Free Radical Research, 2003 Vol. 37 (2), pp. 179–187

Taylor & Francis healthsciences

Xanthine Oxidase Mediates Cytokine-induced, but not Hormone-induced Bone Resorption

JANOS M. KANCZLER[a,*], TIMOTHY M. MILLAR[a], TULIN BODAMYALI[a], DAVID R. BLAKE[a,b] and CLIFF R. STEVENS[a,b]

[a]*Department of Medical Sciences, Bone and Joint Research Group, University of Bath, Bath, BA2 7AY, UK;* [b]*The Royal National Hospital for the Rheumatic Diseases, Upper Borough Walls, Bath, BA1 1RL, UK*

Accepted by Professor F.J. Kelly

(Received 19 June 2002; In revised form 28 August 2002)

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/26/11
For personal use only.

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) have been implicated as mediators of osteoclastic bone resorption. Xanthine oxidase (XO) a ubiquitous enzyme is widely known for its production of these ROS. We therefore evaluated the potential of XO as a source of ROS in cytokine- and hormone-induced bone resorption. XO activity in rat calvarial osteoblasts was found to be significantly elevated upon stimulation by the cytokines, TNFα and IL-1β. These cytokines also caused a dose related increase in bone resorption of mouse calvariae, which was significantly inhibited by catalase (10 IU/ml). Allopurinol, the competitive inhibitor of XO, also caused a dose related (1–50 μM) inhibition of TNFα (20 ng/ml) and (0.01–10 μM) IL-1β (50 IU/ml)-induced bone resorption, respectively. PTH- and 1,25-$(OH)_2$ Vitamin D_3-induced bone resorption could also be inhibited by catalase (100 IU/ml) but was unaffected by allopurinol, indicating that another mediator, other than XO, is required for hormone-induced bone resorption. These results demonstrate, that modulation of the redox balance in the bone microenvironment, which contains XO, can affect the bone resorbing process. Therefore, XO may play a pivotal role in cytokine-induced bone resorption and, if manipulated appropriately, could show a therapeutic benefit in inflammatory bone disorders such as RA.

Keywords: Xanthine oxidase; Bone resorption; Cytokines; Hydrogen peroxide

INTRODUCTION

Bone resorption underlies the pathology of many disabling diseases, ranging from the inflammatory condition rheumatoid arthritis (RA), to the hormonally controlled metabolic disorder osteoporosis.[1] Although the process of pathological bone resorption remains poorly understood, it is widely established that under the influence of factors from the osteoblast, multinucleated giant cells (osteoclasts) resorb bone.[2] A diverse range of agents, including the calcitropic hormones, prostaglandins and the pro-inflammatory cytokines TNFα and IL-1β are known to stimulate bone resorption both *in vitro* and *in vivo*.[3–5,20] Until recently, how this was achieved had not been clearly identified.

It is widely held that in response to stimulation by cytokines, osteoblasts induce osteoclastic bone resorption by a small, soluble, labile factor,[6] which initiates cytokine-induced bone resorption via a prostaglandin-independent mechanism.[7] With the very important recent discovery of osteoprotegerin (OPG) and receptor activator of nuclear factor κB ligand (RANKL) (a novel member of the TNF ligand family, which is involved in osteoclastic differentiation[8]) the mechanism of bone remodelling is starting to be unravelled. Reactive oxygen species (ROS) and other free radicals have also been implicated in the bone resorptive process.[9–11] Indeed, one such species, hydrogen peroxide (H_2O_2) has been shown to directly induce osteoclastogenesis and stimulate osteoclastic activity,[12,13] yet the sources of this H_2O_2 in bone have yet to be determined.

Xanthine oxidase (XO) is a cytosolic and membrane bound complex molybdoflavoprotein

*Corresponding author. Tel.: +44-1225-323835. Fax: +44-1225-323573. E-mail: mpsjmk@bath.ac.uk

ISSN 1071-5762 print/ISSN 1029-2470 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1071576021000040673

RIGHTSLINK

with two Fe-S clusters, which is a constitutive enzyme in purine metabolism of most cells.[14] During the catalytic conversion of xanthine to uric acid, XO can use NAD^+ (dehydrogenase form) or molecular oxygen (oxidase form) as an electron acceptor. The latter results in the generation of the ROS, superoxide anion radical (O_2^-) and H_2O_2. The gene is located on chromosome 2 at band p22 and contains two transcriptional initiation sites consisting of sequences coding for a number of promoter elements associated with the acute phase response genes.[15] These include, four CAAT/Enhancer Binding Protein sites, IL-6 and NF-κB, and potential TNF-, IL-1- and IFN-γ responsive elements.[16]

In this study, we investigated the potential of XO to mediate in part, via the production of ROS (H_2O_2), TNFα-, IL-1β-, PTH- and 1,25-$(OH)_2$ Vitamin D_3-induced mouse calvarial bone resorption.

MATERIALS AND METHODS

Materials

Tissue culture plates, flasks and 50 ml polypropylene tubes were purchased from Falcon (Becton Dickinson, Oxford, UK). PBS (w/o Ca^{2+} and Mg^{2+}), Hank's Balanced Salt Solution, cell culture medium (DMEM, CMRL 1066), antibiotics and L-glutamine were supplied by Gibco Life Technologies (Paisley, Scotland). Foetal calf serum (FCS) was purchased from Globepharm (Esher, UK). TNFα and IL-1β were purchased from Boehringer Mannheim (Lewes, UK). PTH bovine (1–34) was obtained from the National Institute for Biological Standards and Control (Potters Bar, UK). 1,25-$(OH)_2$ Vitamin D_3, collagenase type II, allopurinol, murine catalase and all reagents used in the determination of XO activity were purchased from Sigma (Poole, UK). IL Test™ calcium reagents were purchased from Instrumentation Labs (Warrington, UK).

