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RESEARCH PAPER

Chondroprotective and anti-inflammatory role of melanocortin peptides in TNF-α activated human C-20/A4 chondrocytes

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BACKGROUND AND PURPOSE

Melanocortin MC_1 and MC_3 receptors, mediate the anti-inflammatory effects of melanocortin peptides. Targeting these receptors could therefore lead to development of novel anti-inflammatory therapeutic agents. We investigated the expression of MC_1 and MC_3 receptors on chondrocytes and the role of α-melanocyte-stimulating hormone (α-MSH) and the selective MC_3 receptor agonist, [$DTRP^8$]-γ-MSH, in modulating production of inflammatory cytokines, tissue-destructive proteins and induction of apoptotic pathway(s) in the human chondrocytic C-20/A4 cells.

EXPERIMENTAL APPROACH

Effects of α-MSH, [$DTRP^8$]-γ-MSH alone or in the presence of the $MC_{3/4}$ receptor antagonist, SHU9119, on TNF-α induced release of pro-inflammatory cytokines, MMPs, apoptotic pathway(s) and cell death in C-20/A4 chondrocytes were investigated, along with their effect on the release of the anti-inflammatory cytokine IL-10.

KEY RESULTS

C-20/A4 chondrocytes expressed functionally active $MC_{1,3}$ receptors. α-MSH and [$DTRP^8$]-γ-MSH treatment, for 30 min before TNF-α stimulation, provided a time-and-bell-shaped concentration-dependent decrease in pro-inflammatory cytokines (IL-1β, IL-6 and IL-8) release and increased release of the chondroprotective and anti-inflammatory cytokine, IL-10, whilst decreasing expression of *MMP1, MMP3, MMP13* genes. α-MSH and [$DTRP^8$]-γ-MSH treatment also inhibited TNF-α-induced caspase-3/7 activation and chondrocyte death. The effects of [$DTRP^8$]-γ-MSH, but not α-MSH, were abolished by the $MC_{3/4}$ receptor antagonist, SHU9119.

CONCLUSION AND IMPLICATIONS

Activation of MC_1/MC_3 receptors in C-20/A4 chondrocytes down-regulated production of pro-inflammatory cytokines and cartilage-destroying proteinases, inhibited initiation of apoptotic pathways and promoted release of chondroprotective and anti-inflammatory cytokines. Developing small molecule agonists to MC_1/MC_3 receptors could be a viable approach for developing chondroprotective and anti-inflammatory therapies in rheumatoid and osteoarthritis.

Abbreviations

α-MSH, α-melanocyte-stimulating hormone; ACTH, adrenocorticotropin; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FCS, fetal calf serum; MC, melanocortin receptor; OA, osteoarthritis; RA, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory systemic disorder (Getting *et al.*, 2009); whilst osteoarthritis (OA) is typically associated with obesity, age and abnormal joint loading (Recnik *et al.*, 2009) and was historically considered to be primarily a non-inflammatory arthropathy. However recent studies in both OA and RA patients and in animal

models show a marked elevation in inflammation and pro-inflammatory cytokine levels, including TNF-α, IL-1β IL-6 and IL-8, in the cartilage, which subsequently leads to progressive joint destruction (Fernandes *et al.*, 2002; Rai *et al.*, 2008; Koenders *et al.*, 2011). Chondrocyte activation can lead to increased expression and secretion of cartilage-degrading MMP (Shlopov *et al.*, 2000), such as MMP1 and MMP13, which are highly elevated in OA cartilage (Reboul *et al.*, 1996), leading to degradation of the cartilage extracellular matrix (ECM). In addition to an increase in pro-inflammatory mediators, TNF-α also down-regulates the production of pro-resolving, anti-inflammatory proteins such as IL-10 (Iannone *et al.*, 2001), which may also contribute to an increased proportion of apoptotic cells in OA cartilage compared with healthy cartilage, resulting in further damage to cartilage (Aigner *et al.*, 2001; John *et al.*, 2007).

Melanocortin peptides, including α-melanocyte stimulating hormone (α-MSH), are endogenous anti-inflammatory peptides (Gonzalez-Rey *et al.*, 2007) that exert their effects via activation of melanocortin receptors, of which five (MC_{1-5}; receptor nomenclature follows Alexander *et al.*, 2011) have so far been identified (Getting *et al.*, 2009). Over the last three decades, melanocortin peptides and their MC_1 and MC_3 receptors have been shown to be particularly important as modulators of innate immunity. Consequently, synthetic peptides such as [$DTRP^8$]-γ-MSH have been designed to evaluate the potential of these receptors as targets for therapeutic intervention (Getting *et al.*, 2009; Holloway *et al.*, 2011). For example, melanocortin peptides display efficacy in preclinical models of arthritis (Patel *et al.*, 2010) and models of gout (Getting *et al.*, 2002) where monosodium urate crystal–induced neutrophil migration and pro-inflammatory cytokines and chemokine release are significantly reduced. MC_1 and MC_3 receptors have been proposed to mediate these anti-inflammatory effects (Getting *et al.*, 2009; Holloway *et al.*, 2011) with the selective MC_1 receptor agonist, BMS-470539, inhibiting leukocyte migration in the inflamed vasculature of mice (Leoni *et al.*, 2010); whilst pharmacological approaches have also highlighted a role for the MC_3 receptor as a possible anti-inflammatory target in models of gouty peritonitis (Getting *et al.*, 2006a), ischaemia–reperfusion injury (Leoni *et al.*, 2008) and RA (Patel *et al.*, 2010). In addition, deletion of the MC_3 receptor gene in mice exacerbates the host inflammatory response in a model of ischaemia–reperfusion injury (Leoni *et al.*, 2008), whilst an increase of inflammatory arthritis is also seen in MC_3 receptor null mice compared to wild-type mice (Patel *et al.*, 2010), again confirming the importance of MC_3 receptors as therapeutic targets.

In spite of the substantial evidence suggesting a role of melanocortin peptides in numerous inflammatory pathologies, only a very limited number of studies have evaluated the therapeutic potential of melanocortin peptides in OA. For example, α-MSH down-regulated TNF-α-induced expression of MMPs, by decreasing p38 MAPK phosphorylation and subsequent activation of NF-κB, in a human chondrosarcoma (HTB-94) cell line (Yoon *et al.*, 2008). In human articular chondrocytes, α-MSH decreased IL-1β and TNF-α mRNA levels, mediated via the MC_1 receptor (Grässel *et al.*, 2009). In addition, in rodent chondrocytes, adrenocorticotrophin (ACTH) treatment mediated via the MC_1 receptor promoted the development of the chondrocyte phenotype (Evans *et al.*, 2004).

In this present study, we have demonstrated the expression and functionality of MC_1 and MC_3 receptors on human C-20/A4 chondrocytes and the role of melanocortins in modulating TNF-α induced pro-inflammatory cytokine production, MMP release, caspase-driven chondrocyte apoptosis and chondroprotection.

Methods

The C-20/A4 human chondrocyte cell line was a kind gift of Dr MB Goldring (Hospital for Special Surgery, New York) (Goldring *et al.*, 1994) and is derived from juvenile costal chondrocytes by immortalization via transfection with origin-defective simian virus 40 large T antigen (SV40-Tag) (Finger *et al.*, 2004). Briefly, C-20/A4 cells were cultured in monolayers in complete media [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), penicillin (100 $U \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$)], in a humidified atmosphere supplied with 5% CO_2 and 95% air, until 80% confluent, as previously described (Goldring *et al.*, 1994). Prior to experimentation, FCS concentration was reduced to 1% (v/v) for 24 h, and all subsequent experiments were performed under these conditions.

