Vol. 207, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 6, 1995 Pages 280-287

THE ROLE OF REACTIVE OXYGEN INTERMEDIATES IN OSTEOCLASTIC BONE RESORPTION

T. J. Hall[1], M. Schaeublin, H. Jeker, K. Fuller* and T.J. Chambers*

Ciba-Geigy Ltd., Research Department, Pharmaceuticals Division, Basel, Switzerland

*Department of Histopathology, St. George's Hospital Medical School, London, U.K.

Received December 13, 1994

Osteoclasts have been shown to produce reactive oxygen intermediates (ROI) and it has been suggested that ROI are involved in the process of bone resorption. ROI have also been shown to play a central role in the activation of the multisubunit transcription factor NF-κB that enhances the transcription of genes encoding defence and signaling proteins. Therefore, we have assessed the effect of pyrrolidine dithiocarbamate (PDTC), an oxygen-radical scavenger and metal chelator that is a selective and potent inhibitor of NF-κB activation, on osteoclastic bone resorption in the bone slice assay. PDTC (0.001 - 0.1 mM) dose-dependently and non-cytotoxically inhibited osteoclast activity with an IC_{50} of 0.01 mM. PDTC (0.01 mM) caused no change in the ratio of resorption pit area to resorption pit depth as measured by Lasertec confocal microscopy, indicating that ROI are not involved in the resorptive process *per se*. This view is supported by time-course studies showing that addition of PDTC or N-acetyl cysteine (NAC; an ROI scavenger, but not metal chelator), 6 hr after the start of the assay had no significant effect on subsequent bone resorption. Desferal (100 μM), a chelator of iron and other metal ions, had no significant effect on bone resorption, indicating (along with the results with NAC) that ROI-scavenging rather than metal chelation is responsible for inhibition of osteoclastic bone resorption by PDTC. Taken together these results indicate that ROI produced by osteoclasts in the bone slice assay are not involved in the process of bone resorption, but are important during osteoclast activation for bone resorption, possibly being involved in activation of the transcription factor NF-κB.

[1]Address all correspondence to Dr. Tony J. Hall, Ciba-Geigy Ltd., Pharma Research, R-1056.P.05, CH-4002 Basel, Switzerland.

Abbreviations: hCT, human calcitonin; NAC, N-acetyl cysteine; PDTC, pyrrolidine dithiocarbamate; ROI, reactive oxygen intermediates.

0006-291X/95 $5.00

It has been shown that osteoclasts can produce reactive oxygen intermediates (ROI) such as superoxide (O_2^-) and it has been suggested that ROI may be involved in the bone resorptive process, e.g. by facilitating the degradation of bone collagen [1-3]. However, it is becoming clear that ROI play a central role in cellular activation in response to a variety of stimuli, by activation of the multisubunit transcription factor NF-κB that enhances the transcription of genes encoding defence and signaling proteins [4,5]. Bax et al. have reported that addition of hydrogen peroxide (H_2O_2) to the bone slice assay can increase bone resorption by osteoclasts [6], and discussed the possible involvement of nuclear factor-κB (NF-κB) in osteoclast activation. NF-κB is a multisubunit protein complex consisting of two stimulatory subunits, p50 and p65 and an inhibitory subunit, IκB [5,7]. NF-κB is activated by exposure of cells to mitogens, cytokines, or oxidative stress, and it appears that ROI may represent the final common mechanism for activation of NF-κB by these various stimuli, by releasing IκB from the stimulatory subunits [4,5,7]. Dithiocarbamates have been shown to be potent inhibitors of NF-κB activation in T cells and macrophages, by virtue of their oxygen-radical scavenging activity [8-10]. Pyrrolidine dithiocarbamate has been shown to be a particularly potent and specific inhibitor of NF-κB activation [9,10], and studies examining the effect of PDTC on osteoclastic bone resorption in the bone slice assay are reported here.

MATERIALS AND METHODS

PDTC (Sigma) was dissolved in DMSO at 1M and stored at 4°C. At concentrations of 1 mM and lower, the compound did not affect the pH of the culture medium used in the bone slice assay (pH 6.7-6.8 when incubated at 37°C in a humidified 5% CO_2 in air atmosphere). N-acetyl cysteine (NAC; Sigma) was dissolved in Eagle's MEM, neutralized with 1 M NaOH, made up to to 1 M, sterile filtered and stored at 4°C. Desferal (Ba33112; Ciba Geigy, Basel) was dissolved in deionized water at 100 mM, sterile filtered and stored at 4°C. Human calcitonin (hCT; kindly provided by H. Rink, Ciba-Geigy, Basel) was dissolved in PBS containing 0.1% BSA at 20 μg/ml and stored at -20°C.