Cell Culture

Rat (male Wistars, Harlene Olac, UK) calvarial osteoblasts (RCOBS) were isolated as described previously.[17] Briefly, calvaria from 5-day-old rats were dissected, minced and incubated with 1 mg/ml type II collagenase. Cells isolated from enzymatic digestion populations III–V were pooled together and cultured in flasks (75 cm^2) containing 10 ml of DMEM supplemented with 10% heat-inactivated FCS, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells from passages 2–4 that were greater than 85% alkaline phosphatase positive were used for XO activity assays.

Effects of TNF-α and IL-1β on RCOB XO Activity

XO enzyme activity in RCOB homogenates were measured using a fluorometric assay monitoring the conversion of pterin (a xanthine substitute) to fluorescent isoxanthopterin.[18] RCOBs were stimulated with the cytokines TNFα (0–10 ng/ml) and IL-1β (0–100 IU/ml) for 24 h. The cell monolayers were then washed with cold PBS and homogenised in an ice cold buffered solution (pH 7.4), containing 0.05 M potassium phosphate, 1 mM EDTA, 0.1 mM PMSF, 10 mM DTT, 1 μg/ml pepstatin A, 1 μg/ml antipain and 1 μg/ml leupeptin (Sigma, Poole, UK). The homogenates were then centrifuged at 10,000 rpm for 10 min at 4°C. The resulting lysates (200 μl of lysate + 800 μl of PBS) were placed in a cuvette containing 20 μM pterin and introduced to a F-4500 fluorescence spectrophotometer (Hitachi Scientific Instruments, Wokingham, UK) with an excitation wavelength of 345 nm and an emission wavelength of 390 nm. A 50 μM concentration of allopurinol was added to inhibit XO activity in these samples. Values were normalised to the amount of total protein in the sample, as estimated by the method of Bradford.[19]

Bone Resorption Assay

Calvarial bones excised from 5-day old Swiss albino, CD-1 neonatal mice (Harlene Olac, UK) were divided into equal halves along the saggital suture and cultured in 6-well plates containing 1.5 ml of TCM (CMRL-1066 supplemented with 5% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine), After a pre-incubation period of 18–24 h, the TCM was changed and replaced with fresh medium, which contained one of the following test substances, TNFα (20 ng/ml), IL-1β (50 IU/ml), PTH (1 U/ml) or 1,25-$(OH)_2D_3$ (as determined by optimal dose response analysis). The inhibitors, allopurinol or catalase were also added, or omitted from the TCM and the calvariae were incubated in an environment of 5% CO_2/95% air for a period of 96 h. At the end of this period, the extent of resorption was assessed by Ca^{2+} release into the TCM by reaction with cresolphthalein complexone forming a coloured complex, which was detected using a Monarch 2000 centrifugal analyser (Instrumentation Labs, Warrington, UK).

Statistics

Dose response relationships were assessed using one way ANOVA and where significant, mean values in individual groups were compared to the cytokine/hormone control values by Dunnet's test. Comparisons of inhibition of XO activity in RCOBs by allopurinol was by Student's *t*-test.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/26/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/26/11
For personal use only.

RESULTS

Effects of TNFα and IL-1β on XO Activity in RCOBs

The activity of XO in the cell lysates of RCOBs (as determined by the fluorometric pterin assay) showed an approximate three-fold increase when stimulated with TNFα (0.1, 1 and 5 ng/ml, respectively) and a two-fold increase when stimulated with IL-1β (10 and 50 IU/ml, respectively) (Table I). The measured activity was confirmed to be due to XO within these cells by the complete inhibition of activity by allopurinol (50 μM) (Table I).

Effects of the XO Inhibitor, Allopurinol and the H_2O_2 Scavenger, Catalase on TNFα and IL-1β-induced Mouse Calvarial Bone Resorption

The functional role of XO in cytokine-induced bone resorption was confirmed by the potent inhibitory effect of allopurinol, a competitive inhibitor of XO, on mouse calvarial bone calcium release *in vitro*. As reported previously by other groups[4,5] we confirmed that TNFα and IL-1β caused dose related increases in bone resorption, as determined by Ca^{2+} release into the culture medium from neonatal mouse calvaria. Allopurinol had no significant effect on basal resorption (data not shown), but caused a significant dose-related (1–50 μM and 0.01–10 μM) inhibition of the resorption induced by the sub optimal doses of both TNFα (20 ng/ml) and IL-1β (50 IU/ml), respectively (Fig. 1A and B). A combination of the doses of TNFα (20 ng/ml) and IL-1β (50 IU/ml) had an additive increase on mouse calvarial bone resorption, and again resorption was inhibited by allopurinol down to basal levels of resorption (Fig. 1C)

Catalase (10 U/ml) had no effect on basal resorption, but significantly inhibited bone resorption induced by TNFα (20 ng/ml), IL-1β (50 IU/ml) and a combination of both cytokines, respectively (Fig. 2A–C). This indicated that H_2O_2 was involved in the signalling mechanism of cytokine-induced bone resorption, confirming other reports, which showed H_2O_2 to directly induce osteoclastogenesis.[12,13]

Effects of Allopurinol and Catalase on PTH and 1,25-$(OH)_2$ Vitamin D_3-induced Mouse Calvarial Bone Resorption

The addition of allopurinol (0.01–10 μM) to the culture medium which contained either PTH (1 U/ml) or 1,25-$(OH)_2$ Vitamin D_3 (100 nM), respectively, had no significant inhibitory effect in these hormone-induced bone resorption assays (Fig. 3A and B). In contrast, catalase significantly inhibited both PTH (1 U/ml)-induced and 1,25-$(OH)_2$ Vitamin D_3 (100 nM)-induced mouse calvarial bone resorption (Fig. 4A and B).