In vitro *chondrocyte stimulation*

C-20/A4 chondrocytes were treated for 30 min with either PBS (control), α-MSH (1–30 $\mu g \cdot mL^{-1}$; Sigma-Aldrich, Poole, Dorset, UK) or the selective MC_3 receptor agonist, [$DTRP^8$]-γ-MSH (1–30 $\mu g \cdot mL^{-1}$) (synthesized by Dr P Grieco, University of Naples, Italy) (Grieco *et al.*, 2000; Getting *et al.*, 2006b) and stimulated with human recombinant TNF-α (0–80 $pg \cdot mL^{-1}$: Sigma-Aldrich) (Kaneva *et al.*, 2010); and cell-free supernatants were collected and stored at –20°C. In some experiments (as indicated), cells were also pretreated for 1 h with the $MC_{3/4}$ receptor antagonist, SHU9119 (10 $\mu g \cdot mL^{-1}$; Phoenix Pharmaceuticals Inc, Karlsruhe, Germany) (Getting *et al.*, 2006a), before addition of α-MSH or [$DTRP^8$]-γ-MSH.

cAMP accumulation in C-20/A4 chondrocytes

To assess receptor function, a cAMP accumulation assay (RPN225, GE Healthcare, Amersham, UK) was used as previously described (Getting *et al.*, 2006a; 2008). Briefly, C-20/A4 chondrocytes (2×10^5 cells·per well) were seeded in 96-well-plates in 100 μL complete media and incubated for 2 h to allow cell adhesion. Cells were then incubated for 30 min in serum-free medium in a 37°C humidified atmosphere of 5% CO_2 and 95% air, in the presence of the direct adenylate activator, forskolin (3 μM, positive control; Sigma-Aldrich), α-MSH (1–30$\mu g \cdot mL^{-1}$) or [$DTRP^8$]-γ-MSH (1–30$\mu g \cdot mL^{-1}$) alone or in the presence of the $MC_{3/4}$ receptor antagonist, SHU9119 (10 $\mu g \cdot mL^{-1}$), all in the presence of 1 mM IBMX (Sigma-Aldrich). A negative control (cells incubated alone) was incubated under identical conditions. Cell supernatants were removed, adhered cells were lysed according to the manufacturer's instructions and intracellular cAMP concentration was determined (Getting *et al.*, 2006a; 2008).

Molecular analysis

RT-PCR analysis. C-20/A4 chondrocyte RNA was extracted and isolated using the NucleoSpin® RNA II Kit (Macherey-Nagel, Duren, Germany), and RNA concentrations were determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer ($A_{260nm/280\ nm}$: 1.9–2.1). cDNA was synthesized by taking 1 μg of DNase-treated total RNA, PolyT, Random Primers and RNase inhibitor in a total volume of 20 μL using the Improm II Reverse Transcription System (Promega, Southampton, UK). 2 μL aliquots of the cDNA (in a final volume of 25 μL) were used as a template for PCR amplification using specific primer pairs (see below) for *MMP1*, *MMP3*, *MMP13* using the GoTaq® Green Mastermix system (Promega). The oligonucleotide primer sequences were as follows:

MMP1_FWD: 5′-CGACTCTAGAAACAGAAGAGCAAGA-3′ and

MMP1_REV: 5′-AAGGTTAGCTTACTGTCACACACGCTT-3′;

MMP3_FWD: 5′-GGAAATCAGTTCTGGGCTATACGAGG-3′ and

MMP3_REV: 5′-CCAACTGCGAAGATCCACTGAAGAAG-3′;

MMP13_FWD: 5′-GTGGTGTGGGAAGTATCATCA-3′ and

MMP13_REV: 5′-GCATCTGGAGTAACCGTATTG-3′.

The PCR parameters were as follows: initial denaturing for 5 min at 95°C, followed by 25 cycles of denaturation (95°C for 60 s), annealing (55–59°C, depending on the primers used for 90 s) and extension (72°C for 90 s), with a single final extension of 72°C for 10 min. Oligonucleotide primers for human β-actin (FWD: 5′-GTCCCGGCATGTGCAA-3′; REV: 5′-AGGATGTTCATGAGGTAGT-3′) were used as a control. Amplification products were separated by agarose gel electrophoresis and stained with ethidium bromide. Densitometry analysis was performed using Image J software (NIH, Bethesda, MD).

Western blotting. C-20/A4 chondrocyte expression of MC_1 and MC_3 receptors was determined as previously described (Getting *et al.*, 2008). Following electrophoresis in a 10% SDS-polyacrylamide gel, proteins were transferred onto nylon membrane by electroblotting, blocked overnight in 5% nonfat milk solution in Tris–HCl-buffered saline, pH 7.5 (TBS) containing 0.1% (v/v) Tween-20 and then incubated with either specific anti-MC_1 or anti-MC_3 receptor (1:2000 dilution M9193 and M4937; Sigma-Aldrich, Dorset, UK) rabbit antibodies in blocking solution. Blots were washed in TBS prior to the addition of a secondary goat anti-rabbit HRP-conjugated antibody (1:2000 dilution), and specific antibody binding was detected by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL). Following detection, bound antibodies were removed by incubating the membranes in 100 mM glycine–HCl, pH 2.5 for 30 min, and the blot was re-probed to detect α-tubulin as described previously (Getting *et al.*, 2008). Densitometry analysis was performed using Image J software (NIH).

Biochemical and cell viability analyses

MTT cytotoxicity assay. Cell viability was determined using an MTT assay (Lam *et al.*, 2006). Briefly, C-20/A4 cells plated at 2×10^5 cells·per well in 96-well plates (in 200 μL of complete medium) and allowed to adhere, prior to treatment as described above (see *In vitro* chondrocyte stimulation section). Following stimulation, cell culture medium was aspirated, and a 1:10 dilution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium (MTT; Sigma-Aldrich) solution was added for 2 h at 37°C in a humidified chamber with 5% CO_2 and 95% air at 37°C. Following incubation, the supernatant was replaced with DMSO (100 μL per well) and incubated for 15 min, and the absorbance was determined at 570 nm.

Cytokine quantification by ELISA. Human IL-1β, IL6, IL-8 and IL-10 concentrations in cell-free supernatants were determined using commercially available ELISA kits (R&D Systems Europe Ltd, Oxford, UK). These ELISAs showed negligible (<1%) cross-reactivity with other cytokines and chemokines (data furnished by manufacturer).

Caspase-Glo 3/7 apoptosis assay. Apoptosis was determined by measuring caspase-3 and -7 activity following cell stimulation as detailed above (see *In vitro* chondrocyte stimulation section). Briefly, C-20/A4 chondrocytes were plated at 2×10^5 cells per well in 96-well plates, and 100 μL of caspase-Glo 3/7 Reagent (Promega) was added to each well and incubated at room temperature for 1 h, after which luminescence was measured as per the manufacturer's instructions.

Statistics

All data are reported as mean ± SEM of *n* observations, using at least three experiments with four determinations per group. Statistical evaluation was performed using ANOVA (Prism GraphPad Software, La Jolla, CA, USA) incorporating either Dunnett's or Bonferroni's multiple comparison tests to allow for *post hoc* analyses, with values of $^*P \leq 0.05$ taken as significant.

Results

TNF-α up-regulates pro-inflammatory cytokine release from C-20/A4 chondrocytes

C-20/A4 chondrocytes were stimulated with different concentrations of TNF-α (0–80 $pg{\cdot}mL^{-1}$) to evaluate its ability to promote the release of IL-1β, IL-6 and IL-8 over 24 h. TNF-α stimulation at all concentrations led to a significant release of IL-1β, IL-6 and IL-8 over basal levels at all time points evaluated (Figure 1A–C). IL-1β synthesis increased in a concentration-dependent manner with a maximal release at 6 h, with a plateau observed between 60 and 80 $pg{\cdot}mL^{-1}$ TNF-α, whilst IL-6 and IL-8 increased in a concentration-dependent manner, peaking at 24 h, with 60 $pg{\cdot}mL^{-1}$ TNF-α, causing maximal stimulation (Figure 1A–C).

C-20/A4 chondrocytes express functionally active MC_1 and MC_3 receptors

Western blotting showed the presence of both MC_1 and MC_3 receptor proteins on C-20/A4 human chondrocytes with product sizes of 35 and 40 kDa being observed (Figure 2A). α-MSH and [$DTRP^8$]-γ-MSH were used to test for functional receptors on these cells by measuring cAMP production.