Bone resorption assay

The isolation and culture of osteoclasts for use in the bone resorption assay has been described in detail previously [11,12]. Briefly, femora and tibiae from neonatal rats were cleaned and the osteoclasts released by curetting the bones, followed by pipetting in HEPES-buffered medium 199 (Gibco). The cell suspension was allowed to sediment and attach onto bovine cortical bone slices (3 x 3 x 0.1 mm) for 15 min at 37°C, when the non-adherent cells were washed off and the bone slices cultured in 96-well plates in Eagle's MEM supplemented with 2 mM glutamine and 5% fetal calf serum (Gibco). The compounds diluted in supplemented Eagle's MEM were added to the bone slices at the beginning of culture or various times thereafter. After incubation for 24 hr, the bone slices were formalin-fixed and stained with toluidine blue for assessment by reflected light microscopy [12], using a Leitz Laborlux microscope fitted with a Kappa CF15/2 video camera (Gleichen, Germany). Bone resorption was quantified by measuring the total number and surface area of excavations in each bone slice. Osteoclasts on bone slices were identified and counted by virtue of their

size, multinuclearity, morphology and characteristic brown staining colour. Statistical differences between groups were analysed by non-parametric two-tailed Mann-Whitney test using the InStat™ program (GraphPad Software, San Diego, CA).

Lasertec confocal microscopy

Bone slices incubated with 0.01 mM PDTC (which inhibited the area resorbed per bone slice by 48% in this experiment) or medium alone were sputter-coated with gold palladium. The first 25 resorption pits observed (5 per bone slice) were assessed for pit depth and plan area, and the pit volume was then calculated.

RESULTS

Effect of PDTC on osteoclastic bone resorption

As shown in Fig. 1, PDTC inhibited bone resorption in a concentration-dependent manner, with maximal non-toxic inhibition of ~90% at 0.1 mM and an IC_{50} = 0.01 mM (from number of pits or area resorbed per bone slice). The area per resorption pit was a less sensitive parameter (IC_{50} = 0.05 mM), but indicates that the compound acts at the level of individual osteoclasts. At 1 mM, PDTC was cytotoxic and significantly decreased osteoclast survival on bone slices (Table 1), but at lower concentrations the compound did not affect osteoclast survival or morphology.

Effect of N-acetyl cysteine and desferal on osteoclastic bone resorption

PDTC is both an anti-oxidant and a metal chelator [9]. Therefore, we examined the effects of NAC (anti-oxidant, not metal chelator) and desferal (metal chelator, not anti-oxidant) in the bone slice assay. As shown in Fig. 2, NAC (30 mM [9]) strongly inhibited osteoclastic bone resorption assessed by area resorbed per bone slice, and was not cytotoxic at this concentration (Table 2). Desferal, on the other hand, had no significant effect on osteoclastic bone resorption at 100 μM, a concentration which strongly inhibits UMR-106 cell proliferation (by chelating iron [13]) and is not cytotoxic

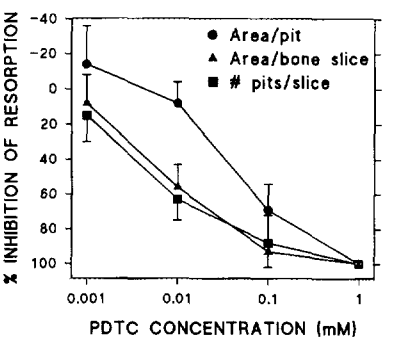

Figure 1. Effect of PDTC on osteoclastic bone resorption.
PDTC was added at the start of the 24-hr bone slice assay. The results shown are the mean ± SEM of 4 separate experiments (15-20 bone slices per point). Control values (mean ± SEM) were: Area resorbed per bone slice = 6360 ± 1790 μm^2; Number of pits per bone slice = 6.9 ± 1.2; area/pit = 890 ± 170 μm^2.

