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Novel anti-inflammatory and chondroprotective effects of the human melanocortin MC1 receptor agonist BMS-470539 dihydrochloride and human melanocortin MC3 receptor agonist PG-990 on lipopolysaccharide activated chondrocytes

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Abstract

Human melanocortin MC1 and MC3 receptors expressed on C-20/A4 chondrocytes exhibit chondroprotective and anti-inflammatory effects when activated by melanocortin peptides. Nearly 9 million people in the UK suffer from osteoarthritis, and bacterial infections play a role in its development. Here, we evaluate the effect of a panel of melanocortin peptides with different selectivity for human melanocortin MC1 (α-MSH, BMS-470539 dihydrochloride) and MC3 receptors ([DTrp^{8}]-γ-MSH, PG-990) and C-terminal peptide α-MSH$_{11-13}$(KPV), on inhibiting LPS-induced chondrocyte death, pro-inflammatory mediators and induction of anti-inflammatory proteins. C-20/A4 chondrocytes were treated with a panel of melanocortin peptides prophylactically and therapeutically in presence of LPS (0.1 μg/ml). The chondroprotective properties of these peptides determined by cell viability assay, RT-PCR, ELISA for detection of changes in inflammatory markers (IL-6, IL-8 and MMP-1, -3 and -13) and western blotting for expression of the anti-inflammatory protein heme-oxygenase-1. C-20/A4 expressed human melanocortin MC1 and MC3 receptors and melanocortin peptides elevated cAMP. LPS stimulation caused a reduction in C-20/A4 viability, attenuated by the human melanocortin MC1 receptor agonist BMS-470539 dihydrochloride, and MC3 receptor agonists PG-990 and [DTrp^{8}]-γ-MSH. Prophylactic and therapeutic regimes of [DTrp^{8}]-γ-MSH significantly inhibited LPS-induced modulation of cartilage-damaging IL-6, IL-8, MMPs -1,-3 and -13 mediators both prophylactically and therapeutically, whilst human melanocortin MC1 and MC3 receptor agonists promoted an increase in HO-1 production. In the presence of LPS, activation of human melanocortin MC1 and MC3 receptors provided potent chondroprotection, upregulation of anti-inflammatory proteins and downregulation of inflammatory and proteolytic mediators involved in cartilage degradation, suggesting a new avenue for osteoarthritis treatment.

Key words: anti-inflammatory; apoptosis; chemokines; chondroprotective; melanocortins; matrix metalloproteinases

1. Introduction

The bacterial endotoxin lipopolysaccharide (LPS) contributes to the development of Osteoarthritis (OA) and intra-articular infections (Mue et al., 2013; Huang et al., 2016) via activation of Toll-Like Receptors. Activation, results in rapid expression of a variety of pro-inflammatory cytokines and cytotoxic mediators, destructive matrix metalloproteinases (MMPs) and reactive oxygen species (ROS) (Ziskoven et al., 2010; Schmal et al., 2016). Chondrocytes directly activated by LPS up-regulate expression of pro-inflammatory cytokines including Interleukin(IL)-6, IL-8 and cartilage destructive proteases including matrix metalloproteinases (MMPs) -1,-3,-13, increasing chondrocyte death (Capsoni et al., 2015) and disrupting chondrocyte biosynthetic abilities compromising cartilage stability (Huang and Kraus, 2016).

Melanocortin receptors are G-Protein-coupled receptors (GPCR's), positively coupled to adenylate cyclase activating the cAMP signalling pathway (Patruno et al., 2018). They display diverse biological functions ranging from modulating inflammatory pathways, skin pigmentation, energy metabolism/food intake and sexual health (Getting 2006; Loram et al., 2015). Over the past two decades, in addition to studies with the parent hormone Adrenocorticotrophic hormone-(ACTH) (Getting et al., 2009) and alpha-melanocyte stimulating hormone-(α-MSH) (Getting et al., 2009; Horrell et al., 2016), several selective peptides have been developed including the human melanocortin MC3 receptor agonist [DTrp^{8}]-γ-MSH (Grieco et al., 2000), human melanocortin MC1 receptor agonist BMS-470539 dihydrochloride (Leoni et al., 2010), enabling a clearer understanding of the role of these receptors in modulating inflammation in arthritis and stroke (Getting et al., 2006; Holloway et al., 2015). The importance of targeting these receptors in inflammatory models, has been demonstrated by using naturally occurring melanocortin MC1 receptor mutants

(recessive yellow e/e mice) (Leoni et al., 2010) and gene deletion of melanocortin MC3 receptor in mice (Getting et al., 2006), demonstrating exacerbation of the host inflammatory response (Patel et al., 2010; Holloway et al., 2015).

Although the role of melanocortin peptides has been established in many models of disease only a few studies highlight the role of melanocortin peptides in OA models providing chondroprotection, inhibition of pro-inflammatory markers and induction of anti-inflammatory proteins. These studies demonstrate that melanocortin peptides repress NF-κB pathways in the human sarcoma cell line-(HTB-94) downregulating transcriptional activation of MMPs (Yoon et al., 2008), whilst, in C-20/A4 chondrocytes, melanocortin peptides provide chondroprotection modulating MMPs, IL-6 and IL-8 (Kaneva et al., 2012). These findings identified in cell-lines and confirmed in primary cells and tissue, with α-MSH inhibiting IL-1β and Tumour necrosis factor alpha-(TNF-α) mRNA levels in human articular chondrocytes (Capsoni et al., 2015), and IL-1β, IL-6 and IL-8 from primary bovine chondrocytes and cartilage (Kaneva et al., 2014). In this study, we determined whether LPS activation caused expression of IL-1β, IL-6, IL-8, MMP1, MMP3 and MMP13 and whether melanocortin peptides increased the anti-apoptotic protein, heme-oxygenase-1-(HO-1) in C-20/A4 chondrocytes. We also determined if targeting human melanocortin MC1 and MC3 receptors using the peptides α-MSH, BMS-470539 dihydrochloride, [DTrp^{8}]-γ-MSH, PG-990 and C-terminal peptide α-MSH$_{11\text{-}13}$(KPV) would modulate the pro-inflammatory and catabolic effects of LPS, prophylactically and therapeutically, whilst inducing the pro-resolving anti-inflammatory protein HO-1, thus demonstrating a role for these peptides in modulating inflammation and promoting the resolution phase in cartilage repair.

2. Materials and methods

2.1. Materials

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium), lipopolysaccharide (*E. coli* serotype 111.60) (LPS) and α-MSH were purchased from Sigma-Aldrich (Poole, Dorset, UK), BMS-470539 dihydrochloride purchased from Tocris Bioscience (Bristol, UK), C-terminal peptide α-MSH_{11-13} (KPV) were purchased from Bachem (UK) Ltd (Saint Helens, UK.), [$DTrp^8$]-γ-MSH and PG-990 synthesised by Prof. P. Grieco (University of Naples, Italy) (Grieco et al., 2000; Carotenuto et al., 2015). Primers *IL6, IL8, MMP1, MMP3* and *MMP13* purchased from Eurofins Genomics. Anti-HO-1 antibody purchased from Enzolifesciences. ELISA kits purchased from R & D Systems (Oxon, UK).