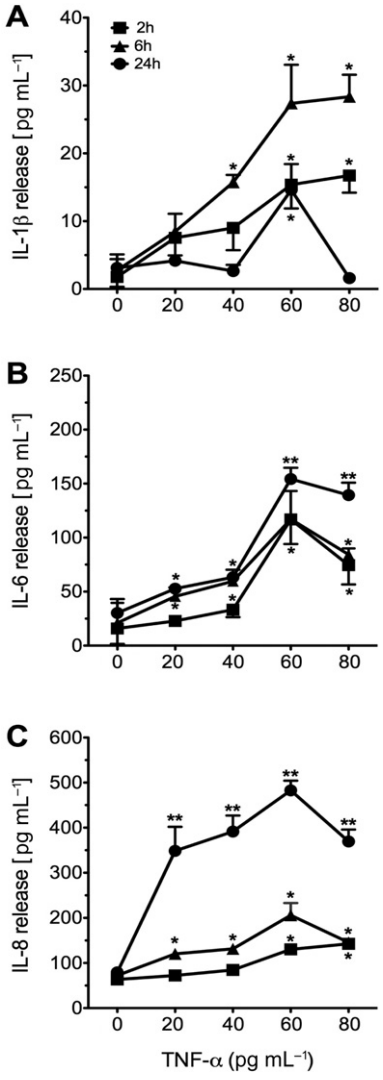

Figure 1

TNF-α stimulates IL-1β, IL-6 and IL-8 release from C-20/A4 chondrocytes. C-20/A4 chondrocytes were stimulated with TNF-α (0–80 pg·mL^{-1}), and cell-free supernatants were collected 0–24 h post-stimulation and analysed for IL-1β (A), IL-6 (B) and IL-8 (C) levels by ELISA. Data are presented as mean ± SEM of $n = 4$ independent experiments repeated in triplicate, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, significant effect of TNF-α.

Addition of the pan-melanocortin receptor agonist, α-MSH (1–30 μg·mL^{-1}) or the selective MC_3 receptor agonist, [DTRP^8]-γ-MSH (1–30 μg·mL^{-1}), both provoked a significant accumulation of intracellular cAMP. The direct adenylate cyclase stimulator, forskolin (FSK, 3 μM), caused a ninefold increase in cAMP (2230 ± 74 fmol·per well) over control (249 ± 11 fmol·per well), with α-MSH treatment causing a significant increase in cAMP accumulation at all concentrations tested, with a maximal increase being observed at 10 μg·mL^{-1} (Figure 2B). The selective MC_3 receptor agonist, [DTRP^8]-γ-MSH, also caused a marked increase in cAMP accumulation with a maximal effect observed at 3 μg·mL^{-1} (Figure 2C). Higher concentrations of both α-MSH and [DTRP^8]-γ-MSH

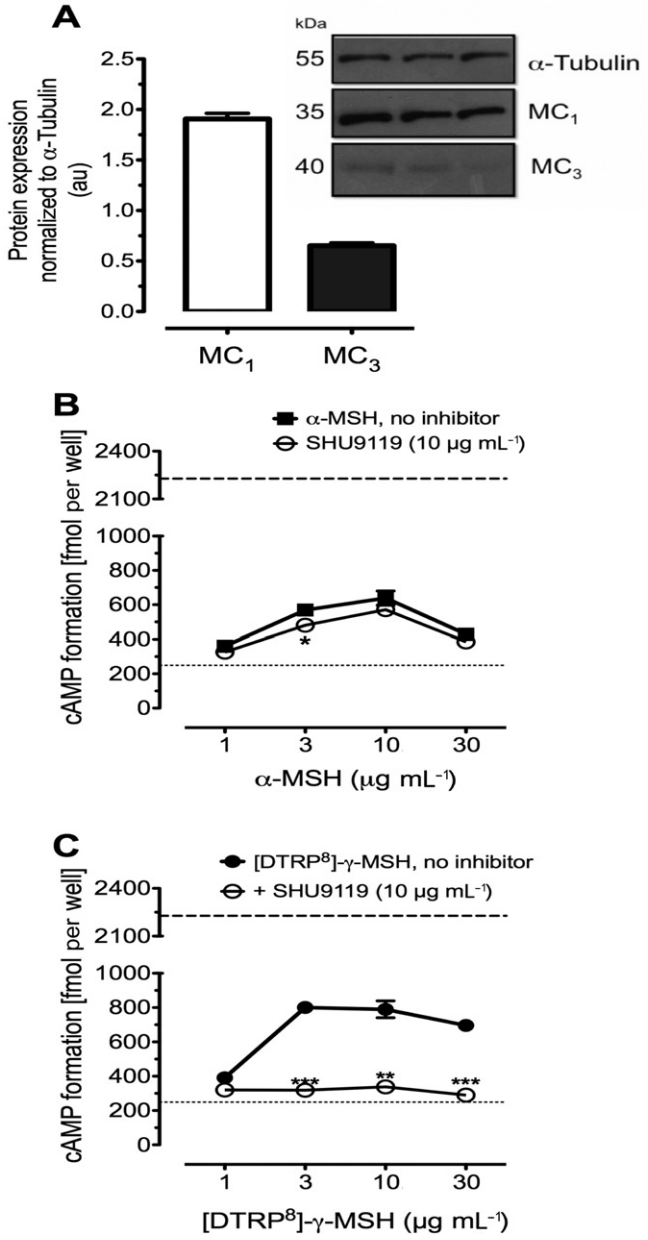

Figure 2

Endogenous expression of functionally active MC_1 and MC_3 receptors in C-20/A4 chondrocytes. Western blotting was used to determine MC_1 and MC_3 protein levels using rabbit anti-MC_1 and rabbit anti-MC_3 mAbs (1:2000). Bands with sizes corresponding to MC_1 (35 kDa), MC_3 (40 kDa) and α-tubulin (55 kDa) were detected and densitometrically quantified (A). C-20/A4 chondrocytes were stimulated with 1–30 μg·mL^{-1} α-MSH (B) or [DTRP^8]-γ-MSH (C) alone or in the presence of SHU9119 (10 μg·mL^{-1}) for 30 min before measuring cAMP concentration by EIA. Dotted lines indicate basal cAMP accumulation in PBS-treated cells, whilst dashed lines indicate maximal accumulation of cAMP in forskolin-treated C-20/A4 cells. Data are presented as mean ± SEM of three independent experiments for Western blot analysis and $n = 6$ samples for cAMP accumulation, $^{*}P \leq 0.05$, $^{***}P \leq 0.01$ significantly different from PBS-treated control cells.

caused a less pronounced cAMP accumulation. (Figure 2 B and C).

The selectivity of responses to α-MSH and [DTRP^8]-γ-MSH on cAMP production was also evaluated by co-stimulating the chondrocytes with the peptides alone or in the presence of the $MC_{3/4}$ receptor antagonist SHU9119 (10 μg·mL^{-1}). SHU9119 treatment caused no significant inhibition of α-MSH-induced cAMP production (Figure 2B) but in contrast caused a ~84% reduction in the cAMP accumulation elicited by [DTRP^8]-γ-MSH, at all concentrations tested ($P \leq 0.01$; Figure 2C).

α-MSH and D[TRP^8]-γ-MSH inhibit TNF-α-induced cytokine but not PGE_2 release from C-20/A4 chondrocytes

As C-20/A4 chondrocytes express functionally active MC_1 and MC_3 receptors and TNF-α (60 pg·mL^{-1}) stimulation caused significant release of the pro-inflammatory mediators IL-1β, IL-6 and IL-8, we tested the effects of α-MSH and [DTRP^8]-γ-MSH (0.1–30 μg·mL^{-1}) on the release of these cytokines and PGE_2.