Table 1. Effect of PDTC, NAC and desferal on osteoclast survival

COMPOUND	% INHIBITION OF BONE RESORPTION	No. OC/BONE SLICE (Mean ± SD, n = 15)
Control	-	6.4 ± 2.3
PDTC 1 mM	100	0.3 ± 0.3*
PDTC 0.1 mM	95	5.5 ± 1.7
PDTC 0.01 mM	50	6.3 ± 1.3
Control	-	6.3 ± 2.2
NAC 30 mM	92	5.0 ± 1.8
Control	-	5.5 ± 1.4
Desferal 0.1 mM	-4	6.0 ± 2.1

*p = 0.017 compared to control.

to osteoclasts (see Table 2). Taken together, these results suggest that it is the anti-oxidant activity of PDTC, rather than its metal chelating properties, that is responsible for its inhibitory activity on osteoclasts.

Effect of time of addition of PDTC and NAC to the bone slice assay on osteoclastic bone resorption in comparison to human calcitonin (hCT)

We have previously reported on the kinetics of osteoclastic bone resorption [12] and we have shown that hCT inhibits all osteoclast activity subsequent to its addition at any time after the start of the 24 hr bone slice assay [14]. We have used this kinetic system to assess the effect of addition of PDTC (0.1 mM) or NAC (30 mM) at various times after the start of the bone slice assay. As shown in Fig. 3, both PDTC and NAC

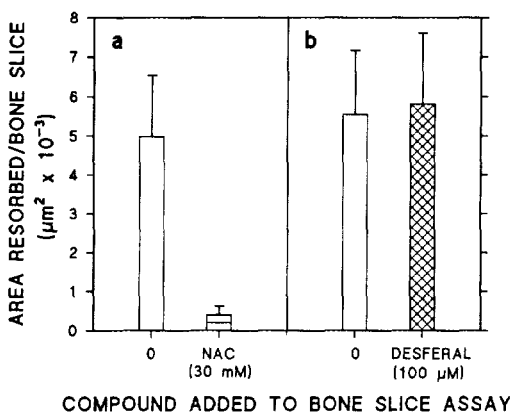

Figure 2. Effect of NAC and desferal on osteoclastic bone resorption. NAC and desferal were added at the start of the 24-hr bone slice assay. The results are mean ± SEM from 3 experiments with each compound (15 bone slices per point).

Table 2. Lack of effect of PDTC on the process of osteoclastic bone resorption assessed by Lasertec confocal microscopy

	CONTROL		PDTC (0.01 mM)
CONFOCAL MICROSCOPY (Mean ± SEM, n = 25 pits)			
Pit depth (μm)	5.99 ± 0.4	(9%)	5.46 ± 0.5
Pit area (μm^2)	1957 ± 338	(-7%)	2103 ± 372
Pit volume (μm^3)	6224 ± 1387	(5%)	5934 ± 1233
LIGHT MICROSCOPY (Mean ± SD, n = 5 bone slices)			
Area resorbed / bone slice (μm^2)	8510 ± 2450	(48%)*	4390 ± 1720

In confocal microscopy, resorption pits of similar plan surface area were measured. Figures in parentheses are percent inhibition by PDTC compared to control values. *p = 0.015.

inhibited osteoclastic bone resorption by ~90% when added at the start of the assay, while hCT (1 ng/ml) completely inhibited osteoclast activity. Addition of PDTC and NAC at 3 hr when ~10% of resorption had already occured only inhibited the subsequent 90% of resorption by ~60%, while hCT completely inhibited all subsequent resorption. The loss of activity of PDTC and NAC was even more striking

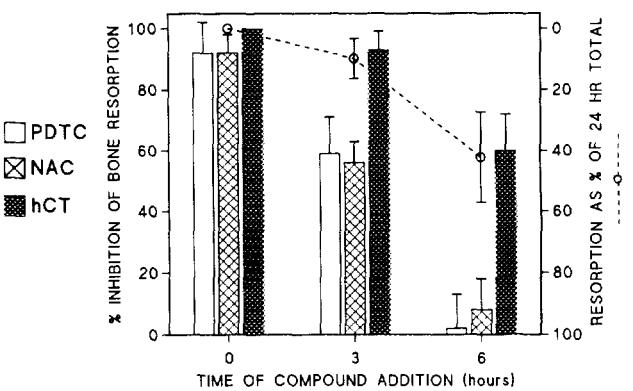

Figure 3. Effect of time of addition of PDTC and NAC to the bone slice assay on osteoclastic bone resorption.
The results shown are the mean ± SEM plan area resorbed per bone slice from 3 or 4 experiments (15 - 20 bone slices) for each compound at each time point. The control area resorbed per bone slice in 10 experiments was 6170 ± 1470 μm^2 (mean ± SEM). Compound concentrations were: PDTC 0.1 mM, NAC 30 mM, hCT 1 ng/ml. The data for bone resorption as a percent of that at 24 hr is taken from reference [12].