2.2. Cell culture

The human chondrocyte cell-line C-20/A4, derived from juvenile costal chondrocytes, a kind gift of Prof. M.B. Goldring, (Hospital for Special Surgery, New York, USA) (Kaneva et al., 2012) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated Foetal Calf Serum (FCS), 1% (v/v) L-glutamine and Penicillin/Streptomycin (100.0 U/ml and 100.0 µg/ml), incubated at 37ºC in a humidified atmosphere supplied with 5% CO_2 until 80% confluent, as previously described (Kaneva et al., 2012). Prior to experimentation, FCS concentration was reduced to 1% (v/v) (DMEM supplemented with 1% (v/v) FCS, 1% (v/v) L-glutamine and Penicillin/Streptomycin (100.0 U/ml and 100.0 µg/ml)) for 24 h and all subsequent experiments were performed under these conditions using chondrocytes between passages number 4-7 (from frozen stock).

2.3. C-20/A4 chondrocyte stimulation/ treatment

C-20/A4 cells were treated either 30 min prior to (prophylactic regime) or 2 h post (therapeutic regime) 0.1 μg/ml LPS (*E. coli;* 111.60) stimulation (concentration selected from preliminary experiments), Phosphate Buffered Saline (PBS) (control), pan human melanocortin MC1 receptor agonist α-MSH (3.0 μg/ml) (Kaneva et al., 2012), human melanocortin MC3 receptor agonist [DTrp^{8}]-γ-MSH (3.0 μg/ml) (Kaneva et al., 2012), KPV (4.0 μg/ml) (Getting et al., 2009), human melanocortin MC1 receptor agonist BMS-470539 dihydrochloride (10.0 μg/ml) (Leoni et al., 2010), or selective human melanocortin MC3 receptor agonist PG-990 (3.0 μg/ml) (Carotenuto et al., 2015). Receptor nomenclature for melanocortin receptors was used in accordance with the IUPHAR receptor database.

2.4 Molecular analysis

2.4.1. RT-PCR analysis

C-20/A4 chondrocyte RNA extracted and isolated using the NucleoSpin® RNA II Kit (Macherey-Nagel, Duren, Germany) as previously described (Kaneva et al., 2012). Briefly, RNA concentrations were determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer ($A_{260nm/280nm}$: 1.9-2.1). cDNA was synthesised by taking 1.0 μg of DNase-treated total RNA, Oligo(dT), Random Primers and RNase inhibitor in a total volume of 20.0 μl using the Improm II Reverse Transcription System (Promega, UK). 2.0 μl aliquots of the cDNA (in a final volume of 25 μl) were used as a template for PCR amplification using specific primer pairs for *IL6*, *IL8*, *MMP1*, *MMP3 and MMP13* with the GoTaq® Green Mastermix system (Promega, UK). Oligonucleotide primers for human β-actin were used as a control. The oligonucleotide primer sequences used shown in Table 1. The PCR parameters were as follows: initial denaturation for 5 min at 95°C, followed by 25 cycles of denaturation

(95°C for 60 s), annealing (53.7-64.8°C, depending on the primers used for 90 s), extension (72°C for 90 s), with a single final extension of 72°C for 10 min. Amplification products were separated by agarose gel electrophoresis and stained with ethidium bromide. Densitometry analysis was performed using Image J software (NIH, Bethesda, Maryland, USA).

2.4.2. Intracellular cAMP accumulation

Intracellular cAMP in C-20/A4 chondrocytes was determined by Enzyme Immunoassay (RPN225, GE Healthcare, Amersham, UK). 2 x 10^5 chondrocytes·per well were seeded into a 96-well plate as previously described (Getting et al., 2005; Kaneva et al., 2012) and incubated for 24 h at 37ºC in a humidified incubator supplied with 5% CO_2 to allow cell adhesion. Medium was discarded and chondrocytes were treated with the panel of melanocortin peptides, 3.0 μM forskolin (used as a positive control) or PBS (negative control) for 30 min in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, UK). Adhered cells were lysed according to the manufacturer's guidelines and intracellular cAMP concentration was determined (Lam et al., 2005a; Kaneva et al., 2012).

2.4.3. Western blotting

C-20/A4 chondrocytes protein expression of HO-1 was determined as previously described (Lam et al., 2005b). Following electrophoresis in a 10% SDS-polyacrylamide gel, proteins were transferred onto a nitrocellulose membrane, blocked overnight in 5% non-fat milk solution in Tris-HCl buffered saline (TBS) pH 7.5 containing 0.1% (v/v) Tween-20 and then incubated with either rabbit anti-HO-1 antibody (1:2000, Enzolifesciences, UK) or mouse anti-α-Tubulin antibody (1:2000, Sigma-Aldrich, Poole, Dorset, UK) in 5% non-fat milk

solution. Blots were washed in TBS prior to the addition of a secondary goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG antibody (1:2000, DakoCytomation, USA) or goat anti-mouse HRP-conjugated IgG antibody (1:2000, DakoCytomation, USA) and signal was detected by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA). Densitometry analysis performed using Image J software (NIH, Bethesda, Maryland, USA).

2.5. Biochemical and cell viability analysis

2.5.1. Cytokine and MMP quantification by ELISA

Human IL-1β, IL-6, IL-8, MMP-1, MMP-3 and MMP-13 concentrations in C-20/A4 chondrocyte cell-free supernatants following LPS and peptide treatments were determined using commercially available ELISA kits (R&D Systems Europe Ltd, Oxford, UK) according to the manufacturer's instructions.

2.5.2. MTT assay

Cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium (MTT) assay, as previously described (Kaneva et al., 2012). Briefly, C-20/A4 cells were plated at 1.5 x 10^6 cells/well in 24-well plates and allowed to adhere overnight prior to serum reduction. Cells were treated as described in the individual figure legends. Media was aspirated and MTT solution added for 2h, chondrocytes were then incubated at 37°C in a humidified chamber with 5% CO_2. Following incubation with the MTT solution, the supernatant was replaced with DMSO and samples incubated for 15 min, 100 µl was then transferred to a 96 well plate and the absorbance determined at 570 nm.

2.5.3. Caspase-Glo 3/7 apoptosis assay.

Apoptosis was determined by measuring caspase-3 and -7 activity following cell stimulation as detailed above (see section 2.3. C-20/A4 chondrocyte stimulation/ treatment). Briefly, C-20/A4 chondrocytes were plated at 2.0 $x10^3$ cells per well in 96 well plates, and 100 μl of Caspase-GLO 3/7 Reagent (Promega, UK) was added to each well and incubated at room temperature for 1 h, after which luminescence was measured as per manufacturer's instructions.

2.6. Statistics

All data are reported as mean ± S.E.M of *n* observations performed in triplicate unless otherwise stated. Statistical evaluation was performed using analysis of variance ANOVA (Prism GraphPad Software) incorporating either Dunnetts' or Bonferroni's Multiple Comparison tests to allow for post-hoc analyses with a probability *P≤ 0.05; **P≤ 0.01; ***P≤0.001 taken as significant (Figures) and ^{a}P≤ 0.05; ^{b}P≤ 0.01; ^{c}P≤0.001 taken as significant (Tables).