α-MSH and [DTRP^8]-γ-MSH inhibited IL-1β release in a bell-shaped manner, with a maximal reduction of 85% and 73%, respectively, observed at 3 μg·mL^{-1} (Figure 3A and B); whilst higher concentrations did not sustain this level of inhibition. IL-6 release was inhibited by α-MSH, with 3.0 μg·mL^{-1} being the most effective concentration causing a 72.1% reduction (Figure 3C), whilst [DTRP^8]-γ-MSH (1 and 3 μg·mL^{-1}) caused a similar maximal degree of inhibition of approximately 60% (Figure 3D), with higher concentrations not sustaining this level of inhibition. IL-8 levels were reduced in a bell-shaped fashion for α-MSH, with a maximal inhibition of 60% observed at 3 μg·mL^{-1} (Figure 3E), whilst [DTRP^8]-γ-MSH caused a similar reduction in IL-8 release with a maximal inhibition of 76% detected at 10 μg·mL^{-1} [DTRP^8]-γ-MSH, higher concentrations of either peptide did not sustain this level of inhibition (Figure 3F). In order to further elucidate the effects of the melanocortin peptides, α-MSH and [DTRP^8]-γ-MSH, on TNF-α stimulated cytokine production, the specific $MC_{3/4}$ receptor antagonist, SHU9119, was used to selectively block the function of the MC_3 receptor. As expected, SHU9119 did not significantly block α-MSH (3 μg·mL^{-1}) mediated inhibition of IL-1β, IL-6 and IL-8 release (Figure 4A–C), but did abolish the effect of [DTRP^8]-γ-MSH (3 μg·mL^{-1}) (Figure 4A–C). Following identification that α-MSH and [DTRP^8]-γ-MSH inhibited cytokine release, their effect on TNF-α (60 pg·mL^{-1}) induced PGE_2 release was determined, as PGE_2 has been shown to inhibit *MMP1* and *MMP13* expression in chondrocytes (Nishitani *et al.*, 2010). TNF-α (60 pg·mL^{-1}), significantly increased PGE_2 release, compared with control cells. However, in contrast to their effects on pro-inflammatory cytokine release, neither α-MSH nor [DTRP^8]-γ-MSH (3 μg·mL^{-1}) significantly altered TNF-α stimulated PGE_2 levels, inducing only a ~20% and ~14% reduction respectively (Table 1).

α-MSH and D[TRP^8]-γ-MSH inhibit MMP1, MMP3 and MMP13 gene expression in TNF-α-activated chondrocytes

Given the role *MMP1*, *MMP3* and *MMP13* play in the pathogenesis of OA (Lawyer *et al.*, 2011), we determined the effects of α-MSH and D[TRP^8]-γ-MSH on *MMP1*, *MMP3* and *MMP13* gene expression. TNF-α treatment caused significant increases in *MMP1*, *MMP3* and *MMP13* gene expression 6 h post challenge, whilst pretreatment with α-MSH (3 μg·mL^{-1}) led to a significant fivefold reduction in mRNA levels for *MMP1* as well as a 1.9-fold reduction in *MMP3* and a threefold reduction in *MMP13* gene expression compared with control (Figure 5A–D). Similarly, [DTRP^8]-γ-MSH (3 μg·mL^{-1}) treatment also dramatically reduced *MMP1*, *MMP3* and *MMP13* gene expression by 9-, 4.2- and12.5-fold, respectively, compared with control (Figure 5A–D). Treatment of cells with the $MC_{3/4}$ receptor antagonist, SHU9119 (10 μg·mL^{-1}) synergistically enhanced the effect of α-MSH (3 μg·mL^{-1}) in down-regulating *MMP13* gene expression (Figure 5A and D) with an 8.3-fold decrease compared with control ($P \leq 0.001$). In contrast, it had no significant effect on α-MSH's ability to reduce *MMP1* and *MMP3* gene expression; whilst SHU9119 abolished the inhibitory effects of [DTRP^8]-γ-MSH on *MMP1*, *MMP3* and *MMP13* gene expression (Figure 5A–D).

α-MSH and [DTRP^8]-γ-MSH induce IL-10 release from chondrocytes

Following identification that α-MSH and [DTRP^8]-γ-MSH inhibited the release of both pro-inflammatory cytokines and metalloproteinases, we evaluated their ability to promote release of the anti-inflammatory cytokine IL-10.

α-MSH and [DTRP]-γ-MSH (0.1–30 μg·mL^{-1}) both significantly increased IL-10 release compared with untreated chondrocytes, with α-MSH at 1 μg·mL^{-1} causing a maximal 29-fold increase (Figure 6A) and [DTRP^8]-γ-MSH at 3.0 μg·mL^{-1} causing a maximal 21-fold release (Figure 6B), although higher concentrations of both peptides did not sustain the same levels of increase. These increases in IL-10 release were antagonized by the $MC_{3/4}$ receptor antagonist, SHU9119 for [DTRP^8]-γ-MSH but not for α-MSH (Figure 6C).

α-MSH and [D-TRP^8]-γ-MSH inhibit caspase-3/7 activation and cell death in TNF-α-activated chondrocytes

As melanocortin peptides both inhibited production of pro-inflammatory IL-1β, IL-6, IL-8 and induced release of the anti-inflammatory and chondroprotective cytokine IL-10, we examined their effects on chondrocyte death and apoptosis. TNF-α (60 pg·mL^{-1}) caused a 26% reduction in chondrocyte viability and increased caspase-3/7 activity 5.7-fold, compared with unstimulated (control) cells (Figure 7A). α-MSH and [DTRP^8]-γ-MSH alone had no effect on chondrocyte viability as detected via MTT assay, or caspase-3/7 activity (data not shown). However, each peptide inhibited TNF-α-induced chondrocyte death, with a maximal protection of 25% observed at 3 μg·mL^{-1} α-MSH (Figure 7A), with a similar protective effect observed for 3–30 μg·mL^{-1} [DTRP^8]-γ-MSH ($P \leq 0.01$; Figure 7B). TNF-α stimulation caused a 25% ($P \leq 0.05$) increase in the production of cleaved caspase-3, with α-MSH and [DTRP^8]-γ-MSH (3 μg·mL^{-1}) reducing activated caspase-3 by 50% and 42% respectively ($P \leq 0.01$; Figure 7C). The $MC_{3/4}$ receptor antagonist, SHU9119, failed to antagonize the anti-apoptotic properties of α-MSH but reduced the effectiveness of the selective MC_3 receptor agonist, [DTRP^8]-γ-MSH (Figure 7C).