DISCUSSION

The destruction of joints by bone erosion in diseases such as RA and postmenopausal osteoporosis has been associated with increased production of the cytokines TNFα and IL-1β,[21,22] which are potent inducers of bone resorption.[4,5] TNFα is currently perceived to hold a pivotal position in the hierarchy of cytokine responses in RA, as evidenced by the clinical studies of Maini *et al.*[23] Some of the actions of TNFα have been shown to involve the generation of low levels of ROS and other free radicals.[24] Furthermore, recent findings have indicated a biochemical link that exists between an increase in oxidative stress and a reduction in bone density.[39] Radical scavengers, antioxidants and metal chelators can inhibit the cytotoxicity and gene expression in response to TNFα in numerous cell types.[25] In addition, TNFα and IL-1β can upregulate

TABLE I XO activity in RCOB cells treated with TNF-α and IL-1β

TNF-α	XO activity (ρmoles min^{-1} mg $protein^{-1}$ ± SEM)	IL-1β	XO activity (ρmoles min^{-1} mg $protein^{-1}$ ± SEM)
Control	2.15 ± 0.00	Control	2.15 ± 0.00
0.01 ng/ml	3.28 ± 0.53	0.1 (U/ml)	3.02 ± 0.26
0.1 ng/ml	5.67 ± 1.58*	1 (U/ml)	4.09 ± 0.49
1 ng/ml	6.45 ± 1.01***	10 (U/ml)	4.36 ± 0.43*
5 ng/ml	5.56 ± 1.03*	50 (U/ml)	4.62 ± 0.21**
10 ng/ml	4.08 ± 0.54	100 (U/ml)	3.59 ± 1.07
5 ng/ml + 50 μM allopurinol	$0.21 \pm 0.05^{+++}$	50 U/ml + 50 μM allopurinol	$0.19 \pm 0.19^{++}$

The effects of TNFα and IL-1β on XO activity in RCOBs. Cell lysates from RCOBs treated with cytokines TNFα (0.01–10 ng/ml) and IL-1β (0.1–100 U/ml) for 24 h were tested for XO activity using the pterin assay, as described in "Materials and Methods" section. Both TNFα and IL-1β significantly increased the activity of XO in RCOBs cultured for 24 h. Units of activity were normalised to total protein present in the lysate. (*$P < 0.05$, **$P < 0.01$; ***$P < 0.001$ increase in XO activity vs. control). Addition of 50 μM of allopurinol significantly inhibited XO activity in cells treated with 5 ng/ml TNF-α ($^{+++}P < 0.001$) and 50 U/ml IL-1β ($^{++}P < 0.01$), respectively. Data are mean ± SEM, $n = 3$ separate experiments).

