Urocortin is a novel regulator of osteoclast differentiation and function through inhibition of a canonical transient receptor potential 1-like cation channel

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Abstract

This study investigated the role of urocortin (UCN), a member of the corticotrophin-releasing factor (CRF) family of peptides, in osteoclast maturation and function. We found that 10^{-7} M UCN significantly ($P < 0.05$) suppressed osteoclast differentiation from bone marrow precursor cells in culture and reduced the expression of several osteoclastic markers. Furthermore, UCN potently suppressed osteoclast bone resorption, by significantly inhibiting both the plan area of bone resorbed by osteoclasts and actin ring formation within osteoclasts at 10^{-9} M ($P < 0.05$), with complete inhibition at 10^{-7} M ($P < 0.001$). UCN also inhibited osteoclast motility (10^{-7} M) but had no effect on osteoclast survival. Osteoclasts expressed mRNA encoding both UCN and the CRF receptor 2 β subtype. Pre-osteoclasts however,

expressed CRF receptor 2 β alone. Unstimulated osteoclasts contained constitutively active cation channel currents with a unitary conductance of 3–4 pS, which were inhibited by over 70% with UCN (10^{-7} M). Compounds that regulate calcium signalling and energy status of the cell, both crucial for osteoclast activity were investigated. The non-selective cation channel blockers, lanthanum (La^{3+}) and gadolinium (Gd^{3+}), inhibited actin ring formation in osteoclasts, whereas modulators of voltage-dependent Ca^{2+} channels and K_{ATP} channels had no effect. These findings show for the first time that UCN is a novel anti-resorptive molecule that acts through a direct effect on osteoclasts and their precursor cells.

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Introduction

Urocortin (UCN) is a member of the corticotrophin-releasing factor (CRF) family of peptides. It was originally cloned from a rat brain cDNA library, using a probe homologous to CRF (Vaughan *et al.* 1995). Since then, two new members have been isolated, UCN 2 and 3, which are equivalent to human stresscopin-related peptide and stresscopin respectively (Hsu & Hsueh 2001, Lewis *et al.* 2001, Reyes *et al.* 2001, Fekete & Zorrilla 2007). All three UCNs are synthesised as precursors of ~ 200 amino acids, which are then subsequently cleaved to yield the active peptides, which in the case of UCN is 40 amino acids (Fekete & Zorrilla 2007). The level of functional availability of UCN is closely regulated by a high affinity CRF-binding protein (CRF-BP), expressed at both release and activation sites of UCN, which acts as a decoy receptor to terminate the ligand signal (Behan *et al.* 1995, Seasholtz *et al.* 2002).

UCN binds to two G-protein-coupled receptor subtypes, CRF receptor 1 (CRFR1) and CRFR2, with further

receptor diversity produced by alternative RNA splicing (Chen *et al.* 1993, Lovenberg *et al.* 1995, Perrin & Vale 1999, Hauger *et al.* 2003, Fekete & Zorrilla 2007). Radioligand binding studies have demonstrated that CRF and UCN can bind to both CRFR1 and CRFR2, whereas UCN 2 and 3 bind exclusively to CRFR2. UCN has been shown to act predominantly through both $\text{G}_{\alpha\text{s}}$ and $\text{G}_{\alpha\text{q}}$ G-protein subunits to stimulate diverse signalling cascades, through respectively, adenylate cyclase (AC) and phospholipase C (PLC) pathways, depending on the tissue type (Grammatopoulos *et al.* 2000, Cantarella *et al.* 2001, Graziani *et al.* 2002, McEvoy *et al.* 2002, Bayatti *et al.* 2003, Karteris *et al.* 2005). UCN-mediated activation of these signal transduction pathways modulates a variety of end effectors including ion channels involved in calcium signalling and energy metabolism, such as L-type voltage-gated calcium channels, store-operated channels and K_{ATP} channels (Lawrence *et al.* 2002, Tao *et al.* 2004, 2006, Smani *et al.* 2007).

The CRF family members have been linked to a number of physiological and pathological conditions. CRF is the classic

mediator of the hypothalamic stress response, initiating an anxiogenic, neuroendocrine and behavioural stress phenotype. In contrast, UCN plays a more anxiolytic role in this response and is also involved in appetite suppression and energy metabolism (Spina *et al.* 1996, Smagin *et al.* 1998, Tanaka *et al.* 2009). The peptides are also expressed in peripheral tissue, where they appear to be cytoprotective through CRFR2, in models of cellular stress (Brar *et al.* 2002, Lawrence & Latchman 2006). Recently, UCN has been found to be elevated in the synovial fluid of patients with rheumatoid arthritis (Kohno *et al.* 2001, Uzuki *et al.* 2001) and has also been linked to a reduction in inflammation and bone erosion in a mouse model of this disease (Gonzalez-Rey *et al.* 2007). However, there is little information on the role of UCN in the physiology or pathology of bone. Because UCN is the only member of this family capable of binding to both CRFR1 and 2, its use would give the best chance of detecting an effect on osteoclasts.