3. RESULTS

3.1 C-20/A4 chondrocytes express functionally active human melanocortin MC1 and MC3 receptors

RT-PCR confirmed endogenous expression of the human melanocortin MC1 and MC3 receptors in C-20/A4 cells (Data not shown). Receptor functionality was determined by cAMP accumulation, direct adenylate cyclase stimulator, Forskolin (3.0 μM) caused a 7.5-fold increase in cAMP over control. A panel of melanocortin peptides were used to determine receptor functionality by measuring cAMP accumulation. α-MSH (3.0 μg/ml) and [DTrp^{8}]-γ-MSH (3.0 μg/ml) caused an elevation in cAMP (Data not shown). Whilst, the highly selective human melanocortin MC1 receptor agonist BMS-470539 dihydrochloride (10.0 μg/ml) caused a 2.0-fold increase and selective human melanocortin MC3 receptor agonist PG-990 (3.0 μg/ml) caused a 3.7-fold increase in intracellular cAMP accumulation (Table 2).

3.2. Melanocortin peptides provide chondroprotection against LPS-induced activation of chondrocytes

Previous studies indicate that melanocortin peptides can modulate TNF-α induced cell death (Kaneva et al., 2012); here we wanted to determine the effect of the melanocortin peptides in the presence of LPS. LPS stimulation caused a 13.0% ($P \leq 0.001$ *vs* untreated control) reduction in cell viability compared to untreated control (Table 3). Prophylactic treatment with α-MSH, [DTrp^{8}]-γ-MSH and KPV significantly protected against LPS-induced cell death by causing a 74.0%, 39.0% and 38.0% ($P \leq 0.001$ *vs* LPS) increase in cell viability,

respectively, compared to LPS stimulation. To evaluate if this effect was via human melanocortin MC1 receptor and/or human melanocortin MC3 receptor, the selective human melanocortin MC1 receptor and human melanocortin MC3 receptor agonists BMS-470539 dihydrochloride and PG-990 were evaluated and caused a 26.0% and 41.0% increase in cell viability, respectively ($P \leq 0.001$ *vs* LPS). Therapeutic treatment with α-MSH, [DTRP^8]-γ-MSH and PG-990 caused a 22.0%, 18.0% and 21.0% ($P \leq 0.001$ *vs* LPS) increase in cell viability, respectively above LPS stimulation. Whilst KPV and BMS-470539 dihydrochloride increased cell viability above LPS stimulation by 14.0% and 13.0% *(*$P \leq 0.01$ *vs* LPS) respectively, returning values to basal levels (Table 3). Previous studies have shown that melanocortin peptides can modulate TNF-α induced caspase 3/7 activity (Kaneva et al., 2012); here we ascertained the effect of the melanocortin peptides in the presence of LPS. LPS stimulation caused a significant increase in caspase 3/7 activity compared to untreated control ($P \leq 0.001$ *vs* untreated control) (Fig. 1A-B). Prophylactic treatment with α-MSH, [DTrp^8]-γ-MSH and KPV significantly reduced the caspase 3/7 activity by 15.9%, 7.3% and 9.7% ($P \leq 0.01$ *vs* LPS) respectively compared to LPS stimulation. To evaluate if this effect was via human melanocortin MC1 receptor and/or human melanocortin MC3 receptor, the selective human melanocortin MC1 receptor and human melanocortin MC3 receptor agonists BMS-470539 dihydrochloride and PG-990 were evaluated and caused a 7.1% and 8.0% decrease in caspase 3/7 activity, respectively ($P \leq 0.01$ *vs* LPS). Therapeutic treatment with α-MSH, [DTRP^8]-γ-MSH and PG-990 caused an 8.4%, 9.0% and 11.3% ($P \leq 0.001$ *vs* LPS) decrease in caspase 3/7 activity following LPS stimulation. Whilst KPV and BMS-470539 dihydrochloride decreased caspase 3/7 activity by 8.6% and 8.9% *(*$P \leq 0.01$ *vs* LPS) respectively (Fig. 1A-B).

3.3. Prophylactic and therapeutic treatment of C-20/A4 chondrocytes with α-MSH, [DTrp^8]-γ-MSH and KPV reduced LPS-induced IL-1β, IL-6 and IL-8 production.

The pro-inflammatory cytokines IL-1β, IL-6 and IL-8 play an important role in the development of OA (Kaneva et al., 2012, 2014; Ni et al., 2019); here we determined the effects of LPS on C-20/A4 chondrocytes on these markers. LPS stimulation caused a significant 3.1-fold and 3.0-fold upregulation of *IL6* and *IL8* mRNA levels respectively (P≤0.001 *vs* untreated control; **Fig. 2B-C**). Prophylactic treatment with α-MSH abrogated *IL6* gene expression and significantly reduced *IL8* gene expression by 5.0-fold ±0.05 (P≤0.001 *vs* LPS). [DTrp^8]-γ-MSH abrogated both *IL6* and *IL8* gene expression, whilst, KPV abrogated *IL6* gene expression and significantly reduced *IL8* gene expression by 10.0-fold ±0.03 (P≤0.001 *vs* LPS). Therapeutic treatment with α-MSH caused a 1.7-fold ±0.02 and 1.2-fold ±0.04 decrease in LPS-induced *IL6* and *IL8* gene expression respectively (P≤0.01, P≤0.001 *vs* LPS), whilst [DTrp^8]-γ-MSH caused a significant decrease in LPS-induced *IL6* and *IL8* gene expression by a 3.4-fold ±0.05 and 3.8-fold ±0.04 respectively (P≤0.001 *vs* LPS). The C-terminal peptide KPV caused a 1.9-fold ±0.05 and 1.2-fold ±0.04 decrease in LPS-induced *IL6* and *IL8* gene expression respectively (P≤0.01, P≤0.001 *vs* LPS; Fig. 2A-C). Following observation of a reduction in *IL6* and *IL8* gene expression by the melanocortin peptides, ELISAs were performed to determine effects on protein level. LPS (0.1 μg/ml) stimulation caused a significant increase in IL-1β, IL-6 and IL-8 protein release at 6h, with 33 ±0.5 (pg•mL^{-1}), 258 ±7.1 (pg•mL^{-1}) and 378 ±1.8 (pg•mL^{-1}) respectively above control levels (P≤0.001 *vs* untreated control) (Table 4). Prophylactic treatment with α-MSH, [DTrp^8]-γ-MSH and KPV caused an 45.5% ±7.8, 54.5% ±5.3 and 45.5% ±8.9 reduction in IL-1β respectively (P≤0.01 *vs* LPS), 84.0% ±3.0, 88.0% ±5.4 and 77.0% ±7.7 reduction in IL-6 respectively (P≤0.001 *vs* LPS). α-MSH and [DTrp^8]-γ-MSH caused a 77.0% ±6.9 and

68.0% ±7.0 reduction in IL-8 protein release respectively (P≤0.001 *vs* LPS), whilst KPV failed to significantly reduce IL-8 levels when administered prophylactically. Following determination that prophylactic treatment with certain melanocortin peptides resulted in significant reductions in the expression of IL-1β, IL-6 and IL-8, their efficacy in a therapeutic scenario was determined. α-MSH, [DTrp^{8}]-γ-MSH and KPV caused a 33.3% ±5.5, 54.5% ±6.0 and 42.4% ±6.3 reduction in IL-1β release (P≤0.01 *vs* LPS), 74.0% ±4.0, 81.0% ±1.1 and 22.0% ±1.2 reduction in IL-6 release (P≤0.001 *vs* LPS), α-MSH and [DTrp^{8}]-γ-MSH caused a 59.0% ±4.3 and 76.0% ±4.2 reduction respectively in IL-8 (P≤0.001 *vs* LPS), whilst KPV failed to inhibit IL-8 protein release when administered therapeutically (Table 4).