RIGHTSLINK

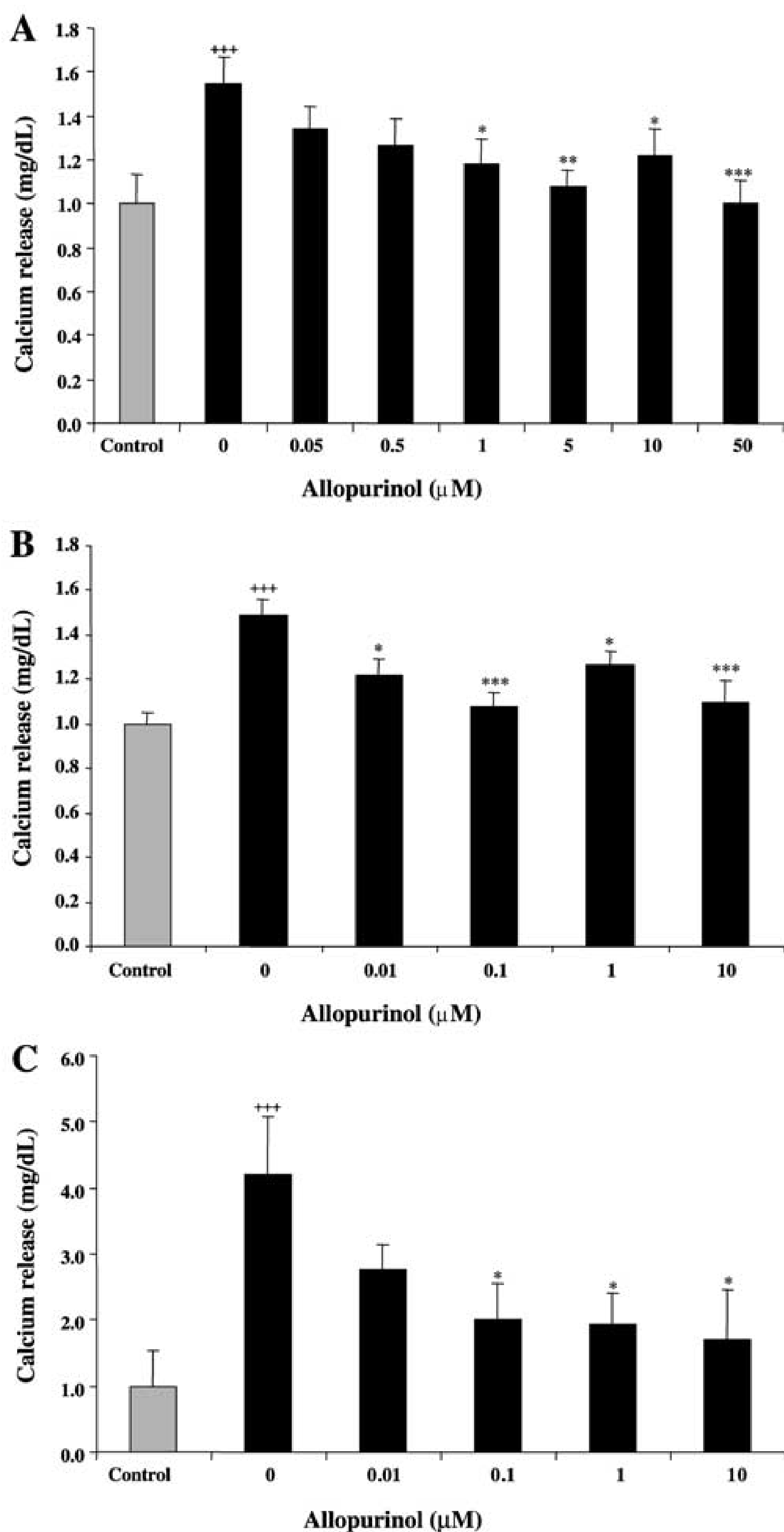

FIGURE 1 The effect of allopurinol on cytokine-induced bone resorption. Allopurinol dose dependently inhibited (A) TNFα (20 ng/ml) and (B) IL-1β (50 IU/ml)-induced bone resorption of 5-day old mouse calvariae. A combination of the two cytokines (C) TNFα (20 ng/ml) and IL-1β (50 IU/ml) had an additive effect, which was inhibited by allopurinol back to basal levels of bone resorption. $^{*}P < 0.05$; $^{**}P < 0.01$ $^{***}P < 0.001$ inhibition of resorption by allopurinol. $^{+++}P < 0.001$ increase in resorption vs. control (Data is representative of three separate experiments; ± SEM, $n = 6$–10 calvariae per treatment).

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/26/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/26/11
For personal use only.

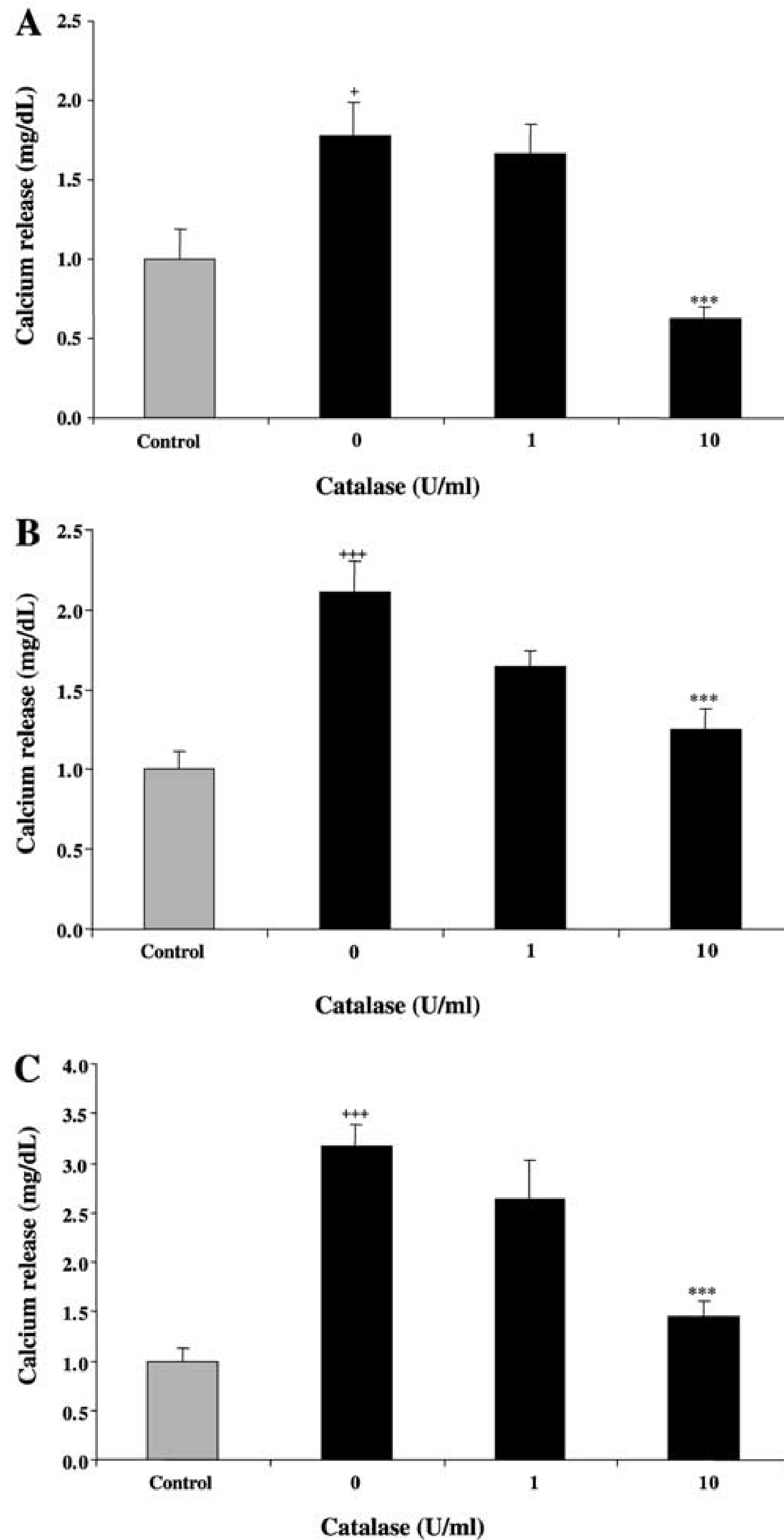

FIGURE 2 The effect of catalase on cytokine-induced bone resorption. Catalase (10 IU/ml) significantly inhibited bone resorption induced both by (A) TNFα (20 ng/ml) and (B) IL-1β (50 IU/ml) respectively. A combination of the two cytokines (C) TNFα (20 ng/ml) and IL-1β (50 IU/ml) had an additive effect, which was inhibited by catalase back to basal resorptive levels. $^{***}P < 0.005$ inhibition of resorption by catalase. $^{+}P < 0.05$; $^{+++}P < 0.005$ increase in resorption vs. control (Data is representative of three separate experiments; ± SEM, $n = 6$ calvariae per treatment).