Osteoclasts are derived from the monocyte macrophage lineage; they are large multinucleated cells and are the only cells of the body capable of resorbing bone. They mature to form large tartrate-resistant acid phosphatase (TRAP) positive, multinucleated cells in the presence of receptor activator of NF κ B ligand (RANKL) and macrophage colony stimulating factor (M-CSF). Upon activation, osteoclasts locate to the bone surface and initiate resorption by creating a sealing zone, which is accompanied by a ruffled border and actin reorganisation (Väänänen *et al.* 2000). Excessive resorption by these cells is the cause of several common diseases of bone, including osteoporosis.

In this study we investigated the effects of UCN on the formation and function of osteoclasts. Our results clearly demonstrate that UCN is a regulator of osteoclast resorption, by suppressing osteoclast maturation and potentially inhibiting osteoclastic function. Furthermore, we provide evidence that the inhibitory action of UCN on osteoclasts is mediated by suppression of constitutively active cation channels that have properties characteristic of a canonical transient receptor potential 1 (TRPC1) channel.

Materials and Methods

Culture media and reagents

Cells were incubated in minimum essential medium (MEM) with Earle's salts, supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml benzylpenicillin and 100 μ g/ml streptomycin, UCN (all from Sigma), unless stated otherwise. Recombinant human M-CSF and soluble recombinant murine RANKL were purchased from PeproTech EC (London, UK). Recombinant murine interleukin 1 α (IL1 α) and purified human transforming growth factor- β 1 (TGF- β 1) were obtained from R&D Systems (Abingdon, Oxon, UK). Osteoblastic MC3T3-E1 cell line was from Bioresource Centre (Ibaraki, Japan). Cell incubations were

performed at 37 °C in 5% CO₂ in humidified air, unless stated otherwise. Slices of bovine cortical bone were prepared as previously described (Fuller *et al.* 2006).

Generation of osteoclasts

Osteoclast suspensions were prepared from murine bone marrow cells as previously described (Fuller *et al.* 2006). Briefly, MF1 mice (4–8 weeks old) were killed by cervical dislocation in accord with Home Office Ethical Guidelines. Femora and tibiae were aseptically removed and dissected free of adherent soft tissue. The bone ends were removed and the marrow cavity was flushed out into a Petri dish by slowly injecting PBS at one end of the bone using a sterile 25-gauge needle. The bone marrow suspension was passed repeatedly through a 21-gauge needle to obtain a single cell suspension. Bone marrow cells were then washed, re-suspended in MEM/FCS, and incubated at a density of 3×10^5 cells/ml for 24 h in a 75 cm² flask (Greiner Bio-One, Stonehouse, Gloucestershire, UK) containing M-CSF (50 ng/ml), to deplete the cell preparations of stromal cells. Non-adherent cells were collected by centrifugation and used immediately (pre-osteoclasts) or added to 90 mm diameter cell culture dishes (Greiner) in MEM/FCS, containing M-CSF (50 ng/ml), RANKL (30 ng/ml) and TGF- β 1 (0.1 ng/ml) (7.2×10^6 cells in 25 ml for each dish). Cultures were incubated for 5 days, when osteoclast numbers are maximal. Cells were fed every 2–3 days by replacing 15 ml of culture medium with an equal volume of fresh medium and cytokines. When multinuclear cells had formed, the medium was removed and the cell layer washed three times with PBS without calcium and magnesium. Six millilitres of 0.02% EDTA were added to the dish and cells incubated for 20 min at room temperature. The EDTA was then removed from the dish and replaced with 4 ml calcium/magnesium-free PBS. A cell scraper (Greiner) was used to scrape the cells into the PBS, and the resulting cell suspension was agitated using a pipette to ensure uniform cell dispersal for further use as described below.

Assessment of bone resorption and actin ring formation

Seventy-five microlitres of osteoclast-containing cell suspension were added to wells of a 96-well plate (Greiner), each of which contained a bone slice in 75 μ l MEM/FCS. Cells were allowed to sediment for 20 min at 37 °C before the bone slices were washed in PBS and transferred to fresh wells. Cells for assessment of resorption were incubated for 4 h in 200 μ l MEM/FCS in the presence of M-CSF (50 ng/ml), RANKL (30 ng/ml), IL1 α (10 ng/ml) with UCN (10^{-7} – 10^{-10} M) or vehicle. Osteoclasts for assessment of actin ring formation were incubated for 4 h in M-CSF, RANKL and IL1 α as above, before addition of UCN (10^{-7} – 10^{-10} M) or vehicle. Incubation was then continued for 30 min. In some actin ring formation experiments, ion channel modulators were also included.

To measure bone resorption, bone slices were immersed in 10% (v/v) sodium hypochlorite for 10 min to remove cells, washed, air-dried, mounted onto stubs for scanning electron microscopy and sputter coated with gold. The entire surface of each bone slice was examined blind and the total area resorbed per bone slice was quantified by scanning electron microscopy (S90: Cambridge Instruments, Cambridge, UK).