at 6 hr where they had no significant effect, despite the fact that ~60% of resorption took place during the following 18 hrs, as shown by the inhibitory effect of hCT (~60%). These results show that ROI are not involved in the process of osteoclastic bone resorption, but rather suggest that they play a role in osteoclast activation which occurs during the early stages ($\leq$3 hr) of the bone slice assay [12].

Lack of effect of PDTC on the process of osteoclastic bone resorption

If ROI are involved in the process of osteoclastic bone resorption, then it would be expected that, for pits of similar plan surface area, the depth of the pits would be reduced by PDTC. Therefore, bone slices from an experiment where PDTC (0.01 mM) inhibited bone resorption by 48% (Table 2), were analysed by Lasertec confocal microscopy. When the depth and volume of pits are compared (Table 2), these parameters were similar in control and PDTC treated cultures. Thus, the ratio of pit area to pit depth was unaffected by PDTC, indicating by direct measurement of resorption pits, that ROI are involved in the process of bone resorption *per se*.

DISCUSSION

In this study we have examined the effects of compounds with anti-oxidant and/or metal chelating properties on osteoclastic bone resorption. The results with PDTC and NAC show that compounds with anti-oxidant activity can strongly inhibit osteoclast activity (Figs. 1 and 2), whereas the metal chelator desferal was without effect (Fig. 2). These results support previous suggestions that ROI play a role in osteoclastic bone resorption [1-3]. However, we have examined the effects of PDTC and NAC in kinetic experiments in order to ascertain more precisely the role played by ROI in osteoclastic bone resorption. We found that anti-oxidants inhibit osteoclastic bone resorption when added early to the bone slice assay ($\leq$3 hr), but not when added later (6 hr) at a time when bone resorption is taking place. These results indicate that ROI play a role in early events associated with osteoclast activation for bone resorption, but do not play a major role in the process of bone resorption by osteoclasts. This was supported by the confocal microscopy studies which showed that PDTC caused no change in the ratio of resorption pit area to resorption pit depth.

It is interesting to note that both PDTC and NAC have been shown to inhibit the activation of the transcription factor NF-κB by ROI in various cell types [8-10], and thus it may be that NF-κB is involved in osteoclast activation for bone resorption. Data from Baeuerle's group indicate that H_2O_2 is the ROI involved in NF-κB activation [5,7,9], and it has been reported that H_2O_2 can stimulate osteoclastic bone resorption [6], however we have been unable to reproduce these latter results (unpublished observations). It has also been shown that serine protease inhibitors such as tosyl-Phe-chloromethylketone (TPCK) can inhibit NF-κB activation [15,16], and this may represent an alternative mechanism to ROI for activating NF-κB. We attempted to obtain additional evidence for a role of NF-κB in osteoclast activation by testing the

effect of TPCK in the bone slice assay, but at concentrations used to inhibit IκB proteolysis (20 μM), we found TPCK was cytotoxic to osteoclasts (data not shown).

In many cell types, NF-κB activation leads to the transcription of genes encoding signaling and defence proteins [4,5,9]. Thus, activation of NF-κB in osteoclasts may result in the expression of proteins required for bone resorption (carbonic anhydrase II, V-ATPase etc. [17]), and proteins involved in protecting the osteoclast against ROI-induced damage (glutathione synthase, superoxide dismutase, catalase etc. [18]). In addition, it has been suggested that ROI-activated NF-κB may be involved in osteoclastogenesis by stimulating the expression of interleukin-6 and its receptor in osteoclast precursors [19].