3.4. Effect of prophylactic and therapeutic treatment of α-MSH, [DTrp^{8}]-γ-MSH and KPV on MMP1, MMP3 and MMP13 gene expression and protein release from LPS activated C-20/A4 chondrocytes

MMP-1, MMP-3 and MMP-13 play an important role in the pathogenesis of OA (Kannengiesser et al., 2008; Getting et al., 2009; Kaneva et al., 2012), the effect of LPS stimulation of C-20/A4 chondrocytes on these markers was determined. Chondrocytes were stimulated with LPS and *MMP1, MMP3* and *MMP13* gene expression and protein release determined at 6h post stimulation. LPS (0.1 μg/ml) stimulation caused a 27.0-fold ±0.05, 2.0-fold ±0.01 and 1.7-fold ±0.04 increase in *MMP1, MMP3* and *MMP13* mRNA levels respectively (P≤0.001 *vs* untreated control) (**Fig. 3B-D**). α-MSH, [DTRP^{8}]-γ-MSH and KPV treatment on gene expression was determined both prophylactically and therapeutically. Prophylactic treatment with α-MSH, [DTRP^{8}]-γ-MSH and KPV abrogated *MMP1* gene expression, whilst, therapeutic treatment with α-MSH, [DTRP^{8}]-γ-MSH and KPV significantly decreased *MMP1* gene expression by 27.0-fold ±0.02, 27.0-fold ±0.05 and 27.0-

fold ±0.05 (P≤0.001 *vs* LPS) respectively (Fig. 3B). Prophylactic treatment with α-MSH and [DTRP^8]-γ-MSH completely abrogated LPS-induced *MMP3* gene expression, whilst, KPV caused a 1.0-fold ±0.02 decrease in *MMP3* mRNA level (P≤0.05 *vs* LPS) (Fig. 3C). Therapeutic treatment with α-MSH decreased *MMP3* mRNA level by 8.0-fold ±0.05 and [DTRP^8]-γ-MSH completely abrogated LPS-induced *MMP3* mRNA gene expression (P≤0.001 *vs* LPS). KPV had no effect on *MMP3* gene expression following both prophylactic and therapeutic administration (Fig. 3C). Prophylactic treatment with α-MSH, [DTrp^8]-γ-MSH and KPV completely abrogated LPS-induced *MMP13* gene expression (Fig. 3D), as did therapeutic treatment with [DTrp^8]-γ-MSH. Therapeutic treatment with α-MSH and KPV significantly down-regulated *MMP13* gene expression by 8.5-fold ±0.02 and 34.0-fold ±0.05 (P≤0.001 *vs* LPS) respectively (Fig. 3D). Since, certain melanocortin peptides were able to completely abrogate or reduce *MMP1, MMP3* and *MMP13* gene expression, their effect was determined at protein level by ELISA. LPS stimulation caused an increase in MMP-1, MMP-3 and MMP-13 protein release at 6h, with 988 ±1.8 (pg•mL^{-1}), 408 ±2.2 (pg•mL^{-1}) and 149 ±1.8 (pg•mL^{-1}) respectively (P≤0.001 *vs* untreated control) (Table 4). Prophylactic treatment with α-MSH, [DTRP^8]-γ-MSH and KPV caused a 44.0% ±3.1, 90.0% ±4.2 and 29.0% ±4.9 (P≤0.001 *vs* LPS) reduction respectively in LPS-induced MMP-1, whilst, a 73.0% ±3.6, 73.0% ±3.5 and 71.0% ±3.1 (P≤0.001 *vs* LPS) reduction respectively was observed for MMP-3, and a 66.0% ±6.1, 72.0% ±6.3 and 49.0% ±6.5 (P≤0.001 *vs* LPS) reduction for MMP-13 protein release (Table 4). Following determination that prophylactic treatment of the melanocortin peptides resulted in significant reductions in MMP-1, MMP-3 and MMP-13 protein release, their effect when administered therapeutically was determined. α-MSH, [DTrp^8]-γ-MSH and KPV caused a 69.0% ±5.6, 97.0% ±6.7 and 66.0% ±8.2 (P≤0.001 *vs* LPS) reduction in LPS-induced MMP-1 protein release, with a 15.0% ±2.3, 90.0% ±2.2 and

13.0% ±2.2 reduction in MMP-3 (P≤0.001, P≤0.001 *vs* LPS) and a 60.0% ±1.3, 74.0% ±1.1 and 60.0% ±4.2 reduction in MMP-13 respectively (P≤0.001 *vs* LPS) (Table 4).

3.5. Melanocortin peptides induce the pro-resolving protein HO-1

Since, melanocortin peptides inhibited both pro-inflammatory and tissue destructive mediators, we next evaluated their effect on their ability to induce the anti-inflammatory protein HO-1. LPS stimulation caused a 1.9-fold ±0.01 decrease in HO-1 production (P≤0.001 *vs* untreated control), whilst, prophylactic treatment with α-MSH, [D-Trp^{8}]-γ-MSH and KPV caused a 6.1-fold ±0.09, 7.9-fold ±0.08 and 4.1-fold ±0.06 increase respectively (P≤0.001 *vs* LPS) abrogating the inhibitory effect of LPS and increasing expression of HO-1 above that of the control (Fig. 4B). Therapeutic treatment with α-MSH, [DTRP^{8}]-γ-MSH and KPV caused a 3.1-fold ±0.003, 3.1-fold ±0.03 and 2.7-fold ±0.06 increase respectively (P≤0.001 *vs* LPS; Fig. 4B).