For assessment of actin rings, bone slices were fixed in 10% formalin. Cells were permeabilised with 0.1% Triton X-100 for 5 min, washed three times in PBS and then incubated with FITC-conjugated phalloidin (1 µg/ml) for 45 min at 37 °C. Bone slices were washed three times in PBS and mounted onto slides in fluorescence mounting medium (Dako, Ely, Cambridge, UK). Actin rings were visualized using a Zeiss fluorescent microscope and the numbers of actin rings in ten fields per bone slice were counted.

Assessment of osteoclast motility

Four hundred and fifty microlitres of osteoclast-containing cell suspension were added to 24-well plate wells (Greiner) containing 13 mm diameter glass coverslips in 450 µl MEM/FCS and cells were allowed to sediment for 20 min in an incubator. Coverslips were washed and transferred to 25 cm² tissue flasks (Greiner). Cells were allowed to equilibrate for a minimum of 1 h in 4.5 ml MEM/FCS then flasks were sealed and placed in an incubation chamber of an Olympus 1MT-373 inverted microscope (Gallenkamp and Co., Ltd, London, UK). A suitable field was chosen and recorded for 30 min by a time-lapse video recorder at 1/60 normal speed. UCN (10⁻⁷ M final concentration) was added in 0.5 ml pre-warmed MEM/FCS and recording was continued for a further 60 min.

Assessment of osteoclast apoptosis and number

Osteoclasts containing M-CSF (50 ng/ml) and RANKL (30 ng/ml) were incubated for 18 h with UCN (10⁻⁷ M), vehicle, or exposed to u.v. light (100 mJ/cm² for 5 min, as a positive control for the induction of apoptosis) and then incubated for 18 h. Proteins were extracted and the extent of cleaved caspase 3 (Asp175) and cleaved poly (ADP-ribose) polymerase (PARP (Asp214)) (Cell Signalling) NEB, was assessed by western blotting.

Osteoclasts underwent the same experimental conditions as those in the actin ring experiments and were either untreated or treated with UCN (10⁻⁷ M) for 30 min and then TRAP-positive cells displaying three or more nuclei were counted.

Western blotting

Osteoclasts were lysed in RIPA buffer: Tris-HCl (50 mM), pH 8.0, NaCl (150 mM), 0.5% (w/v) SDS and 1% (v/v) Nonidet P-40, containing 10 mM dithiothreitol (DTT) and protease inhibitor cocktail (Roche). Proteins were extracted

and resolved on a 10% SDS-PAGE gel and transferred onto Hybond-C nylon membrane (GE Healthcare, Chalfont St Giles, Bucks, UK). Membranes were blocked with PBS containing 4% (w/v) Marvel (milk powder) for 30 min. The blocking solution was removed and replaced with PBS containing 1% (w/v) Marvel. TRPC1, actin (Alomone Labs, Jerusalem, Israel), caspase 3 and PARP primary antibodies were incubated overnight at 4 °C. The next day, membranes were washed two times for 1 min in PBS containing 0.5% Tween and then the appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was applied in PBS containing 1% (w/v) Marvel and incubated for 2 h at room temperature. To visualise the proteins of interest, membranes were washed three times for 5 min in PBS containing 0.5% Tween and then exposed to ECL reagent (GE Healthcare) for 1 min before exposure to light sensitive film.

Preparation of calvaria-derived osteoblastic cells

Calvaria were removed from 3-day-old neonatal mice and dissected free of soft tissue and periosteum. They were incubated in PBS containing type II collagenase (1 mg/ml; type II) for 2 h at 37 °C with periodic agitation. After incubation, the calvaria were further disrupted using a wide bore Pasteur pipette. The cell suspension was then combined with DMEM containing 10% FCS and centrifuged at 400 g for 5 min. The cell pellet was resuspended in DMEM/FCS and plated at the desired density for 3 h after which the non-adherent cells were removed and fresh media added. The cells were incubated overnight.

MC3T3-E1 cells

MC3T3-E1 osteoblastic cell line was propagated in MEM/FCS supplemented with non-essential amino acids. Cells were allowed to reach 80% confluency before experiments were conducted.

Assessment of osteoclast differentiation

M-CSF-dependent, non-adherent murine bone marrow cells were prepared as described above and harvested. 3 × 10⁴ Cells were added to the wells of a 96-well plate (Greiner) containing 6 mm Thermanox coverslips (VWR International Ltd, Lutterworth, Leicester, UK) and incubated in 200 µl MEM/FCS with M-CSF (50 ng/ml), RANKL (30 ng/ml) and TGF-β1 (0.1 ng/ml) in the presence or the absence of UCN (10⁻⁹–10⁻⁷ M) from 1 to 5 days. After 5 days incubation, cells were fixed in 10% formalin for 10 min and stained for TRAP using the Leucognost-AP cytochemical reagent kit (VWR International Ltd). Osteoclast numbers were evaluated 'blind' by quantification of the number of TRAP-positive cells with three or more nuclei per square centimetre.

RT-PCR

Total RNA was extracted from cells and tissues using Trizol reagent (Invitrogen), DNase treated and purified using RNA MinElute columns (Qiagen). One microgram of total RNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen) and the cDNA amplified using REDTaq ReadyMix PCR Mix, in the presence of the relevant primer sets.