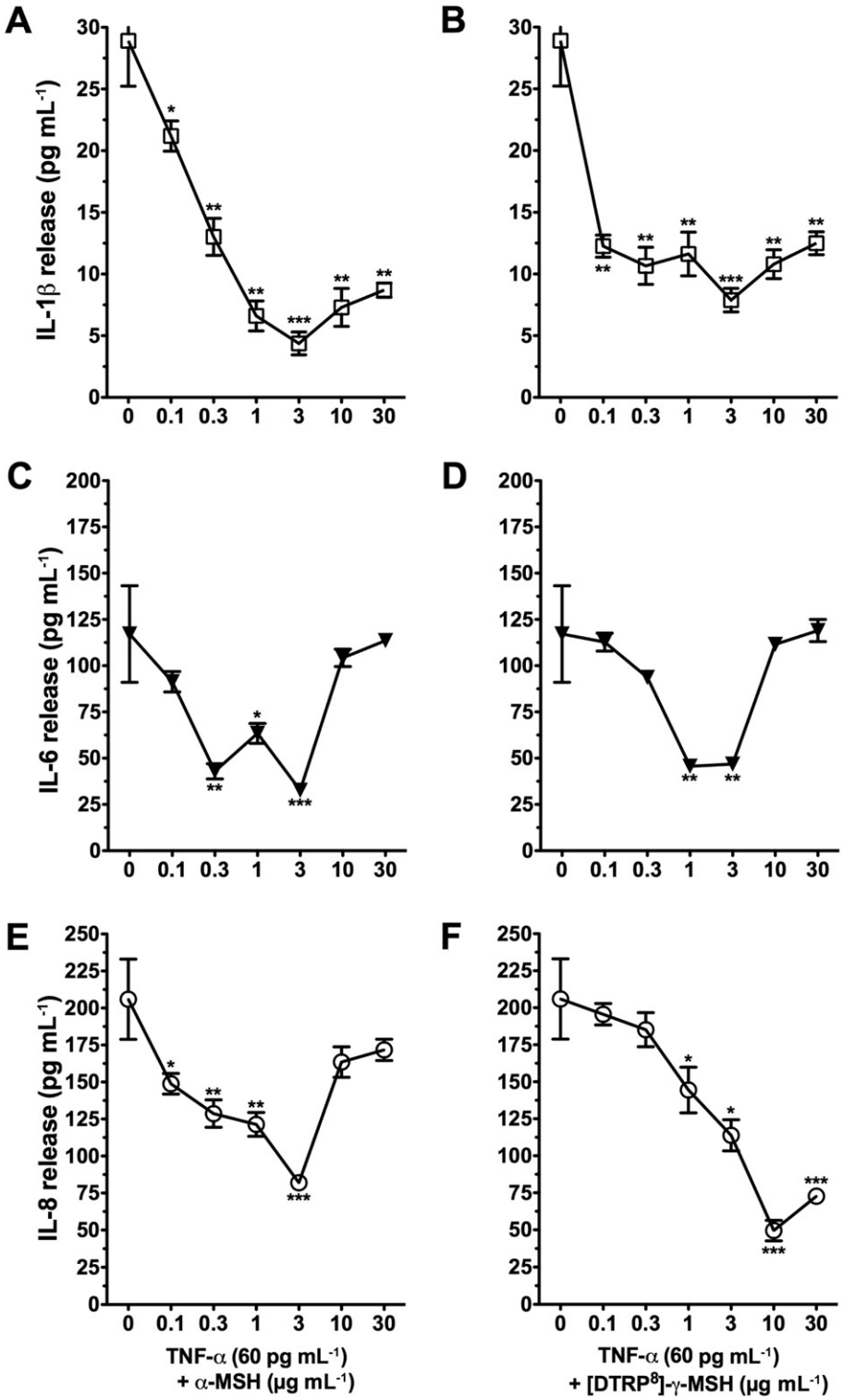

Figure 3

[DTRP^8]-γ-MSH and α-MSH inhibit IL-1β, IL-6 and IL-8 release from C-20/A4 chondrocytes. C-20/A4 chondrocytes were pretreated for 30 min with PBS, α-MSH or [DTRP^8]-γ-MSH (0.1–30 μg·mL^{-1}) prior to stimulation with TNF-α (60 pg·mL^{-1}); and cell-free supernatants were collected 6 h post stimulation and analysed for IL-1β (A and B), IL-6 (C and D) and IL-8 (E and F) concentration by ELISA. Data are presented as mean ± SEM of n = 4 independent experiments repeated in triplicate, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, significantly different from TNF-α alone.

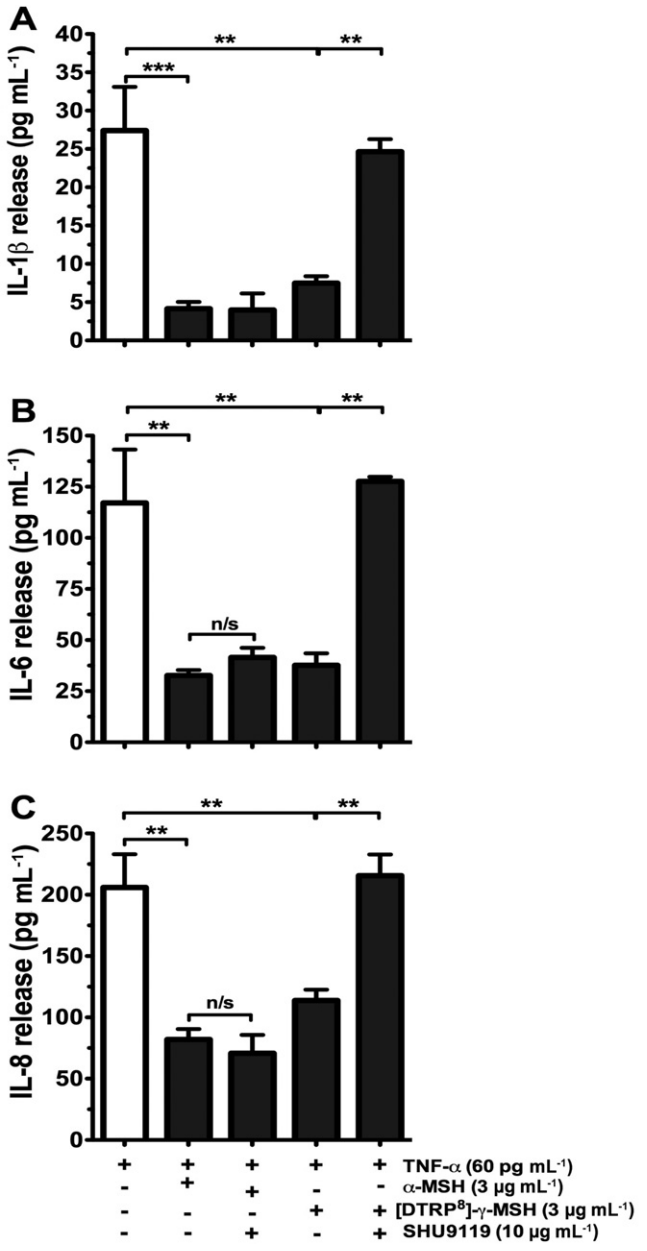

Figure 4

SHU9119 antagonizes [DTRP^8]-γ-MSH but not α-MSH inhibition of IL-1β, IL-6 and IL-8 release from TNF-α stimulated C-20/A4 chondrocytes. C-20/A4 chondrocytes were left untreated or were pre-incubated for 1 h with SHU9119 (10 μg·mL^{-1}) before α-MSH or [DTRP^8]-γ-MSH (3 μg·mL^{-1}) treatment for 30 min. Cells were then stimulated with TNF-α (60 pg·mL^{-1}), and cell-free supernatants were collected 6 h later and analysed for IL-1β (A), IL-6 (B) and IL-8 (C) levels by ELISA. Data are presented as mean ± SEM of n = 4 independent experiments repeated in triplicate, *$P \leq 0.05$, **$P \leq 0.01$, significantly different as shown.

Discussion

In this study we have determined the chondroprotective and anti-inflammatory properties of the selective MC_3 receptor agonist D[TRP^8]-γ-MSH and the pan-MC receptor agonist α-MSH on TNF-α induced pro-inflammatory cytokine release, MMP gene expression, caspase-3/7 activity and cell viability in C-20/A4 chondrocytes. The data produced indicated that α-MSH and [DTRP^8]-γ-MSH reduced pro-inflammatory cytokine release and MMP gene expression whilst preventing C-20/A4 chondrocyte death via reduction in caspase-3 and caspase-7 activity.

Melanocortin peptides have been shown to possess potent anti-inflammatory, anti-pyretic (Getting *et al.*, 2009) and pro-resolving properties (Patel *et al.*, 2010; Montero-Melendez *et al.*, 2011) in a a number of experimental models of inflammation, including cell lines (Lam *et al.*, 2006), primary human cells (Capsoni *et al.*, 2009) and rodent models of both rheumatoid (Patel *et al.*, 2010) and gouty (Getting *et al.*, 2002; 2006a) arthritis. These peptides down-regulate the host inflammatory response by inhibiting both leukocyte migration and the release of pro-inflammatory cytokines and chemokines, including TNF-α, IL-1β, IL-6 and IL-8 (Getting *et al.*, 2002, Grässel *et al.*, 2009). To date, two melanocortin receptors, MC_1 and MC_3, have been shown to mediate these anti-inflammatory effects via the adenylate cyclase–PKA pathway (Getting *et al.*, 2006a; 2008; Lam *et al.*, 2006), leading to a reduction in pro-inflammatory cytokine production (Delgrado *et al.*, 1998) as well as an induction of anti-inflammatory and pro-resolving proteins IL-10 and haem oxygenase 1 (Lam *et al.*, 2005; 2006), which aid in the resolution of inflammation (Montero-Melendez *et al.*, 2011). However, to date, only two studies have evaluated the role of melanocortin peptides on chondrocytes (Evans *et al.*, 2004; Grässel *et al.*, 2009), which is somewhat surprising given the role that chondrocytes play in the development of OA and the anti-inflammatory and pro-resolving effects of melanocortin peptide treatment in other models of arthritis (Getting *et al.*, 2002; Patel *et al.*, 2010).