3.6. Prophylactic and therapeutic effect of the selective human melanocortin MC1 and MC3 receptor agonists on IL-1β, IL-6 and IL-8 gene expression and protein release in LPS activated chondrocytes

Following identification of the chondroprotective, anti-inflammatory and pro-resolving properties of α-MSH, [DTrp^{8}]-γ-MSH and KPV *vs* LPS activated chondrocytes, we next determined if human melanocortin MC1 receptor and /or human melanocortin MC3 receptor was responsible for eliciting these effects using the selective human melanocortin MC1 receptor agonist BMS-470539 dihydrochloride and selective human melanocortin MC3 receptor agonist PG-990 on pro-inflammatory cytokine release. Prophylactic treatment with

BMS-470539 dihydrochloride significantly decreased LPS-induced *IL6* and *IL8* gene expression by a 30.0-fold ±0.07 and 10.2-fold ±0.07 (P≤0.001 *vs* LPS), whilst, therapeutic treatment with BMS-470539 dihydrochloride caused a 15.0-fold ±0.05 and 30.5-fold ±0.01 decrease in LPS-induced *IL6* and *IL8* gene expression respectively (P≤0.001 *vs* LPS; Fig. 5B-C). Prophylactic treatment with PG-990 caused a 30.0-fold ±0.04 and 10.2-fold ±0.01 decrease in LPS-induced *IL6* and *IL8* gene expression respectively (P≤0.001 *vs* LPS), whilst, therapeutic treatment displayed significant reductions in LPS-induced *IL6* and *IL8* mRNA levels by a 30.0-fold ±0.01 and 30.5-fold ±0.02 respectively (P≤0.001 *vs* LPS; Fig. 4B-C). At the protein level, LPS stimulation caused a significant increase in IL-1β, IL-6 and IL-8 protein release at 6h, with 33 ±0.5 ($pg \bullet mL^{-1}$), 258 ±7.1 ($pg \bullet mL^{-1}$) and 378 ±1.8 ($pg \bullet mL^{-1}$) (P≤0.001 *vs* untreated control) respectively above control levels (Table 4). Prophylactic treatment with BMS-470539 dihydrochloride and PG-990 caused a 3.0% ±4.0 (n.s. *vs* LPS) and 45.5% ±6.1 (P≤0.01 *vs* LPS) reduction in LPS-induced IL-1β; 41.0% ±1.2 and 81.0% ±2.9 (P≤0.001 *vs* LPS) reduction in LPS-induced IL-6, and a, 58.0% ±5.2 and 89.0% ±4.4 reduction respectively for LPS-induced IL-8 release (P≤0.001 *vs* LPS) (Table 4). Following determination that prophylactic administration of the selective melanocortin peptides results in significant reductions in the secretion of IL-1β, IL-6 and IL-8, their effect therapeutically was determined. BMS-470539 dihydrochloride and PG-990 caused a 3.0% ±2.8 (n.s. *vs* LPS) and 54.5% ±4.7 (P≤0.01 *vs* LPS) reduction in LPS-induced IL-1β; 21.0% ±3.1 and 58.0% ±4.4 reduction in LPS-induced IL-6 protein release respectively (P≤0.01; P≤0.001 *vs* LPS) and a 21.0% ±1.9 and 19.0% ±7.2 reduction respectively in IL-8 release (P≤0.01, P≤0.001 *vs* LPS; Table 4).

3.7. Prophylactic and therapeutic effect of the selective human melanocortin MC1 and MC3 receptor agonists on MMP-1, MMP-3 and MMP-13 gene expression and protein release from LPS-activated chondrocytes

Since, the panel of melanocortin peptides were able to completely abrogate or reduce pro-inflammatory cytokine release at gene expression and protein levels, the effects of the selective melanocortin peptides BMS-470539 dihydrochloride and PG-990 on MMP-1, MMP-3 and MMP-13 gene expression and protein release was investigated prophylactically and therapeutically. Prophylactic treatment with BMS-470539 dihydrochloride significantly decreased *MMP1* gene expression by 1.6-fold ±0.05, whilst, PG-990 abrogated *MMP1* gene expression ($P \leq 0.001$ *vs* LPS) (Fig. 6B). BMS-470539 dihydrochloride significantly reduced *MMP3* gene expression by 1.3-fold ±0.02, whilst, PG-990 abrogated *MMP3* gene expression ($P \leq 0.001$ *vs* LPS) (Fig. 6C). BMS-470539 dihydrochloride significantly reduced *MMP13* mRNA level by 2.4-fold ±0.06 ($P \leq 0.001$ *vs* LPS), whilst, PG-990 again completely abrogated *MMP13* gene expression (Fig. 6D). Therapeutic evaluation of BMS-470539 dihydrochloride showed a decrease of *MMP1*, *MMP3* and *MMP13* gene expression by 21.0-fold ±0.03, 2.4-fold ±0.06 and 2.4-fold ±0.05 respectively, whilst, PG-990 reduced *MMP1*, *MMP3* and *MMP13* gene expression by 21.0-fold ±0.01, 2.4-fold ±0.01 and 2.4-fold ±0.02 respectively ($P \leq 0.001$ *vs* LPS) (Fig. 6B-D). At the protein level, LPS stimulation caused a significant increase in MMP-1, MMP-3 and MMP-13 protein release at 6 h (Table 4). Prophylactic treatment with BMS-470539 dihydrochloride reduced MMP-1, MMP-3 and MMP-13 protein release by 9% ±5.5, 72.0% ±3.9 and 61.0% ±6.5 respectively, whilst, PG-990 reduced MMP-1, MMP-3 and MMP-13 protein release by 29.0% ±1.9, 33.0% ±3.1 and 68.0% ±6.7 respectively ($P \leq 0.01$, $P \leq 0.001$ *vs* LPS) (Table 4). Therapeutic treatment with the selective melanocortin peptides BMS-470539 dihydrochloride reduced LPS-induced MMP-1 and MMP-13 protein release by 69.0% ±2.3*** and 59.0% ±1.2*** respectively but did not

significantly inhibit MMP-3 protein release (P≤0.001 *vs* LPS). PG-990 reduced MMP-1, MMP-3 and MMP-13 protein release by 77.0% ±4.7, 57.0% ±2.5 and 72.0% ±1.3 respectively (P≤0.01, P≤0.001 *vs* LPS) (Table 4).

3.8. Determination of whether the selective human melanocortin MC1 and MC3 receptor agonists induce the pro-resolving protein HO-1 when administered either prophylactically or therapeutically.

In the final section of this study, we evaluated whether the selective human melanocortin MC1 receptor agonist BMS-470539 dihydrochloride and the selective human melanocortin MC3 receptor agonist PG-990 could induce HO-1 expression. Fig. 7 reports the data obtained at 6h for both prophylactic and therapeutic treatment. LPS caused a 1.7-fold ±0.05 decrease in HO-1 expression (P≤0.01 *vs* untreated control), whilst, prophylactic treatment of BMS-470539 dihydrochloride and PG-990 caused a 2.1-fold ±0.06 and 3.1-fold ±0.05 (P≤0.001 *vs* LPS) increase, respectively. Therapeutic treatment of the selective melanocortin peptides BMS-470539 dihydrochloride and PG-990 caused a 1.9-fold ±0.04 and 2.0-fold ±0.03 (P≤0.001 vs LPS) increase in HO-1 expression respectively (Fig. 7B), again abrogating LPS-induced reductions of HO-1 expression and increasing levels above control.