Assessment of osteoclastic markers

Non-adherent bone marrow cells were incubated in MEM/FCS, M-CSF (50 ng/ml), RANKL (30 ng/ml) and TGF- β (0.1 ng/ml), with or without UCN (10^{-10} – 10^{-7} M). After 5 days osteoclasts were assessed for expression of the calcitonin receptor (CT-R), cathepsin K (Cath K), TRAP and dendritic cell-specific transmembrane protein (DC-STAMP) (Nicholson *et al.* 1986) and GAPDH by RT-PCR using the following primers:

CT-R:

Forward: 5'-GTCTTGCAACTACTTCTGGATGC-3'

Reverse: 5'-AAGAAGAAGTTGACCACCAGAGC-3'

Cath K:

Forward: 5'-CACGGGAAGCAGTACAACAG-3'

Reverse: 5'-AGTCATCTTCTGAACCACTTCTTC-3'

TRAP:

Forward: 5'-TCCCCTGGTATGTGCTGG-3'

Reverse: 5'-GCATTTGGGCTGCTGA-3'

DC-STAMP:

Forward: 5'-CTAGCTGGCTGGACTTCATCC-3'

Reverse: 5'-TCATGCTGTCTAGGAGACCTC-3'

GAPDH:

Forward: 5'-GGTCTCGCTCCTGGAAGATG-3'

Reverse: 5'-CGGATTTGGCCGTATTGG-3'.

Expression of the UCN system and TRPC1 in bone cells

The expression of UCN, CRF receptors and CRF-BP was investigated using semi-quantitative RT-PCR as above in pre-osteoclasts, mature osteoclasts, MC3T3-E1 cells, calvaria-derived primary osteoblasts, heart and brain. Total RNA was extracted and cDNA synthesised as above and PCR was performed using the following primers:

UCN:

Forward: 5'-ACTGTCCATCGACCTCACCTTCCA-3'

Reverse: 5'-ACTGAGACAGCTCCGGTTGTGC-3'

All CRFR1 subtypes:

Forward: 5'-GGTGTGCCTTTCCCCATCATT-3'

Reverse: 5'-CAACATGTAGGTGATGCCCAG-3'

CRFR2 α specific:

Forward: 5'-CCTGGACGGCTGGAGTC-3'

Reverse: 5'-CAGAATGAAGGTGGTGTGATGAGGTT-3'

CRFR2 β specific:

Forward: 5'-CAGGCCAGGCACCCAGGAC-3'

Reverse: 5'-ACCACGCGATGTTTCTCAG-3'

CRF-BP:

Forward: 5'-AGACCATCCTCACCACAGCATT-3'

Reverse: 5'-ACTTTGACCTGGTCTCCATCGACTGC-CAG-3'

TRPC1:

Forward: 5'-CAAGATTTTGGGAAATTTCTGG-3'

Reverse: 5'-TTTATCCTCATGATTTGCTAT-3'.

Effect of channel blockers on actin ring formation

Osteoclasts were incubated for 4 h in 200 μ l MEM/FCS in the presence of M-CSF (50 ng/ml), RANKL (30 ng/ml), IL1 β (10 ng/ml) and with one of the following: UCN (10^{-7} M), nicardipine (10^{-6} M), BayK6844 (10^{-6} M), La³⁺ (10^{-4} M), Gd³⁺ (10^{-4} M), glibenclamide (10^{-6} M) for 30 min or vehicle and actin rings assessed as above.

Electrophysiology

Single cation channel currents were recorded from cell-attached patches with an AXOPatch 200B patch-clamp amplifier (Axon Instruments, Sunnyvale, CA, USA) at room temperature. Composition of the external solution was (mM) KCl (126), CaCl₂ (1.5), HEPES (10) and glucose (11), pH to 7.2 with 10 M KOH, to set the membrane potential at $\sim$ 0 mV. The patch pipette solution contained (mM): NaCl (126), CaCl₂ (1.5), HEPES (10), glucose (11), TEA (10), 4-AP (5), iberiotoxin (0.0002), DIDS (0.1), niflumic acid (0.1) and nicardipine (0.005), pH to 7.2 with NaOH. Under these conditions voltage-dependent Ca²⁺ channels, K⁺ currents, Cl⁻ currents were abolished and non-selective cation currents were recorded in isolation. Patch pipettes were manufactured from borosilicate glass to produce pipettes with resistances of about 10 M Ω when filled with patch pipette solution. *I/V* relationships of single channel currents were determined by manually altering the membrane potential between -90 and $+50$ mV. For off-line analysis, single channel currents were filtered at 100–200 Hz (-3 db, low pass 8-pole Bessel filter, Frequency Devices, model LP02, Scensys Ltd, Aylesbury, UK) and acquired using a Digidata 1322A and pCLAMP 9.0 at a sample rate of 1–2 kHz. Single channel current amplitudes were calculated from idealised traces of at least 60 s in duration using the 50% threshold method and analysed using pCLAMP v.9.0 software, with events lasting for 3.332–6.664 ms ($\times 2$ rise time for a 100–200 Hz, -3 db, low pass Bessel filter) being excluded from analysis. Single channel current amplitude histograms were plotted and fitted with Gaussian curves with the peak of these curves determining the unitary amplitude of the single channel currents. Open probability (NP_o) was calculated automatically using pCLAMP 9. Figure preparation was carried out using MicroCal Origin software 6.0 (Origin Lab

Inc, Northampton, MA, USA) where inward single cation channel openings are shown as downward deflections.