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/26/11
For personal use only.

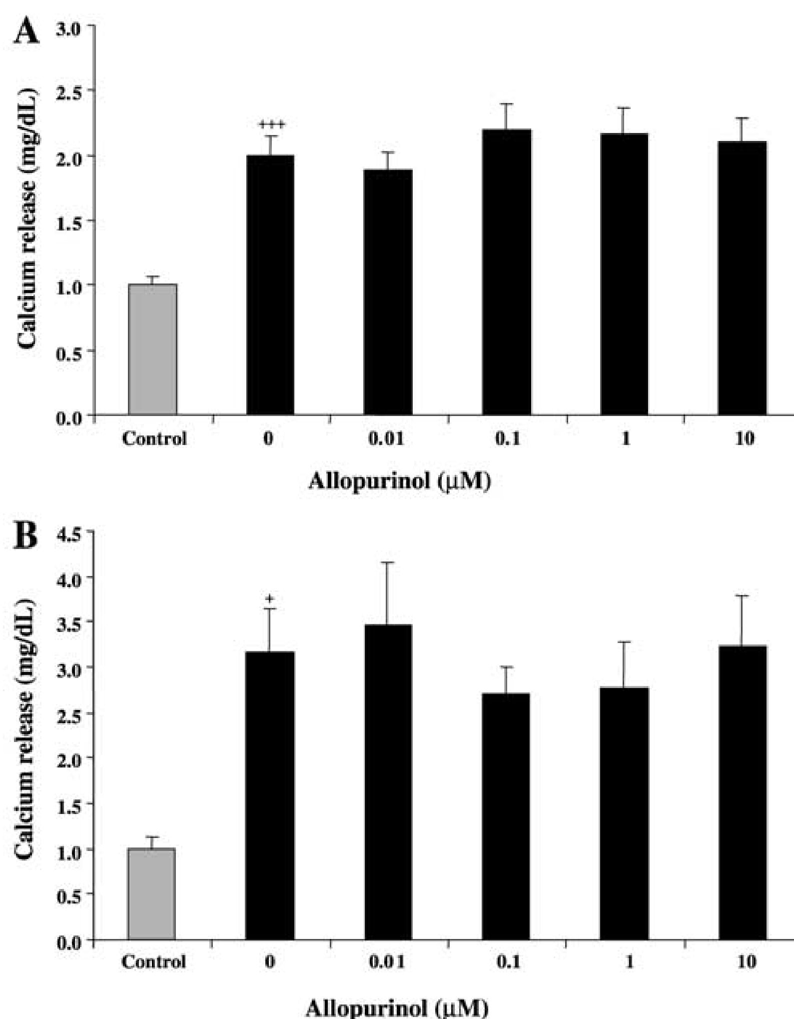

FIGURE 3 The effects of allopurinol on PTH and 1,25-$(OH)_2$Vitamin D_3-induced bone resorption. Incubation with allopurinol had no effect on (A) PTH (1 U/ml)-or (B) 1,25-$(OH)_2$Vitamin D_3 (100 nM)-induced bone resorption. $^{+}P < 0.05$; $^{+++}P < 0.001$ increase in resorption vs. control (Data is representative of three separate experiments; ± SEM, $n = 6$ calvariae per treatment).

mRNA and protein expression of XO in renal epithelial cells.[26]

Using ^{1}H NMR spectroscopy osteoblasts were found to generate H_2O_2 in response to TNFα.[28] Our studies have demonstrated that XO plays a role in cytokine-induced bone resorption and that the intermediate molecule H_2O_2 mediates induction of bone resorption. However, XO does not seem to be involved in PTH and 1,25-$(OH)_2$ Vitamin D_3-induced bone resorption, but H_2O_2 does appear to be a critical effector molecule in these pathways. Additionally, we have shown that the bone resorptive effects of these cytokines can be suppressed by the XO inhibitor, allopurinol. The results from this study showing H_2O_2 to be an important mediator of cytokine-induced bone resorption, concurs with published work, demonstrating that H_2O_2 directly stimulates osteoclastogenesis and mature osteoclasts to resorb bone.[12,13,27,37] We believe the functional correlate of these findings implicate H_2O_2 as one of the factors required for cytokine-induced bone resorption and XO as a possible source.