4. DISCUSSION

We report the effects of prophylactic and therapuetic administration of melanocortin peptides on cell viability, pro-inflammatory cytokines, MMPs and anti-inflammatory pro-resolving, HO-1 following LPS activation of C-20/A4 human chondrocytes. Chondrocyte cell survival is pivitol in OA, being the only cell type within cartilage and having limited capacity for replication and being solely responsible for the synthesis and maintenance of the extraceullar

matrix (Grässel et al., 2009). A shift towards a catabolic phenotype over an anabolic one leads to increased chondrocyte death and burden on surviving cells to maintain normal matrix turnover. Understanding the role-played by endogenous compounds that maintain a balance between catabolic and anabolic processes within the joint is therefore of interest. In this study the pan-melanocortin agonist α-MSH, human melanocortin MC3 receptor agonist [DTrp^{8}]-γ-MSH, C-terminal peptide α-MSH$_{11-13}$(KPV), selective human melanocortin MC1 and MC3 receptors agonists BMS-470539 dihydrochloride and PG-990 displayed chondroprotection and abrogated LPS induced IL-6, IL-8, MMP-1, MMP-3 and MMP-13, and induced the pro-resolving anti-inflammatory protein HO-1.

A number of mediators released from chondrocytes include IL-1β, IL-6 and IL-8 are implicated in OA (Kaneva et al., 2012) and drive the inflammatory response leading to pro-catabolic in-balance within the joint. Chondrocyte apoptosis and degradation of the articular cartilage matrix occur by IL-8 (Grässel et al., 2009; Chauffier et al., 2012), MMP-1 and MMP-13 (Goldring et al., 2011; Kaneva et al., 2012; 2014) with murine MMP13 knockdown demonstrating a decrease in OA development (Yoon et al., 2008) is of particular interest. Therefore, determining if melanocortin peptides display anti-inflammatory and pro-resolving effects in LPS activated chondrocytes were evaluated.

Numerous studies have highlighted the potential for development of melanocortin peptides for modulating the inflammatory response, including the non-selective pan agonists α-MSH and NDP-α-MSH displaying anti-inflammatory, anti-pyretic effects (Ahmed et al., 2013; Patruno et al., 2018), whilst, more selective compounds [DTrp^{8}]-γ-MSH (Kaneva et al., 2012; Patruno et al., 2018), BMS-470539 dihydrochloride (Leoni et al., 2010; Holloway et al.,

2015) and AP214 (Montero-Melendez et al., 2011) modulate inflammatory responses in primary chondrocytes (Capsoni et al., 2015), experimental inflammatory arthritis (Patel et al., 2010), and ischaemic reperfusion injury (Holloway et al., 2015), exerting their effects via human melanocortin MC1 and MC3 receptors via adenylate cyclase as demonstrated here and in agreement with Kaneva et al., 2012 for α-MSH and [DTrp^{8}]-γ-MSH, whilst inhibiting pro-inflammatory, tissue destructive mediators and inducing pro-resolving anti-inflammatory proteins (including IL-10) (Kaneva et al., 2012), HO-1 (Mawatari et al., 2013) and clearance of apoptotic neutrophils (Patruno et al., 2018). In spite of the potential of melanocortin peptides in modulating inflammation elicited by LPS (Sun et al., 2017), no studies to date have evaluated their effect in LPS (0.1 μg/ml) activated chondrocytes. In this study, we tested the hypothesis that melanocortin peptides displaying different receptor selectivities, can modulate LPS induced chondrocyte activation and death.

Previous studies show that LPS causes chondrocyte death and upregulates IL-1β, IL-6, IL-8, MMP-1, MMP-3 and MMP-13 that play a role in cartilage degradation and arthritic progression. In this model, LPS caused a modest reduction in cell viability (13%) in agreement with other studies (Hu et al., 2018) and caused a significant increase in caspase-3 and -7 as previously shown with TNF-α (Kaneva et al. 2012). Melanocortin compounds reduced caspase-3 and -7 activity when administered prophylactically as previously observed for α-MSH and [DTrp^{8}]-γ-MSH (Kaneva et al., 2012) but shown for the first time with KPV, BMS-470539 dihydrochloride and PG-990. However, to our knowledge this is the first demonstration that these peptides can inhibit caspase-3 and -7 when given therapeutically. In addition, LPS activation led to elevations in IL-1β (Ni et al., 2019), IL-6, IL-8, MMP-1, MMP-3 and MMP-13 (Chen et al., 2013), leading to destruction of extracellular matrix and chondrocyte apoptosis via caspase 3 and 7 (Goldring et al., 2011; Kaneva et al., 2012), whilst IL-1β causes synovial inflammation and OA progression (Ni et al., 2019), upregulating the

disintegrin and metalloproteinases with thrombospondin motifs (ADAMTS)-4 and ADAMTS-5 (Zhang et al., 2018). A role for human melanocortin MC1 and/or MC3 receptors in modulating cell death, pro-inflammation and tissue destructive mediators has previously been seen (Kaneva et al., 2012, 2014). In this model, prophylactic and therapeutic treatments with α-MSH, [DTrp^8]-γ-MSH, KPV, BMS-470539 dihydrochloride and PG-990, in the presence of LPS, led to a significant increase in cell viability, abrogating the LPS induced cell death. Whilst, prophylactic chondroprotective effects were observed with α-MSH and [DTrp^8]-γ-MSH (Kaneva et al., 2012), we report for the first time the therapeutic effect of these agonists on LPS induced cell death. Moreover, we report for the first time that both prophylactic and therapeutic administration of the C-terminal peptide KPV (Luger and Brzoska 2007), BMS-470537 dihydrochloride and PG-990, display chondroprotection opening up new avenues for targeting OA.

Following determination of the peptides chondroprotective effects, the peptides in the presence of LPS was evaluated against OA markers. LPS stimulation of C-20/A4 chondrocytes led to increases in IL-1β, IL-6, IL-8, MMP-1, MMP-3 and MMP-13 as previously seen in rat (Chen et al., 2013; Hu et al., 2018) and human chondrocytes (Mawatari et al., 2013; Ni et al., 2019). LPS causes a significant upregulation of TNF-α and IL-1β (Chen et al., 2013; Ni et al., 2019), upregulating MMPs gene expression and pro-inflammatory cytokine, driving apoptosis via the effector caspases, causing cartilage degradation (Kaneva et al., 2012, 2014). Prophylactic treatment with the peptides inhibited expression of IL-1β, IL-6 and IL-8 as previously seen with α-MSH and [DTrp^8]-γ-MSH (Kaneva et al., 2012, 2014). The high degree of inhibition of mRNA pro-inflammatory cytokine expression is reported for the first time following therapeutic treatment, highlighting

the ability of certain melanocortin peptides to modulate NF-κB signalling pathways following LPS chondrocyte activation (Huang and Kraus, 2016). Pro-inflammatory cytokine inhibition was observed with melanocortin peptides inhibiting IL-1β, IL-6 and IL-8 both prophylactically and therapeutically. However, therapeutic treatment with KPV (Kannengiesser et al., 2008; Land, 2012) was not as effective as α-MSH, [DTrp^{8}]-γ-MSH, BMS-470539 dihydrochloride and PG-990 on IL-6 and IL-8 as previously observed (Capsoni et al., 2015). With respect to IL-6 and IL-8, KPV demonstrated a reduced anti-inflammatory effect in this study even though it has previously been shown to have similar effects to α-MSH on elevated intracellular calcium fluxes (Brzoska et al., 2008). In a study in rabbits central effects of KPV were more pronounced than peripheral administration with effects less than α-MSH (Richards and Lipton 1984) suggesting that full activity requires other regions of the hormone (HFRW). However, for IL-1β, KPV appeared to cause a similar degree of inhibition as α-MSH both prophylactically and therapeutically and could be due to the fact that the L form of K(D)PT is similar to IL-1β$_{193\text{-}195}$ and is well tolerated in ulcerative colitis (Kucharzik et al., 2017).