Statistical analysis

Statistical significance was assessed using ANOVA (Dunnett's test). Differences were considered significant if $P < 0.05$. All data are expressed as mean $\pm$ S.E.M. for at least six independent observations.

Results

UCN suppresses osteoclast differentiation, resorption and motility

Osteoclasts are large, multinucleated cells that can be distinguished from undifferentiated precursors by their ability to stain positively for TRAP. In our initial experiments, we investigated the effect of UCN on osteoclast differentiation by measuring the number of multinucleated TRAP-positive

osteoclasts after 5 days incubation. Figure 1A shows that UCN produced a significant reduction in the formation of mature osteoclasts, at 10^{-7} M. Osteoclasts generated in control cultures were large, round and well-spread, whereas following inclusion of UCN (10^{-7} M), osteoclasts contained fewer nuclei and were generally much smaller and less spread (Fig. 1B).

To further characterise the action of UCN on osteoclast differentiation, we investigated the effect of this peptide on expression levels of CT-R, Cath K, TRAP and DC-STAMP in osteoclasts using RT-PCR. UCN was included in the incubation media from 1 to 5 days. These markers are highly expressed in osteoclasts, but virtually absent in macrophages, therefore the expression of these is indicative of osteoclast maturation (Nicholson *et al.* 1986). Figure 1C and D demonstrates that treatment of osteoclasts with UCN produced a significant concentration-dependent reduction in CT-R, Cath K, TRAP and DC-STAMP, mRNA, measured after 5 days. In contrast, UCN had no effect on the expression level of GAPDH mRNA.

Figure 1 Effect of UCN on osteoclast differentiation. (A) Inhibition of the development of multinucleated TRAP-positive osteoclasts by UCN (10^{-7} M) after 5 days, $n=6$ cultures per variable * $P < 0.05$ vs control. (B) Representative photomicrographs at low magnification (left) and higher magnification (right) of TRAP-positive osteoclasts formed in the absence (top panel) or presence (lower panel) of UCN (10^{-7} M) after 5 days. (C) Concentration-dependent suppression of CT-R, Cath K, TRAP and DC-STAMP mRNA levels by UCN assessed by semi-quantitative RT-PCR in developing osteoclasts after 5 days, $n=3$ cultures. (D) Concentration-dependant amplicon density (arbitrary units), for CT-R, Cath K, TRAP and DC-STAMP in developing osteoclasts, expressed as a percentage of untreated values. All values were standardised to GAPDH.

UCN produced a significant concentration-dependent inhibition of osteoclastic bone resorption, with an IC_{50} value of 10^{-9} M and complete abolition at 10^{-7} M (Fig. 2A). Figure 2B depicts representative scanning electron micrographs of bone slices after cells had been exposed to UCN or vehicle. Large pits can be clearly seen in control conditions, but were not seen on bone slices incubated with UCN.

We investigated the effect of UCN on actin ring formation, a marker for osteoclast activation, in osteoclasts sedimented onto bone slices. After 4 h, UCN was added in a dose-dependent manner and the cells were further incubated for 30 min. UCN induced a significant dose-dependent reduction in actin ring formation, with an IC_{50} of $\sim 5 \times 10^{-10}$ M and complete inhibition at 10^{-7} M (Fig. 2C). Untreated osteoclasts produced actin ring structures that were absent after treatment with UCN (Fig. 2D).

The differences seen in the resorption experiments were not due to a toxic effect of UCN, as the number of cells within the untreated and treated groups at the end of the experiments did not differ significantly (Fig. 4B).

Figure 2 Effect of UCN on osteoclast function. (A) Concentration-dependent inhibition of osteoclastic bone resorption by UCN after 30 min, $n=6$ cultures per variable $*P<0.05$, $**P<0.001$ vs control. (B) Representative scanning electron micrographs of resorption pits caused by resorbing osteoclasts in the absence (upper panel) or presence (lower panel) of UCN (10^{-7} M) after 30 min. (C) Concentration-dependent reduction in the number of actin rings per field by UCN. Osteoclasts were incubated on bone slices in the presence of M-CSF, RANKL and IL1 α for 4 h, then UCN was added at the concentrations indicated and incubation continued for 30 min, $n=6$ cultures per variable $**P<0.001$ vs control. (D) Representative photomicrograph of actin rings produced by activated osteoclasts in the absence (upper panel) or presence (lower panel) of UCN (10^{-7} M) after 30 min.

Movement of osteoclasts during the control incubation period was characterised by pseudopodial ruffling. Inclusion of UCN (10^{-7} M) markedly inhibited this ruffling activity (Fig. 3), with complete cessation of cytoplasmic motility, leading to a shape-change characteristic of motility-inhibition, similar to that caused by CT (Chambers 2010).