In this study, we have tested the hypothesis that targeting melanocortin receptors in C-20/A4 chondrocytes may provide a novel therapeutic approach to inhibit pro-inflammatory cytokine production, MMP expression and reduce cell death associated with activation of apoptoic pathways. Current development of therapeutic strategies that prevent cartilage matrix degradation and allow cartilage repair largely depend on the accessibility of human cell

Table 1

Lack of effect of α-MSH and D[TRP^8]-γ-MSH on TNF-α-induced PGE_2 release

Pretreatment	Stimulus	PGE_2 release (pg·mL^{-1})
None (control)	None	93 ± 13
None	TNF-α (60 pg·mL^{-1})	180 ± 6*
α-MSH (3 μg·mL^{-1})	TNF-α(60 pg·mL^{-1})	145 ± 28
[DTRP^8]-γ-MSH (3 μg·mL^{-1})	TNF-α(60 pg·mL^{-1})	154 ± 33

Data are mean ± SEM of n = 4 of three determinations; *$P \leq$ 0.05 compared with control cultures; one-way ANOVA, Dunnett's multiple comparison test.

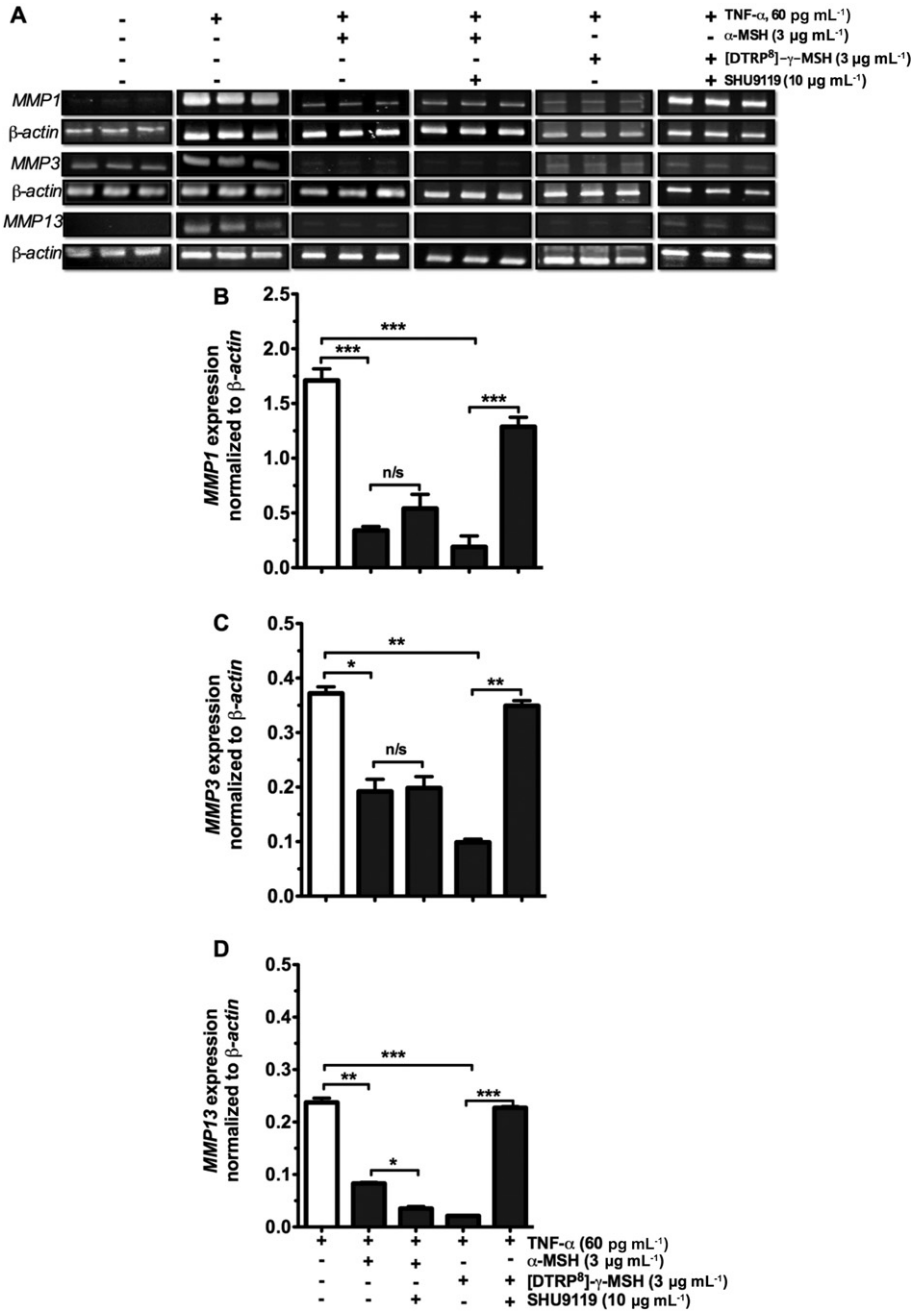

Figure 5

SHU9119 antagonizes the inhibitory effect of [DTRP^8]-γ-MSH but not α-MSH on *MMP1, MMP3* and *MMP13* mRNA expression in C-20/A4 chondrocytes. C-20/A4 chondrocytes were left untreated or pretreated for 1 h with SHU9119 (10 μg·mL^{-1}) before α-MSH or [DTRP^8]-γ-MSH (3 μg·mL^{-1}) treatment for 30 min. Cells were then stimulated with TNF-α (60 pg·mL^{-1}), and total RNA was extracted at 6 h later. Oligonucleotide primers specific for *MMP1, MMP3* and *MMP13* were used to detect and quantify gene expression by PCR followed by analysis on 2% agarose gels in triplicates, with β-actin used as an internal control (A). Comparison of densitometrically quantified *MMP1, MMP3* and *MMP13* gene expression levels for α-MSH, [DTRP^8]-γ-MSH ± SHU9119 (B, C and D) are shown in arbitrary units, each value normalized to the respective β-actin expression. Data are presented as mean ± SEM of $n = 4$ independent experiments *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, significantly different as shown.

culture models. However, primary articular human chondrocytes are difficult to obtain and lose chondrocytic phenotype when expanded in monolayer cultures (Shakibaei *et al.*, 1997), with up-regulation in *COL1A1* and decreases in *COL2A1* expression observed (Benya and Shaffer, 1982; Zwicky and Baici, 2000). The C-20/A4 human chondrocyte cell lines display stable expression of the *COL2A1* gene similar to human primary chondrocytes (Loeser *et al.*, 2000). Therefore, for the purposes of this research, the stably differentiated chondrocytic cell line C-20/A4 was used as a tool for