Although XO is known for its capacity to generate superoxide, there have been conflicting reports in the literature with respect to the role of superoxide (O_2^-) in bone resorption. O_2^- was originally thought to be the main stimulating factor of bone resorption. However, Fraser *et al.*,[13] found that H_2O_2 and not O_2^- stimulated bone resorption. This discrepancy may be explained by efficient dismutation of O_2^- to H_2O_2 by osteoclastic superoxide dismutase, which has been identified as the ligand for the osteoclast-specific monoclonal antibody Mab 121F.[29] Additionally, H_2O_2 can easily pass through the cell membrane unlike O_2^-.[30]

We did not observe any inhibitory effects of allopurinol on PTH and 1,25-$(OH)_2$ Vitamin D_3-induced bone resorption, but catalase did have an

RIGHTSLINK

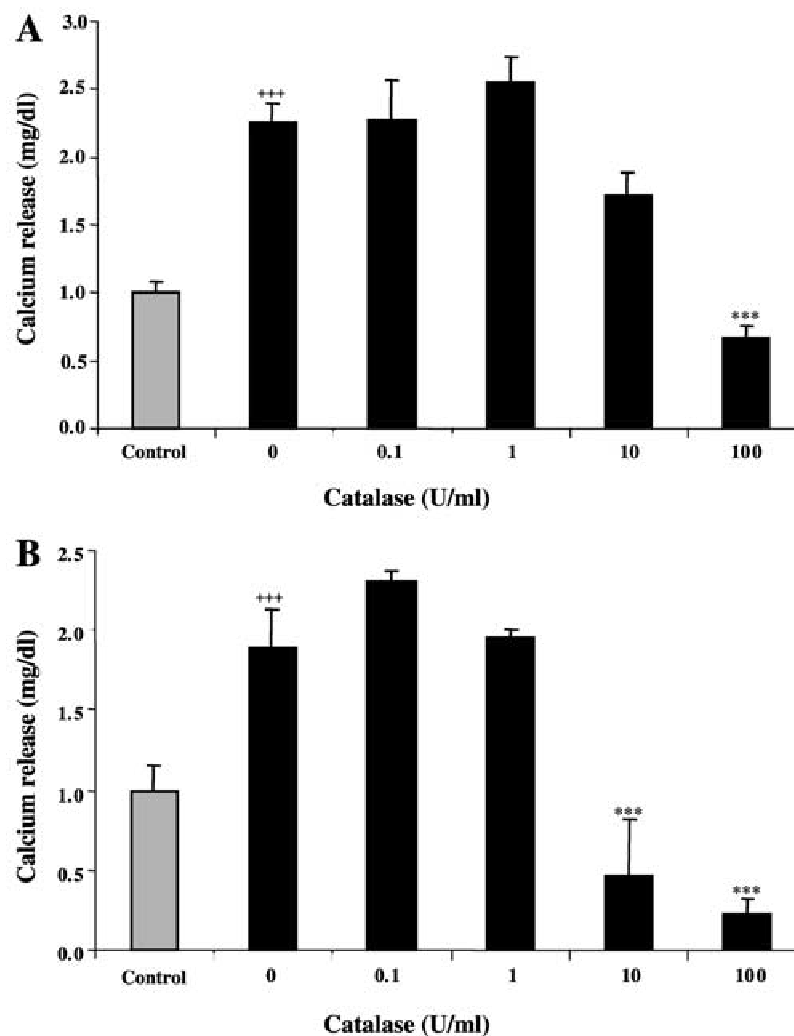

FIGURE 4 The effects of catalase on PTH and 1,25-$(OH)_2$Vitamin D_3-induced bone resorption. Incubation with catalase had a significant inhibitory effect on (A) PTH (1 U/ml)-or (B) 1,25-$(OH)_2$Vitamin D_3 (100 nM)-induced bone resorption. $^{***}P < 0.001$ inhibition of resorption by catalase. $^{+++}P < 0.001$ increase in resorption vs. control (Data is representative of three separate experiments; ± SEM, $n = 4-6$ calvariae per treatment.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/26/11
For personal use only.

inhibitory effect, indicating that H_2O_2 is part of this process. This has been shown to be the case for 1,25-$(OH)_2$ Vitamin D_3 on the process of oxidant stress inducing osteoclastogenesis.[37] O_2^- can also be generated directly by the osteoclast when stimulated with PTH[38] and that NADPH oxidase has the capacity to generate ROS underneath the resorbing osteoclast.[31] As osteoclasts are known to have SOD on their membrane, it is possible that H_2O_2 could be produced from the dismutation of the O_2^- radical. Also, XO can produce ROS via NADH at its FAD site.[33] In our mouse calvarial bone resorption assay we found that diphenyliodonium chloride (DPI), a potent inhibitor of NADPH oxidase[32] and XO,[33] inhibited both PTH and 1,25-$(OH)_2$ Vitamin D_3-induced bone resorption (data not shown).

RANKL induced osteoclastogenesis results in the activation of the transcription factor NF-κB, and the protein kinase JNK. One of the signalling targets of NF-κB is H_2O_2.[8] Activation of NF-κB by H_2O_2 may have a downstream effect in the RANKL-osteoclasteogenesis pathway and XO may bypass this pathway by generating H_2O_2, which can easily diffuse across the cell membrane of osteoclast precursors and directly activate NF-κB, inducing osteoclastogenesis.

Some of our preliminary observations show that osteoclasts isolated from the acetabular reaming tissues of patients undergoing revision of joint hip replacements stain positively for XO. This is of interest, because recently it has been shown that a redox active iron in the binuclear iron centre of TRAP can react with H_2O_2 to produce highly destructive ROS such as the hydroxyl radical ($^\bullet OH$), which in turn, is able to break down the bone matrix.[34] It was hypothesized that proteins

RIGHTSLINK

containing redox-active iron could represent a novel mechanism of fragmenting organic molecules such as collagen and play an important part in mechanisms of tissue remodelling. XO contains two redox active Fe-S sites, and therefore may have a role in matrix degradation in osteoclastic bone resorption by producing $^{\bullet}OH$ from H_2O_2 via the Fenton reaction. However, further work is required to determine if this is the case.