UCN has no effect on osteoclast apoptosis or number

UCN has been documented to have both pro- and anti-apoptotic effects depending on the cell type. Therefore, we examined the effect of UCN on osteoclast apoptosis. We performed western blot analysis to determine the extent of procaspase 3 cleavage into its smaller pro-apoptotic cleaved form and PARP cleavage, both well established markers of late apoptosis. We found no difference in the amount of caspase 3, or PARP cleavage between untreated or UCN-treated osteoclasts, indicating that in these cells UCN had no effect on apoptosis. We did however, find extensive cleavage of both caspase 3 and PARP in osteoclasts undergoing u.v. irradiation (Fig. 4A). Furthermore, there was no difference in osteoclast number between untreated and UCN-treated cells (Fig. 4B).

UCN and CRFR2 β , but not CRF-BP, are expressed in osteoclasts

The expression of genes involved in UCN signalling was investigated in bone cells, we performed RT-PCR on *in vitro*-derived pre-osteoclasts (0 day), osteoclasts, primary calvarial osteoblasts, the osteoblastic cell line MC3T3-E1, and heart and brain tissues as control. Figure 5 demonstrates that mRNA for the CRFR2 β was present, but not UCN, in pre-osteoclasts. However, both UCN and CRFR2 β were expressed in osteoclasts, whereas CRF-BP was absent in both pre-osteoclasts and osteoclasts, but exclusively expressed in osteoblastic cells.

Non-selective cation channel blockers inhibit actin ring formation

In other cell types, UCN has been shown to regulate cellular functions by inhibiting ion channels involved in promoting Ca^{2+} influx, such as voltage-dependent and -independent Ca^{2+} -permeable channels (Tao *et al.* 2004, 2006, Smani *et al.* 2007). Extracellular Ca^{2+} , and hence Ca^{2+} influx pathways, are also important for osteoclast differentiation and function (Boyce & Xing 2007). In particular, intracellular Ca^{2+} levels have been shown to be important for osteoclast differentiation and notably for RANK signalling (Takayanagi *et al.* 2002). Therefore, Ca^{2+} channels are potential targets for UCN in osteoclasts. To investigate whether inhibition of a Ca^{2+} -permeable cation channel is able to mimic UCN we studied the effect of ion channel blockers on actin ring formation. Figure 6A shows that inclusion of the L-type channel modulators BayK6844 and nifedipine, and also the K_{ATP} channel agonist glibenclamide, had no effect on actin ring

Figure 3 UCN suppresses osteoclast motility. Photomicrographs showing motility of an osteoclast before UCN (10^{-7} M) addition and 5, 10, 20, 30 and 50 min after addition. Arrows indicate membrane ruffling, $n=3$ cultures.

formation, whereas the non-selective cation channel blockers La^{3+} and Gd^{3+} reduced osteoclast actin ring formation to a similar degree to that of UCN.

UCN inhibits a constitutively active non-selective cation channel

Given that non-selective cation channel blockers inhibit actin ring formation to a similar level to UCN, this indicates that the inhibition of a cation channel may be involved in the inhibitory mechanism of UCN in osteoclasts. We therefore, investigated whether osteoclasts express constitutively active Ca^{2+} -permeable cation channel activity and whether UCN inhibits this channel activity. To test this, we used standard electrophysiological patch clamp methods to record single cation channel activity in cell-attached patches from mature osteoclasts after 5 days in culture, with recordings made from large, multinucleated osteoclasts. Figure 6B shows that 15 out of 20 un-stimulated patches tested contained downward current deflections, which represented constitutively active cation channel activity in osteoclasts that had a mean NP_o value of 2.42 ± 0.29 ($n=15$) and a mean unitary amplitude of -0.22 ± 0.02 ($n=15$) at -50 mV. Mean I/V relationship of these spontaneous cation channel currents had a slope conductance of 3–4 pS, with a reversal potential of about

+20 mV (data not shown). It is important to note that the activity of these constitutively active cation channel currents was not regulated by altering membrane potential, which indicates that these channels are voltage-independent. Importantly, Fig. 6C shows that bath application of UCN (10^{-7} M) significantly inhibited constitutively active cation channel activity, with the mean NP_o of channel activity being reduced from 2.61 ± 0.59 to 0.78 ± 0.34 ($n=10$, $P<0.01$) at -50 mV, representing a mean inhibition of $70 \pm 7\%$.

Expression of TRPC1 mRNA and channel protein in osteoclasts

The spontaneously active channel currents we have identified have similar unitary conductances to previously studied canonical TRPC1-containing cation channels (Strübing *et al.* 2001, Alfonso *et al.* 2008, Saleh *et al.* 2008, Albert *et al.* 2009), and Fig. 6D shows that both i) mRNA and ii) protein for TRPC1 are highly expressed in osteoclasts.