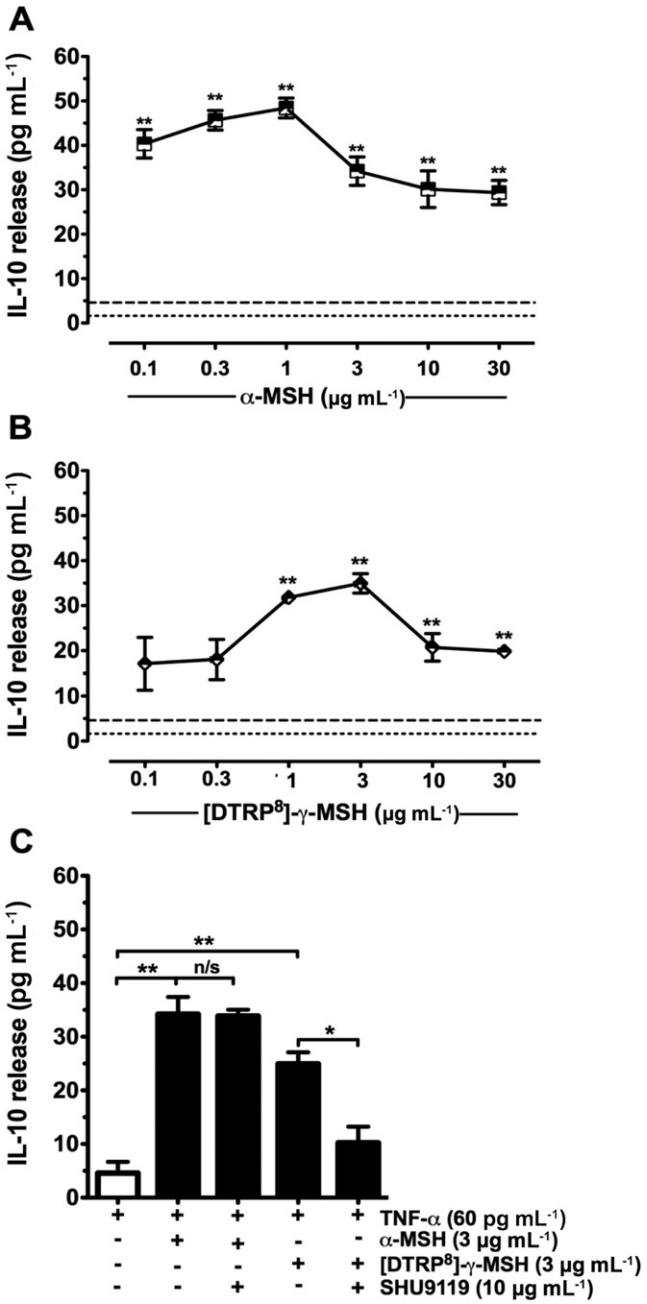

Figure 6

α-MSH and [DTRP^8]-γ-MSH stimulate IL-10 release from C-20/A4 chondrocytes. C-20/A4 chondrocytes were pretreated for 30 min with PBS, α-MSH or [DTRP^8]-γ-MSH (0.1–30 μg·mL^{-1}) before stimulation with TNF-α (60.0 pg·mL^{-1}); and cell-free supernatants were collected 6 h later and analysed for IL-10 (A and B) levels by ELISA. In separate experiments, C-20/A4 chondrocytes were left alone or pretreated for 1 h with SHU9119 (10 μg·mL^{-1}) before α-MSH or [DTRP^8]-γ-MSH (3 μg·mL^{-1}) treatment for 30 min. Cells were then stimulated with TNF-α (60 pg·mL^{-1}), and cell-free supernatants were collected 6 h later and analysed for IL-10 (C) concentration by ELISA. Dotted line indicates control levels and dashed line is TNF-α (60 pg·mL^{-1}) treated cells alone. Data are presented as mean ± SEM of $n = 4$ independent experiments repeated in triplicate. In A and B, $^{**}P \leq 0.01$, significantly different from TNF-α alone; in C, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, significantly different as shown.

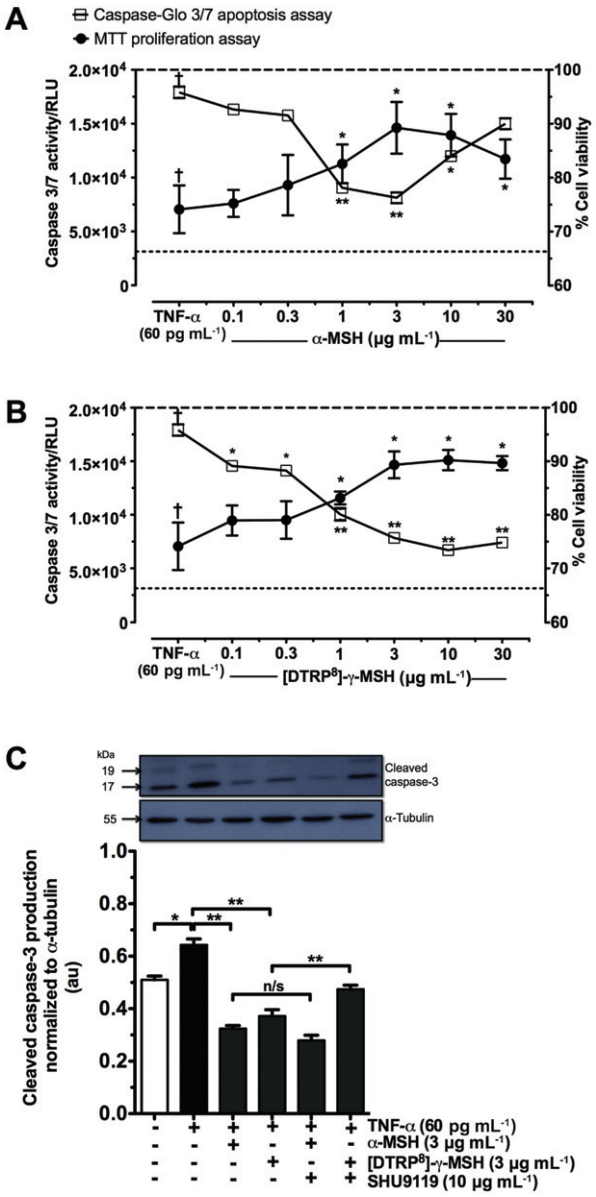

Figure 7

α-MSH and [DTRP^8]-γ-MSH modulate caspase-3/7 activity and protein levels and cell viability in human C-20/A4 chondrocytic cells. C-20/A4 chondrocytes were treated for 6 h with α-MSH or [DTRP^8]-γ-MSH (0.1–30 μg·mL^{-1}) alone or in the presence of SHU9119 (10 μg·mL^{-1}) for 30 min before TNF-α (60 pg·mL^{-1}) stimulation. Caspase-3/7 activity was determined by caspase-3/7 Glo assay, and cell viability was determined by the MTT reduction assay (A and B). The dashed line represents control sample cell viability (i.e. untreated cells as determined by MTT; 100%). The dotted line shows caspase-3/7 activity following DMEM treatment (control) (A and B). Cleaved caspase-3 (Asp^{175}; 17, 19 kDa) and α-tubulin (55 kDa) were detected by Western blotting; the image is representative of four individual experiments (C). Comparison of densitometrically quantified cleaved caspase-3 (Asp^{175}) expression in human C-20/A4 cell lines is shown in arbitrary units (au; C). Dotted line indicates control levels of caspase-3/7 activity, and dashed line is control cell viability. Data are presented as mean ± SEM of $n = 4$ experiments, assessed in triplicate. In A and B, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, significantly different from TNF-α-treated controls; in C, . $^{*}P \leq 0.05$, significantly different as shown.

the characterization and further validation of the role of melanocortin peptides in chondrocytes.

Intital experiments showed expression of MC_1 and MC_3 receptors in C-20/A4 chondrocytes, confirming previous studies highlighting gene expression of MC_1 in the human chondrosarcoma cell line HTB-94 and primary articular chondrocytes (Grässel *et al.*, 2009), although MC_3 receptor expression could not be detected in these cells (Grässel *et al.*, 2009). It is conceivable that the differences between our findings and those of the other groups might be due to variations in the cell lines used, differences in experimental conditions or in the origin of the cells (primary chondrocytes extracted from healthy or OA articular cartilage). It is also plausible that melanocortin receptor expression may be altered depending on resting state or following stimulation as a recent study (Muffley *et al.*, 2011) has demonstrated that MC_1 and α-MSH protein levels are up-regulated in human burn wounds and hypertrophic scars compared with uninjured human skin where both receptor and ligand were absent (Muffley *et al.*, 2011).

TNF-α and IL-1β play a pivotal role in the pathogenesis of OA and RA up-regulating MMP gene expression and pro-inflammatory cytokine production (Fernandes *et al.*, 2002; Lawyer *et al.*, 2011). In this study, TNF-α was selected as it is one of the major cytokines produced by chondrocytes, and it activates effector caspases driving apoptosis (Stanic *et al.*, 2006). TNF-α stimulation led to a marked increase in IL-1β, IL-6 and IL-8 release in a time-dependent fashion as well as an up-regulation in *MMP1*, *MMP3* and *MMP13* gene expression, which are established as important mediators involved in cartilage degradation in OA (Grässel *et al.*, 2009). These data confirm previous findings in primary chondrocytes, where TNF-α triggered a marked upregulation of pro-inflammatory cyokines (Rai *et al.*, 2008) and cartilage destruction (Kobayashi *et al.*, 2005), thereby validating the choice of C-20/A4 chondrocytes as a system for evaluating the effects of melanocortin peptides on these parameters.

