % and primary antibody was diluted 1:100 followed by overnight incubation. Subsequently, secondary antibody AF594 (goat anti-rabbit) (Life Technologies, Paisley, UK) 1:200 was added for one hour, followed by washes and counterstain with DAPI.

MCT/HO-1 double-immunostaining was performed using Vector Immpress kit (Vector, Peterborough, UK). Slides were blocked using Bloxall followed by incubation with 2.5 % normal horse serum and primary HO-1 antibody (Sigma HPA000635, Sigma-Aldrich ®, UK). Washes were performed using PBS and secondary MCT antibody (Abcam 2378,

Cambridge, UK) was used. Toluidine blue histochemistry (Tol) was performed by fixing slides in acetone, followed by washes in water before staining in Tol blue working solution for 3 minutes. The analysis of immunostaining was performed using a Biozero-8000 microscope (Keyence, Milton Keynes, UK) and staining analysis was quantified by ImageJ software (NIH). Degranulated MCs were identified by quantifying 5 or more extracellular metachromatic granules in the direct vicinity of a clearly identifiable perifollicular MCs[5(S5)].

Primary human peripheral blood-derived cultured MCs (PBDMCs)

Human peripheral blood was obtained from a blood bank in Manchester. Mononuclear cells were obtained and CD117+ progenitor cells were isolated by positive selection of FcRI block/CD117+ (Miltenyi Biotec GmbH, Surrey, UK) by magnetic cell sorting[(S17)]. For the first four weeks cells were cultured in StemSpam medium supplement with 1 % penicilin/streptomycin (Invitrogen, Paisley, UK), 50 ng/ml IL-6 (Peprotech, UK), 10 ng/mL IL-3 (Peprotech, UK), 100 ng/mL Stem Cell factor (Peprotech, UK) and 10 µg/mL of LDL (StemCell Technologies, Cambridge, UK). After four weeks cells were cultured in IMDM medium (Thermofisher, Paisley, UK) supplemented with 50 µM 2b-mercaptoethanol (Sigma-Aldrich ®, UK), 0.5 % BSA (Life Technologies, Paisley, UK), 1 % insulin – transferrin – selenium (Life Technologies, Paisley, UK), 1 % penicilin/steptomycin (Invitrogen, Paisley, UK), 50 ng/mL IL-6 (Peprotech,UK) and 100 ng/mL stem cell factor (rhSCF) (Peprotech, UK). Cell viability and maturity after eight weeks was measured using fluorochrome-conjugated antibodies FcεRI (Biolegend, London, UK) and CD117 (Biolegend, London, UK) by FlowCytometry.

LAD2 cell line culture

Laboratory of Allergic Diseases 2 (LAD2) cells (kindly supplied by the NIAID, USA: Arnold S. Kirshenbaum, and Dean D. Metcalfe) were cultured in StemPro-34 serum-free medium (Invitrogen, Paisley, UK) supplemented with 100 U/mL penicilin/streptomycin (Invitrogen, Paisley, UK), 100 U/mL glutamine (Invitrogen, Paisley, UK) and 100 ng/mL rhSCF (Peprotech, UK)[(S7)].

MC degranulation and activity assay

MC activity and degranulation was measured using flow cytometry. Briefly, LAD2 cells and PBDMCs (1.5×10^5) were seeded in 96-well plate and stimulated with 5 µM SFN (Sigma-Aldrich ®, UK) for 1 hr or 24 hrs followed by stimulation with 5µM SP (Milipore, Nottingham, UK) for 1 hr. Subsequently, cells were resuspended in FACS-buffer (2% newborn calf serum, 0.1% NaN3, 2mM EDTA in PBS) and stained with fluorochrome-conjugated antibodies CD63 and CD69 (Biolegend, London,UK) for 30 min at 4^0C followed by wash with Live/Dead® Fixable Blue dead cell stain kit for UV excitation (LifeTechnologies, Paisley, UK). Samples were analysed with a LSR II or Fortessa (BD Biosciences, Oxford, UK).

RNA isolation and RT-PCR

LAD2 cells or PBDMCs (1.5 x 10^5) were treated with 5 µM SFN for 24 hrs followed by stimulation with 5 µM SP for 6 hrs. Total RNA was extracted with an RNeasy Micro kit (Qiagen, Manchester,UK) according to the manufacturer's instructions. Complimentary DNA (cDNA) was reverse transcribed using Tetro®cDNA synthesis kit (Bioline,UK). Quantitative – PCR was performed using StepOne™ real time PCR system (Applied Biosystems, Warrington,UK) using Taqman® fast advance master mix and probes (Applied Biosystems, Warrington,UK). The following probes from AppliedBiosystems were HMOX1 (Hs01110250_m1), NQO1 (Hs02512143_s1), CD69 (Hs00934033_m1) and most important pro-inflammatory mediator in skin disorders IL-1β (Hs00174097_m1). In addition, PTGDS (Hs00168748_m1) and PTGES (Hs00610420_m1) were used. Samples were run using the StepOne Plus™ Real-Time PCR system and associated software (Applied Biosystems, Warrington UK), relative expression was quantified against the housekeeping gene PPIA (Hs04194521_s1).

MCs mediators release assay

PBDMCs (1 x 10^5) were cultured with 5 µM SFN for 1 hr and 24 hrs followed by stimulation with 5 µM SP for 1 hr. Secretion of PGD_2 and PGE_2 were measured according to the manufacturer's instructions (CaymanChemical, Cambridge,UK).

50 µL (25000 cells) from the LAD2 cell or PBDMC cultures were taken and centrifuged to separate the supernatant and cell pellet. Cell pellets were lysed in 50 µL media culture 1% Triton X-100. β-hexosaminidase was measured in supernatant as well as in the cell pellet by adding 100 µL β-hexosaminidase substrate, 10 mM p-nitrophenyl N-acetyl-beta-D-glucosamine (Sigma-Aldrich) in 0.1M Na2HPO4 buffer (pH4.5) for 2h at 37°C, 5% CO2. The reaction was stopped by adding 100 µL of 0.2M glycine buffer (pH10). Optical density was measured at 405 nm. hMC degranulation was assessed as % release of total β-hexosaminidase.