Discussion

This work demonstrates for the first time that UCN produces marked inhibition of osteoclast differentiation and function, indicating that this peptide is a novel regulator of resorption. Our data also provide new information on the expression of a constitutively active Ca^{2+} -permeable cation channel in osteoclasts, which has properties similar to TRPC1-containing channels. Moreover, the action of UCN on osteoclasts is mediated, at least in part, by inhibition of these spontaneously active channels. Our findings illustrate that UCN significantly suppressed osteoclastic differentiation, as measured by the development of TRAP-positive, multinuclear cells at 10^{-7} M and also lowered the mRNA levels of several osteoclastic markers: CT-R, Cath K, TRAP and DC-STAMP. UCN also caused striking inhibition of both osteoclast bone resorption and actin ring formation. In both assays, the IC_{50} for UCN was $\sim 10^{-9}$ M. Furthermore, UCN completely abolished osteoclast motility at 10^{-7} M,

Figure 4 Effect of UCN on osteoclast apoptosis and number. (A) Western blot assessing the extent of caspase 3 (Asp175) and PARP (Asp214) cleavage products in untreated, UCN (10^{-7}) treated 18 h and u.v.-irradiated osteoclasts. (B) Osteoclasts were incubated on bone slices in the presence of M-CSF, RANKL and IL1 α for 4 h, then vehicle or UCN (10^{-7} M) was added for 30 min, $n=3$ cultures.

Figure 5 Expression of a UCN system in bone cells. (A) RT-PCR using primers designed to amplify UCN, CRFR1, CRFR2 α , CRFR2 β , CRF-BP and GAPDH in pre-osteoclasts (pre-Oc), mature osteoclasts (Oc), the osteoblast cell line MC3T3-E1 (MC3T3-E1), heart (H) and brain (B) as positive controls and primary osteoblasts (Ob), $n=3$ cultures.

but had no effect on osteoclast apoptosis as measured by caspase 3 and PARP cleavage, both indicators of late apoptotic events (Ferrer & Planas 2003, Agarwal *et al.* 2009) or osteoclast number.

The plasma concentration of UCN is in a similar range to the IC₅₀ value for the inhibition of actin ring formation (Charles *et al.* 2006, Torricelli *et al.* 2006). Therefore, bone resorption might be regulated *in vivo* by systemic UCN. Alternatively our results raise the possibility that there might

be a local UCN system in bone, and that osteoclasts might be regulated by this.

Our findings show that UCN mRNA is expressed in osteoclasts but not in primary osteoblasts or the osteoblast cell line, MC3T3-E1. We also found, using isoform-specific primers, that osteoclasts express the CRFR2 β receptor subtype, which is commonly expressed in peripheral tissues (Perrin *et al.* 1995, Fekete & Zorrilla 2007). In contrast, CRF-BP was expressed by primary osteoblasts and MC3T3-E1 cells but not osteoclasts. Unlike osteoclasts, we found that pre-osteoclasts expressed CRFR2 β but not UCN itself. This is in contrast to a study by Tezval *et al.* (2009), demonstrating the presence of UCN in human mesenchymal progenitor cells, but these cells had been directed toward an osteoblastic phenotype. Our findings suggest that UCN might represent an autocrine regulator of osteoclast function and a paracrine regulator of maturation and the concentration of UCN in the surrounding milieu could be controlled by osteoblastic CRF-BP release. At present the existence of other UCN members in bone is unknown. It would be of great interest therefore, to determine the presence of UCN 2 and 3 in osteoclasts and osteoblasts and determine their effect on function.

Recently, bone has been found to be intimately involved in the control of energy metabolism (Martin 2007, Fukumoto & Martin 2009, Yadav & Karsenty 2009). From this perspective it is intriguing to note that the actions of UCN on osteoclasts

Figure 6 Osteoclasts express a constitutively active TRPC1-like cation channel currents which are inhibited by UCN. (A) UCN (10⁻⁷ M), La³⁺ (10⁻⁴ M) and Gd³⁺ (10⁻⁴ M) after 30 min significantly inhibited the number of actin rings per field by a similar extent (***) $P<0.001$ vs control). Nicardipine (Nic, 5 × 10⁻⁶ M), BayK8644 (BayK, 10⁻⁵ M) and glibenclamide (Glib, 10⁻⁶ M) had no effect on actin ring formation. $n=3$ cultures per variable. (B) Patch clamp recording from osteoclasts reveal 15 out of 20 un-stimulated patches tested contained downward current deflections, which represented constitutively active cation channel activity in osteoclasts that had a mean NP_o value of 2.42 ± 0.29 ($n=15$) and a mean unitary amplitude of -0.22 ± 0.02 ($n=15$) at -50 mV. (C) Bath application of UCN (10⁻⁷ M) significantly inhibited constitutively active cation channel activity, with the mean NP_o of channel activity being reduced from 2.61 ± 0.59 to 0.78 ± 0.34 ($n=10$, $P<0.01$) at -50 mV, representing a mean inhibition of 70 ± 7%. (D) Osteoclasts express i) TRPC1 mRNA and ii) TRPC1 protein, using RT-PCR and western blotting methods respectively.