It has recently been reported that actively resorbing osteoclasts express NADPH-oxidase [20], which would account for the production of ROI by osteoclasts observed by other groups [1-3]. These groups have suggested that ROI are involved in osteoclastic bone resorption, possibly by damaging bone collagen and making it more amenable to degredation by proteases secreted by osteoclasts. However, such a mechanism would be biologically inefficient since ROI could also be expected to counterproductively damage osteoclast proteases as well. Although NADPH-oxidase represents a host defence mechanism in macrophages and neutrophils [21,22], it is present in other cell types e.g., fibroblasts and endothelial cells where it is activated by growth factors and shear stress, respectively, indicating that physiological production of ROI related to cellular signaling and activation is also a function of this enzyme [5,7,21]. Our data suggest that ROI are not involved in the process of bone resorption by osteoclasts, but rather in osteoclast activation e.g. by activation of NF-κB, prior to bone resorption taking place. However, it is known that osteoclasts undergo apoptosis after bone resorption [23], and it may be that prolonged production of ROI by NADPH-oxidase during bone resorption eventually leads to oxidative stress-mediated apoptosis of osteoclasts, as is seen in other cells [24].

Acknowledgments: We wish to thank Drs. J.R. Green and U. Feige for constructive comments on the manuscript. This work was partly supported by the Wellcome Trust and the ARC.

REFERENCES

1. Garrett, I.R., Boyce, B.F., Oreffo, R.O.C., Bonewald, L., Poser, J. and Mundy, G.R. (1990) J. Clin. Invest. 85, 632-639.
2. Key, L.L., Ries, W.L., Taylor, R.G., Hays, B.D. and Pitzer B.L. (1990) Bone 11, 115-119.
3. Ries, W.L., Key, L.L. and Rodriguiz, R.M. (1992) J. Bone Min. Res. 7, 931-939.
4. Schreck, R. and Baeuerle, P.A. (1991) Trends Cell Biol. 1, 39-42.
5. Schreck, R., Albermann, K. and Baeuerle, P.A. (1992) Free Rad. Res. Comm. 17, 221-237.
6. Bax, B.E., Towhidul, A.S.M., Banerji, B., Bax, C.M.R., Bevis, P.J.R., Stevens, C.R., Moonga, B.S., Blake, D.R. and Zaidi, M. (1992) Biochem. Biophys. Res. Comm. 183, 1153-1158.

7. Grimm, S. and Baeuerle, P.A. (1993) Biochem. J. 290, 297-308.
8. Schreck, R., Rieber, P. and Baeuerle, P.A. (1991) EMBO J. 10, 2247-2258.
9. Schreck, R., Meier, B., Männel, D.N. Dröge, W. and Baeuerle, P.A. (1992) J. Exp. Med. 175, 1181-1194.
10. Zigler-Heitbrock, H.W.L., Sternsdorf, T., Liese, J., Belohradsky, B., Weber, C., Wedel, A., Schreck, R., Baeuerle, P.A. and Ströbel, M. J. Immunol. 151, 6986-6993.
11. Chambers, T.J., McSheehy, P.M.J., Thompson, B.M. and Fuller, K. (1985) Endocrinol. 116, 234-239.
12. Hall, T.J., Schaeublin, M. and Chambers, T.J. (1992) Biochem. Biophys. Res. Comm. 188, 1097-1103.
13. Hall T.J. (1994) Cell Biol. Int. 18,189-193.
14. Hall, T.J., Schaeublin, M. and Missbach, M. (1994) Biochem. Biophys. Res. Comm. 199, 1237-1244.
15. Henkel, T., Machleidt, T., Alkalay, I., Krönke, M., Ben-Nerlah, Y. and Baeuerle, P.A. (1993) Nature 365, 182-185.
16. Saldeen, J. and Welsh, N. (1994) Biochem. Biophys. Res. Comm. 203, 149-155.
17. Chambers, T.J., and Hall, T.J. (1991) Vitamins and Hormones 46, 41-86.
18. Fridovich, I. (1978) Science 201, 875-880.
19. Mee, A.P. and Sharpe, P.T. (1993) BioEssays 15, 783-789.
20. Steinbeck, M.J., Appel, W.H., Verhoeven, A.J. and Karnovsky, M.J. (1994) J. Cell Biol. 126, 765-772.
21. Chanock, S.J., Benna, J.E., Smith, R.M. and Babior, B.M. (1994) J. Biol. Chem. 269, 24519-24522.
22. Segal, A.W and Abo, A. (1993) Trends Biochem. Sci. 18, 43-47.
23. Lin, C.C., Rader, J.I., Gruber, H. and Baylink D.J. (1982) Metab. Bone Dis, Rel. Res. 4, 201-209.
24. Buttke, T.M and Sandstrom, P.A. (1994) Immunol. Today 15, 7-10.