Given the increase in pro-inflammatory mediators by LPS stimulation and their reduction by the melanocortin peptides, the effect of the peptides on MMPs was evaluated. MMP-1, MMP-3, and MMP-13 was reduced following prophylactic administration confirming previous findings with α-MSH and [DTrp^{8}]-γ-MSH (Goldring et al., 2011, Kaneva et al., 2012, 2014). However, a novel finding was observed for KPV (MMP-1 and MMP-13), BMS-470539 dihydrochloride (MMP-1, MMP-3 and MMP-13) and PG-990 (MMP-1, MMP-3 and MMP-13). Another exciting novel observation was the ability of therapeutic treatments with α-MSH, [DTrp^{8}]-γ-MSH and PG-990 to significantly reduce MMP-1, MMP-3 and MMP-13 expression, thus highlighting a more relevant treatment regime for people with OA.

In the final section, we investigated the effect of these peptides on induction of the anti-inflammatory pro-resolving protein HO-1. Previous studies show α-MSH, [DTrp^8]-γ-MSH, human melanocortin MC3/4 receptor agonist MTII increased the anti-inflammatory protein IL-10 (Lam et al., 2005a, Kaneva et al., 2012). The importance of this mediator was demonstrated in healthy primary human chondrocytes, whereby IL-10 was proposed to be the mechanism of action for chondroprotection reducing both *MMP1* and *MMP13* gene expression (Muller et al., 2008; Li et al., 2017). Thus, developing compounds that can induce pro-resolving mediators is an exciting proposal. Here, we determined whether these peptides could induce HO-1, previously shown to be upregulated by ACTH, MTII (Lam et al., 2005b) and α-MSH in cardiovascular tissue (Vecsernyes et al., 2017) and a model of retinal damage (Rossi et al., 2016). LPS reduced the expression of the anabolic protein HO-1, as previously seen (Mawatari et al., 2013; Kaneva et al., 2014) and supports a plausible explanation of why elevations in the catabolic proteins IL-1β, IL-6, IL-8, MMP-1, MMP-3 and MMP-13 occur. Here we report for the first time that prophylactic and therapeutic treatment with the peptides, reversed the inhibition of HO-1 expression by LPS, highlighting the potent anti-inflammatory properties of these peptides. Therefore, the ability of these peptides to signal via HO-1 is an exciting observation in chondrocytes for resolving chondrocyte born pro-inflammatory cytokines and MMPs production in the development of arthritic pathologies.

5. Conclusion

The panel of melanocortin peptides selected for this investigation attenuated the effects caused by LPS at both the molecular and biochemical level. It was observed that the human melanocortin MC3 receptor agonists [DTrp^8]-γ-MSH and PG-990 inhibited pro-inflammatory

cytokine and MMP release to a greater degree than that observed with the human melanocortin MC1 receptor agonists BMS-470539 dihydrochloride and C-terminal KPV when administered prophylactically and therapeutically, suggesting a more prominent role for the human melanocortin MC3 receptor. Evaluating the pro-resolution effects of these peptides demonstrated that [DTrp^{8}]-γ-MSH and PG-990 induced HO-1 and exhibited a greater degree of chondroprotection compared to the more human melanocortin MC1 receptor selective compounds. However, given the protective effects of these peptides targeting both human melanocortin MC1 and MC3 receptors may potentially provide the best therapeutic strategy as observed with AP214, which is in clinical development for acute kidney injury. Whilst it's important to highlight the beneficial effects of peptide therapy with around 150 in current drug development pipelines, we are mindful that a number of issues that exist with this approach, namely oral bioavailability which may limit patient compliance (Lau and Dunn 2018). They are also prone to hydrolysis and oxidation, short half-life and fast elimination, there are also potential threats of immunogenicity (Fosgerau and Hoffmann, 2015). However, they have the advantage of good efficacy, safety and tolerability and predictable metabolism (Fosgerau and Hoffmann 2015). In addition, there are a number of peptides commercially used e.g. Insulin, ACTH (parent hormone of melanocortin peptides) as well as the target for human melanocortin MC1 receptor, Afamelanotide (Melanotan 1) used for erythropoietic protoporphyria (Lau and Dunn 2018). Therefore, development of appropriate drug delivery systems, formulation and improvement in half-life will enhance their biological use. This data, thus further enhances our knowledge of melanocortin peptides in arthritic pathologies highlighting their ability to work both prophylactically and therapeutically.

Conflict of Interest

PG holds a patent on the human melanocortin MC3 receptor agonists - [DTrp^{8}]-γ-MSH and PG-990.

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Figure legends

Fig.1. Effect of α-MSH, [Dtrp^{8}]-γ-MSH, KPV, BMS-470539 and PG-990 on Caspase 3/7 activity on LPS activated C-20/A4 chondrocytes. C-20/A4 chondrocytes were plated at 2.0 x 10^{3} cells/well. Panel A - chondrocytes treated with PBS, α-MSH (3.0 μg/ml), [Dtrp^{8}]-γ-MSH (3.0 μg/ml) or KPV (4.0 μg/ml) for 30 min prior to stimulation with LPS (0.1 μg/ml) for 6 h (prophylactic treatment) or stimulated with LPS (0.1 μg/ml) for 2 h followed by melanocortin peptide treatment for a further 4 h (therapeutic treatment). Panel B, chondrocytes treated with PBS, BMS-470539 dihydrochloride (10.0 μg/ml) or PG-990 (3.0 μg/ml) for 30 min prior to stimulation with LPS (0.1 μg/ml) for 6 h (prophylactic treatment) or stimulated with LPS (0.1 μg/ml) for 2 h followed by melanocortin peptide treatment for a further 4 h (therapeutic treatment). After 6 h, Caspase 3/7 activity was determined by Caspase-3/7 Glo Assay. Dotted line symbolises Caspase 3/7 activity following PBS treatment (control). Data are presented as Mean ± S.E.M of $n = 3$ samples repeated in triplicate. $^{**}P \leq 0.01$ *vs* LPS (0.1 μg/ml).