The pan-agonist α-MSH (Getting *et al.*, 2008), selective MC_3 receptor agonist [$DTRP^8$]-γ-MSH (Grieco *et al.*, 2000; Getting *et al.*, 2006a; b) and $MC_{3/4}$ receptor antagonist SHU9119 (Getting *et al.*, 1999) were used to confirm receptor functionality. α-MSH and [$DTRP^8$]-γ-MSH both caused an accumulation of intracellular cAMP in C-20/A4 chondrocytes, confirming previous findings seen with α-MSH in human primary chondrocytes (Grässel *et al.*, 2009). The MC_3 receptor agonist, [$DTRP^8$]-γ-MSH, previously shown to induce cAMP production in murine peritoneal and alveolar macrophages (Getting *et al.*, 2006a; 2008), also caused increases in cAMP in C-20/A4 chondrocytes, suggesting the presence of functionally active MC_3 receptors. These increases by [$DTRP^8$]-γ-MSH in cAMP levels were abolished by the $MC_{3/4}$ receptor antagonist, SHU9119, in agreement with findings in murine macrophages (Getting *et al.*, 2006a), again confirming the presence of MC_3 receptors on these C-20/A4 chondrocytes.

α-MSH and [$DTRP^8$]-γ-MSH inhibited IL-1β, IL-6 and IL-8 release from C-20/A4 chondrocytes, with the anti-inflammatory properties of D[TRP^8]-γ-MSH antagonized by SHU9119. This ability of melanocortin peptides to reduce cytokine release has been well documented in both *in vitro* and *in vivo* murine studies (Getting *et al.*, 2006a; 2008), and the effect observed here agrees with the previous data obtained using human articular chondrocytes (Grässel *et al.*, 2009) in which IL-1β was reduced by α-MSH. However, in contrast to Grässel *et al.* (2009), we also demonstrated that α-MSH and D[TRP^8]-γ-MSH inhibit IL-6 and IL-8 release. Interestingly a recent study using zymosan-stimulated macrophages showed that a synthetic α-MSH analogue (AP214) caused a 40% reduction in IL-6 (Montero-Melendez *et al.*, 2011), supporting the data observed here. A potential reason for the differences in IL-6 release between our study and the one of Grässel *et al.*, (2009) could be the loss of phenotype in human chondrocyte monolayers (Shakibaei *et al.*, 1997), or that in this study and that of Montero-Melendez *et al.* (2011), the cells were stimulated with an inflammogen rather than freshly isolated.

Cytokine-induced up-regulation of MMP1, MMP3 and MMP13 activities plays a pivotal role in the pathogenesis of OA (Goldring *et al.*, 2011; Lawyer *et al.*, 2011) and has been shown to be reduced by calcitonin in articular chondrocytes via up-regulation of the cAMP–PKA pathway (Karsdal *et al.*, 2007). Following the inhibition of cytokine release by α-MSH and [$DTRP^8$]-γ-MSH, their effects on *MMP1*, *MMP3* and *MMP13* gene expression were determined. Both peptides strongly inhibited *MMP1, MMP3* and *MMP13* gene expression; this effect is novel for [$DTRP^8$]-γ-MSH and confirms previous studies with α-MSH in the human chondrosarcoma cell line, HTB-94 (Yoon *et al.*, 2008), and in human chondrocytes (Grässel *et al.*, 2009) with respect to inhibition of *MMP13* expression. The inhibitory effect of [$DTRP^8$]-γ-MSH was lost following co-treatment with the $MC_{3/4}$ antagonistSHU9119, suggesting again an involvement of MC_3 receptors in regulating MMP expression. α-MSH inhibited all MMPs evaluated; however, co-treatment with SHU9119 caused a synergistic inhibition of *MMP13* gene expression, thus possibly suggesting a compensatory role for the MC_1 receptor following antagonism at MC_3. At present, it is not possible in this model to determine whether melanocortins have a direct effect on MMP expression or via their ability to inhibit cytokine-induced MMP expression (Lawyer *et al.*, 2011). As both [$DTRP^8$]-γ-MSH and α-MSH inhibited pro-inflammatory cytokines and MMP1, MMP3 and MMP13 expression, their effect on the prostanoid PGE_2 was determined as PGE_2 inhibits MMP1 and MMP13 expression induced by IL-1β (Nishitani *et al.*, 2010). A non-significant reduction in PGE_2 was observed following treatment with both melanocortin peptides. This modulatory effect is interesting because increased PGE_2 has been proposed to be chondro-destructive (Nah *et al.*, 2008), whilst other studies show long-term use of NSAIDs reduces PGE_2, leading to accelerated progression of OA (Reijman *et al.*, 2005). Therefore, peptides that increase PGE_2 expression as opposed to decreasing it may be beneficial in the long-term managment of this pathology, thus suggesting activation of melanocortin receptors may play an important role in maintaining cartilage integrity.

Previous studies have shown a role for IL-10 in mediating the anti-inflammatory effects of α-MSH in murine models of eosinophil migration (Grabbe *et al.*, 1996) and RAW264.7 macrophage cell lines (Lam *et al.*, 2006), whilst studies in both TNF-α stimulated OA and healthy primary human chonodrocytes show that IL-10 is chondroprotective by reducing *MMP1* and *MMP13* gene expression (Gonzalez-Rey

et al., 2007; Muller *et al.*, 2008). We therefore addressed the question of whether α-MSH and [DTRP^8]-γ-MSH could induce IL-10 production in C-20/A4 chondrocytes. Our results indicated that both α-MSH and [DTRP^8]-γ-MSH significantly increased IL-10 production, with SHU9119 antagonizing the effect of [DTRP^8]-γ-MSH but not that of α-MSH. These data suggest the exciting idea that melanocortin peptides (in part) exert a homeostatic control over chondrocyte physiology, with an ability to induce chondroprotective and pro-resolving cytokines, and a possible role in resolving chondrocyte-borne inflammation.

Chondrocyte death has also been shown to play an important role in cartilage degradation and progression of diseases such as OA and RA (Aigner and Kim, 2002; Oppenheimer *et al.*, 2012), with TNF-α shown to promote apoptosis (Stanic *et al.*, 2009) via caspase-3 and caspase-7 activation (Luthi and Martin, 2007; Facchini *et al.*, 2011; Lee *et al.*, 2011). In this study, TNF-α significantly increased the production of cleaved caspase-3 and caspase-3 and -7 activities by 30%. Treatment of cells with both α-MSH and [DTRP^8]-γ-MSH led to an inhibition of executioner caspase-3 and -7, with their effects largely mediated by MC_1 and MC_3 receptors, as co-administration with the $MC_{3/4}$ antagonist, SHU9119, prevented [DTRP^8]-γ-MSH but not α-MSH from inhibiting activated caspase-3 production. These pro-survival effects of melanocortin peptides have also been demonstrated by Chai *et al.* (2006), who showed that the non-selective melanocortin peptide, NDP-MSH, inhibited caspase-3 activation in the neuronal cell line GT1-I, whilst α-MSH has also been shown to prevent LPS/INF-γ-induced astrocyte apoptosis (Caruso *et al.*, 2007). However, to our knowledge, this is the first demonstration that α-MSH and [DTRP^8]-γ-MSH can prevent the production and activation of executioner caspases and initiation of chondrocyte death.

In conclusion, this study demonstrated that the anti-inflammatory, chondroprotective and anti-apoptotic effects of melanocortin peptides in C-20/A4 chondrocytes were largely mediated via the MC_1 and MC_3 receptors. These data provide a rationale for the further investigation of these peptides as chondroprotective agents in OA and RA.

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Conflict of interest

PG holds a patent on the MC_3 agonist.

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