Additional evidence leads us to speculate that XO may be involved in bone remodelling. It was demonstrated that calves fed on a high molybdenum diet (6.2 ppm) (molybdenum is a required co-factor at one of the redox sites of XO where the conversion hypoxanthine to xanthine to uric acid takes place) develop bone malformations.[35] Indeed, addition of tungsten (a competitive factor at the molybdenum site of the XO enzyme) to the diets of goats and cows inhibited XO activity in the milk produced. Unfortunately, the authors failed to discuss the skeletal development of these goats and cows,[36] but our group has preliminary data showing that the bone density increases in rats, which have been fed on a high tungsten diet (unpublished observations).

In summary, the elevation of pro-inflammatory cytokines in bone destructive diseases such as RA mediate their pro-resorptive effects via the generation of ROS from XO. Variation in the levels of production of ROS in the bone microenvironment can mediate disruption of cellular interactions, either at the level of the intrinsic antioxidants or at the level of antioxidant/oxidant-responsive transcription factors. This can lead to the dysregulation of the physiological co-ordinated processes of bone remodelling. Such disturbances in this finely tuned phenomenon of oxidant/antioxidant balance may be the underlying reason for both chronic inflammation and uncontrolled bone resorption in RA. Since bone resorption is so closely linked to bone formation, multiple therapies including the manipulation of the redox environment involving XO may be required to offset the altered bone remodelling cycles, that lead to crippling bone deformities.

Acknowledgements

This work was supported by the Arthritis Research Campaign and an infrastructure award from the Royal National Hospital for the Rheumatic Diseases.

References

[1] MacDonald, B.R. and Gowen, M. (1992) "Scientific reviews in rheumatology: cytokines and bone", *Br. J. Rheumatol.* **31**, 149–155.

[2] McSheehy, P.M.J. and Chambers, T.J. (1986) "Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone", *Endocrinology* **118**, 824–828.

[3] Mundy, G.R. (1993) "Role of cytokines in bone resorption", *J. Cell Biochem.* **53**, 296–300.

[4] Bertolini, D.R., Nedwin, G.E., Bringman, T.S., Smith, D.D. and Mundy, G.R. (1986) "Stimulation of bone resorption and inhibition of bone formation *in vitro* by human tumour necrosis factor", *Nature* **319**, 516–518.

[5] Gowen, M., Wood, D.D., Ihrie, E.J., McGuire, M.K. and Russell, R.G. (1983) "An interleukin-1 like factor stimulates bone resorption *in vitro*", *Nature* **306**, 378–380.

[6] McSheehy, P.M. and Chambers, T.J. (1987) "1,25-Dihydroxyvitamin D3 stimulates rat osteoblastic cells to release a soluble factor that increases osteoclastic bone resorption", *J. Clin. Investig.* **80**, 425–429.

[7] Lerner, U.H. and Ohlin, A. (1993) "Tumour necrosis factors alpha and beta can stimulate bone resorption in cultured mouse calvariae by a prostaglandin-independent mechanism", *J. Bone Miner. Res.* **8**, 147–155.

[8] Jimi, E., Akiayama, S., Tsurukai, T., Okahashi, N., Kobayashi, K., Udagawa, N., Nishihara, T., Takahashi, N. and Suda, T. (1999) "Osteoclast differentiation factor as a multifunctional regulator in murine osteoclast differentiation and function", *J. Immunol.* **163**, 434–442.

[9] Garrett, I.R., Boyce, B.F., Oreffo, R.O., Bonewald, L., Poser, J. and Mundy, G.R. (1990) "Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone *in vitro* and *in vivo*", *J. Clin. Investig.* **85**, 632–639.

[10] Ralston, S.H., Ho, L.P., Helfrich, M.H., Grabowski, P.S., Johnston, P.W. and Benjamin, N. (1995) "Nitric oxide: a cytokine-induced regulator of bone resorption", *J. Bone Miner. Res.* **10**, 1040–1049.

[11] Hall, T.J., Shaeublin, M., Jeker, H., Fuller, K. and Chambers, T. (1995) "The role of reactive oxygen intermediates in osteoclastic bone resorption", *Biochem. Biophys. Res. Commun.* **207**, 280–287.

[12] Bax, B.E., Alam, A.S., Banjerji, B., Bax, C.M., Bevis, P.J., Stevens, C.R., Moonga, B.S., Blake, D.R. and Zaidi, M. (1992) "Stimulation of osteoclastic bone resorption by hydrogen peroxide", *Biochem. Biophys. Res. Commun.* **183**, 1153–1158.

[13] Fraser, J.H., Helfrich, M.H., Wallace, H.M. and Ralston, S.H. (1996) "Hydrogen peroxide, but not superoxide, stimulates bone resorption in mouse calvariae", *Bone* **19**, 223–226.

[14] Bray, R.C. (1988) "The inorganic biochemistry of molybdoenzymes", *Q. Rev. Biophys.* **21**, 299–329.

[15] Xu, P., Zhu, X.L., Huecksteadt, T.P., Brothman, A.R. and Hoidal, J.R. (1994) "Assignment of human xanthine dehydrogenase gene to chromosome 2p22", *Genomics* **23**, 289–291.

[16] Xu, P., Huecksteadt, T.P. and Hoidal, J.R. (1996) "Molecular cloning and characterisation of the human xanthine dehydrogenase gene (XDH)", *Genomics* **34**, 173–180.

[17] Bellows, C.G., Aubin, J.E., Heersche, J.N. and Antosz, M.E. (1986) "Mineralized bone nodules formed *in vitro* from enzymatically released rat calvaria populations", *Calcif. Tissue Int.* **38**, 143–154.