Fig. 2. Effect of prophylactic and therapeutic administration of melanocortin peptides on *IL6* and *IL8* gene expression from C-20/A4 cells stimulated with LPS (0.1 μg/ml) for 6 h. C-20/A4 chondrocytes were treated with α-MSH (3.0 μg/ml), [DTrp^{8}]-γ-MSH (3.0 μg/ml) or KPV (4.0 μg/ml) for 30 min prior to LPS (0.1 μg/ml) stimulation for 6 h (prophylactic treatment) or stimulated with LPS (0.1 μg/ml) for 2 h followed by α-MSH (3.0 μg/ml), [DTrp^{8}]-γ-MSH (3.0 μg/ml) or KPV (4.0 μg/ml) treatment for a further 4 h (therapeutic treatment), total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *IL6* and *IL8* was used to detect and quantify gene expression on 2% agarose gels with *β-actin* used as internal control, experiment repeated in triplicate (Panel A). Comparison of densitometrically quantified *IL6* and *IL8* gene expression (Panel B and C) shown in arbitrary units, each value normalised to the respective *β-actin* expression. Data are presented as mean values ± S.E.M of n = 3 samples, experiment repeated in triplicate, $^{\#}P\leq 0.05$, $^{\#\#}P\leq 0.01$, $^{\#\#\#}P\leq 0.001$ *vs* untreated controls or $^{*}P\leq 0.05$, $^{**}P\leq 0.01$, $^{***}P\leq 0.001$ *vs* LPS (0.1 μg/ml).

Fig. 3. Effect of prophylactic and therapeutic administration of melanocortin peptides on *MMP1, MMP3* and *MMP13* gene expression from C-20/A4 cells stimulated with LPS (0.1 μg/ml) for 6 h. C-20/A4 chondrocytes were treated with α-MSH (3.0 μg/ml), [DTrp^{8}]-γ-MSH (3.0 μg/ml) or KPV (4.0 μg/ml) for 30 min prior to LPS (0.1 μg/ml) stimulation for 6 h (prophylactic treatment) or stimulated with LPS (0.1 μg/ml) for 2 h followed by α-MSH (3.0 μg/ml), [DTrp^{8}]-γ-MSH (3.0 μg/ml) or KPV (4.0 μg/ml) treatment for a further 4 h (therapeutic treatment), total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *MMP1*, *MMP3* and *MMP13* was used to detect and quantify gene expression on 2% agarose gels with *β-actin* used as internal control, experiment repeated in

triplicate (Panel A). Comparison of densitometrically quantified *MMP1, MMP3* and *MMP13* gene expression (Panel B, C and D) shown in arbitrary units, each value normalised to the respective *β-actin* expression. Data are presented as mean values ± S.E.M of *n* = 3 samples, experiment repeated in triplicate, $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ *vs* untreated controls or $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ *vs* LPS (0.1 μg/ml).

Fig. 4. Effect of prophylactic and therapeutic administration of α-MSH, [DTrp^{8}]-γ-MSH and KPV in LPS activated C-20/A4 cells on HO-1 expression. A representative western blot of three individual experiments of cells treated with PBS (control), LPS 0.1μg/ml alone or in combination with α-MSH (3.0 μg/ml), [DTrp^{8}]-γ-MSH (3.0 μg/ml) or KPV (4.0 μg/ml) for 6 h (both prophylactic and therapeutic treatment). Western blotting was used to determine HO-1 and α-tubulin using protein levels using rabbit anti-HO-1 (1:2000). Bands with sizes corresponding to HO-1 (32 kDa) and α-tubulin (55 kDa) were detected (Panel A) and densitometrically quantified (Panel B). Data are presented as mean values ± S.E.M of *n* = 3 samples, experiment repeated in triplicate, $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ *vs* untreated controls or $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ *vs* LPS (0.1 μg/ml).

Fig. 5. Effect of prophylactic and therapeutic administration of selective human melanocortin MC1 and MC3 receptor agonists BMS-470539 dihydrochloride and PG-990 on *IL6* and *IL8* gene expression from C-20/A4 cells activated by LPS (0.1 μg/ml) for 6 h. C-20/A4 chondrocytes were treated with selective human melanocortin MC1 receptor agonist BMS-470539 dihydrochloride (BMS; 10.0 μg/ml) or selective human melanocortin MC3 receptor agonist PG-990 (3.0 μg/ml) for 30 min prior to LPS (0.1 μg/ml) stimulation for 6 h (prophylactic treatment) or stimulated with LPS (0.1 μg/ml) for 2 h followed by BMS-

470539 dihydrochloride (BMS; 10.0 μg/ml) or PG-990 (3.0 μg/ml) treatment for a further 4 h (therapeutic treatment), total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *IL6* and *IL8* was used to detect and quantify gene expression on 2% agarose gels with *β-actin* used as internal control, experiment repeated in triplicate (Panel A). Comparison of densitometrically quantified *IL6* and *IL8* gene expression (Panel B and C) shown in arbitrary units, each value normalised to the respective *β-actin* expression. Data are presented as mean values ± S.E.M of $n = 3$ samples, experiment repeated in triplicate, $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ *vs* untreated controls or $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ *vs* LPS (0.1 μg/ml).

Fig. 6. Effect of prophylactic and therapeutic administration of selective human melanocortin MC1 and MC3 receptor agonists BMS-470539 dihydrochloride and PG-990 on *MMP1, MMP3* and *MMP13* gene expression from C-20/A4 cells activated by LPS (0.1 μg/ml) for 6 h. C-20/A4 chondrocytes were treated with selective human melanocortin MC1 receptor agonist BMS-470539 dihydrochloride (BMS; 10.0 μg/ml) or human melanocortin MC3 receptor agonist PG-990 (3.0 μg/ml) for 30 min prior to LPS (0.1 μg/ml) stimulation for 6 h (prophylactic treatment) or stimulated with LPS (0.1 μg/ml) for 2 h followed by BMS-470539 dihydrochloride (BMS; 10.0 μg/ml) or PG-990 (3.0 μg/ml) treatment for a further 4 h (therapeutic treatment), total RNA extracted 6 h post stimulation. PCR amplification with the respective primers for *MMP1*, *MMP3* and *MMP13* was used to detect and quantify gene expression on 2% agarose gels with *β-actin* used as internal control, experiment repeated in triplicate (Panel A). Comparison of densitometrically quantified *MMP1, MMP3* and *MMP13* gene expression (Panel B) shown in arbitrary units, each value normalised to the respective *β-actin* expression. Data are presented as mean values ± S.E.M

of $n = 3$ samples, experiment repeated in triplicate, $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ *vs* untreated controls or $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ *vs* LPS (0.1 μg/ml).

Fig. 7. Effect of prophylactic and therapeutic administration of selective human melanocortin MC1 and MC3 receptor agonists BMS-470539 dihydrochloride and PG-990 in LPS activated C-20/A4 cells on HO-1 expression. A representative western blot of three individual experiments of cells treated with PBS (control), LPS 0.1μg/ml alone or in combination with BMS-470539 dihydrochloride (BMS; 10.0 μg/ml) or PG-990 (3.0 μg/ml) for 6h (both prophylactic and therapeutic treatment). Western blotting was used to determine HO-1 and α-tubulin using protein levels using rabbit anti-HO-1 (1:2000). Bands with sizes corresponding to HO-1 (32 kDa) and α-tubulin (55 kDa) were detected (Panel A) and densitometrically quantified (Panel B). Data are presented as mean values ± S.E.M of $n = 3$ samples, $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ *vs* untreated controls or $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ *vs* LPS (0.1 μg/ml).

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