Figure 7 Schematic representation of the novel UCN/ CRF-BP pathway in bone. In response to unknown stimuli, UCN is released from, and acts upon osteoclasts in an autocrine manner, through binding to cell surface CRFR2 β receptors. This causes the closure of a constitutively active TRPC1-like cation channel, resulting in the inhibition of osteoclast resorption. The biochemical cascade coupling CRFR2 β receptors to closure of TRPC1 channels is currently unknown. The inhibitory effect of UCN is likely to be attenuated by the release, through unknown stimuli, of CRF-BP from osteoblasts.

may represent an additional component of this system. Systemic administration of UCN was shown to inhibit weight gain and decrease visceral fat of obese mice (Asakawa *et al.* 1999, Richard *et al.* 2000, Kuperman & Chen 2008). Furthermore, UCN, which crosses the blood–brain barrier, has potent actions as a regulator of appetite.

CRF receptors have been shown to activate several intracellular pathways, both *in vivo* and *in vitro*. CRFR1 and 2 are coupled to $G_{\alpha s}$ G-proteins, leading to activation of AC and subsequent cAMP-dependent protein kinase A. They can also be linked to $G_{\alpha q}$ proteins, activating PLC that generates inositol-1,4,5-trisphosphate and diacylglycerol, causing the release of Ca^{2+} from internal Ca^{2+} stores and stimulating protein kinase C-dependent pathways respectively (Graziani *et al.* 2002, McEvoy *et al.* 2002). However, other pathways have been reported to be activated by stimulation of CRFRs such as (ERK)–MAP kinase, AKT/protein kinase B and nitric oxide synthase–guanylyl cyclase (Grammatopoulos *et al.* 2000, Cantarella *et al.* 2001, Bayatti *et al.* 2003, Karteris *et al.* 2005). The intracellular signalling pathways activated by UCN in osteoclasts remain unknown, but intracellular Ca^{2+} signalling has been shown to be crucial for maintaining osteoclastic differentiation (Boyce & Xing 2007) and UCN has been shown to inhibit store-operated Ca^{2+} entry in vascular smooth muscle cells (Smani *et al.* 2007, Negishi-Koga & Takayanagi 2009). Therefore, channels that regulate Ca^{2+} entry are potential candidates as a target for UCN in osteoclasts.

The present study shows that osteoclasts express constitutively active cation channel currents, which have

a unitary conductance between 3–4 pS and an E_{rev} of +25 mV that indicates significant permeability to Ca^{2+} ions. The distinctive single channel properties of these channel currents in osteoclasts are similar to previously described TRPC1-containing channels (Strübing *et al.* 2001, Alfonso *et al.* 2008, Saleh *et al.* 2008). Furthermore, we also demonstrate the presence of mRNA, and protein for TRPC1 subunit in mature osteoclasts. This is the first direct evidence that a functional cation conductance with TRPC-like properties is expressed in mature osteoclasts. We currently do not understand the mechanisms driving constitutive channel activity in osteoclasts, although constitutive $G_{\alpha i/o}$ protein activity evokes TRPC3 channel activity through PLD-mediated generation of DAG in vascular smooth muscle (Albert & Large 2003, Albert *et al.* 2005). Furthermore, the function of spontaneously opening Ca^{2+} channels, which are likely to induce Ca^{2+} entry, is also unknown. TRPV4 channel activity has been implicated in the maintenance of intracellular Ca^{2+} needed for nuclear factor of activated T-cells (NFAT) activation for osteoclast differentiation (Masuyama *et al.* 2008), and the RANKL-induced Ca^{2+} increase may also, in part, have extracellular origins, possibly through activation of TRPV5 channels (Bennett *et al.* 2001, van der Eerden *et al.* 2005, Chamoux *et al.* 2010). It is tempting therefore, to speculate that this constitutively active current is also involved in the maintenance of NFAT expression/activity in osteoclasts.

Our results clearly show that UCN, at similar concentrations to those levels producing inhibitory actions on osteoclast differentiation and function, induced a pronounced suppression of the constitutively active channel activity. In addition, La^{3+} and Gd^{3+} , which are non-selective cation channel blockers that inhibit activity of many different cation channels, also produced a marked inhibition of actin ring formation to a similar level to UCN. In comparison, nifedipine, which blocks L-type Ca^{2+} channels, BayK8644 an L-type Ca^{2+} channel opener and glibenclamide a K_{ATP} channel opener, had no effect on actin ring formation. An intriguing hypothesis derived from our work is illustrated in Fig. 7, in which the UCN-mediated system described in the present study shows similarities to the well-established RANKL/osteoprotegerin system in bone, in that both systems employ a decoy receptor derived from osteoblasts to terminate an osteoclast-targeted ligand signal (Chambers 2010). Importantly however, the UCN system demonstrates an alternative regulatory pathway for osteoclastogenesis and activity, in that this system antagonises the effects of RANKL, resulting in the inhibition of osteoclast differentiation and activity.

In conclusion, this study shows that osteoclast differentiation and function are markedly inhibited by UCN, and that UCN, its binding protein and receptor are expressed by bone cells. This suggests that bone contains a local UCN system that might modulate local and/or systemic bone resorption in response to as-yet unidentified signals.

Moreover, our results indicate that the inhibitory actions of UCN on bone resorption are mediated, at least in part, by a reduction in constitutively active cation channel activity that has similar properties to TRPC1-containing channels. Importantly, manipulation of UCN levels, the UCN signalling system and activity of constitutively active channels may represent new therapeutic targets for bone disorders such as osteoporosis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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