[18] Beckman, J.S., Parks, D.A., Pearson, J.D., Marshall, P.A. and Freeman, B.A. (1989) "A sensitive fluorometric assay for measuring xanthine dehydrogenase and oxidase in tissues", *Free Radic. Biol. Med.* **6**, 607–615.

[19] Bradford, M.M. (1976) "A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding", *Anal. Biochem.* **72**, 248–254.

[20] Suda, T., Jimi, E., Nakamura, I. and Takahashi, N. (1997) "Role of 1 alpha, 25-dihydroxyvitamin D3 in osteoclast differentiation and function", *Methods Enzymol.* **282**, 223–235.

[21] Duff, G.W. (1989) "Peptide regulatory factors in non-malignant disease", *Lancet* **1**, 1432–1435.

[22] Brennan, F., Chantry, A., Jackson, R., Maini, R. and Feldman, M. (1989) "Inhibitory effect of TNF alpha antibodies on synovial cell interleukin-1 production in rheumatoid arthritis", *Lancet* **2**, 244–247.

[23] Maini, R.N., Elliott, M.J., Brennan, F.M. and Feldmann, M. (1995) "Beneficial effects of tumour necrosis factor-alpha (TNF-alpha) blockade in rheumatoid arthritis", *Clin. Exp. Immunol.* **101**, 207–212.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/26/11
For personal use only.

RIGHTSLINK

[24] Remacle, J., Raes, M., Toussaint, O., Renard, P. and Rao, G. (1995) "Low levels of reactive oxygen species as modulators of cell function", *Mutat. Res.* **316**, 103–122.
[25] Terada, L.S., Willingham, I.R., Guidot, D.M., Shibao, G.N., Kindt, G.W. and Repine, J.E. (1992) "Tungsten treatment prevents tumour necrosis factor-induced injury of brain endothelial cells", *Inflammation* **16**, 13–19.
[26] Pfeffer, K.D., Huecksteadt, T.P. and Hoidal, J.R. (1994) "Xanthine dehydrogenase and xathine oxidase activity and gene expression in renal epithelial cells. Cytokine and steroid regulation", *J. Immunol.* **153**, 1789–1797.
[27] Steinbeck, M.J., Kim, J.K., Trudeau, M.J., Hauschka, P.V. and Karnovosky, M.J. (1998) "Involvement of hydrogen peroxide in the differentiation of clonal HD-11EM cells into osteoclast-like cells", *J. Cell. Physiol.* **176**, 574–587.
[28] Stevens, C.R., Abbot, S.E., Harley, S.L., Sahinoglu, T., Grootveld, M.C., Zaidi, M. and Blake, D.R. (1993) "TNF-alpha-stimulated osteoblasts induce oxidative modification of their culture environment consistent with hydrogen peroxide generation-a proton NMR study", *J. Bone Miner. Res.* **8**(S1), 124.
[29] Oursler, M.J., Li, L. and Osdoby, P. (1991) "Purification and characterization of an osteoclast membrane glycoprotein with homology to manganese superoxide dismutase", *J. Cell. Biochem.* **46**, 219–233.
[30] Okabe, E., Takahashi, K.K., Norisue, M. and Ito, H. (1990) "Oxygen free radicals as a mediator of bone resorption", *Bull. Kanagawa Dent. Coll.* **18**, 151–159.
[31] Darden, A.G., Ries, W.L., Wolf, W.C., Rodriguiz, R.M. and Key, Jr., L.L. (1996) "Osteoclastic superoxide production and bone resorption: stimulation and inhibition by modulators of NADPH oxidase", *J. Bone Miner. Res.* **11**, 671–675.
[32] Cross, A.R. and Jones, O.T. (1986) "The effect of the inhibitor diphenylene iodonium on the superoxide-generating system of neutrophils. Specific labelling of a component polypeptide of the oxidase", *Biochem. J.* **237**, 111–116.
[33] Sanders, S.A., Eisenthal, R. and Harrison, R. (1997) "NADH oxidase of human xanthine oxidoreductase generation of superoxide anion", *Eur. J. Biochem.* **245**, 541–548.
[34] Halleen, J.M., Raisanen, S., Salos, J.J., Reddy, S.V., Roodman, G.D., Hentunen, T.A., Lehenkari, P.P., Kaija, H., Vihko, P. and Vaananen, H.K. (1999) "Intracellular fragmentation of bone resorption products by reactive oxygen species generated by osteoclastic tartrate-resistant acid phosphatase", *J. Biol. Chem.* **274**, 22907–22910.
[35] Smith, B.P., Fisher, G.L., Poulos, P.W. and Irwin, M.R. (1975) "Abnormal bone development and lameness associated with secondary copper deficiency in young cattle", *J. Am. Vet. Med. Assoc.* **166**, 682–688.
[36] Owen, E.C. and Proudfoot, R. (1968) "The effect of tungstate ingestion on xanthine oxidase in milk and liver", *Br. J. Nutr.* **22**, 331–340.
[37] Suda, N., Morita, I., Kuroda, T. and Murota, S. (1993) "Participation of oxidative stress in the process of osteoclast differentiation", *Biochem. Biophys. Acta* **1157**, 318–323.
[38] Datta, H.K., Rathod, H., Manning, P., Turnbull, Y. and McNeil, C.J. (1996) "Parathyroid hormone induces superoxide anion burst in the osteoclast: evidence for the direct instantaneous activation of the osteoclast by the hormone", *J. Endocrinol.* **149**, 269–275.
[39] Samar, B., Michaelson, K., Olofsson, H., Johansson, S. and Melhus, H. (2001) "Association between oxidative stress and bone mineral density", *Biochem. Biophys. Res. Commun.* **288**, 275–279.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/26/11
For personal use only.

RIGHTSLINK