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Autocrine and Paracrine Nitric Oxide Regulate Attachment of Human Osteoclasts

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Abstract Nitric oxide (NO) can reduce bone loss in chronic bone diseases. NO inhibits or kills osteoclasts, but the mechanism of action of NO in human bone turnover is not clear. To address this, we studied effects of NO on attachment and motility of human osteoclasts on mineralized and tissue culture substrates under defined conditions. Osteoclasts were differentiated in vitro from CD14 selected monocytes in RANKL and CSF-1, and characterized by cathepsin K expression, tartrate-resistant acid phosphatase (TRAP) activity, acid secretion, and lacunar resorption. Cell attachment was labeled with monoclonal antibody 23C6, specific for a binding domain of a key osteoclast attachment protein, the CD51/CD61 integrin dimer ($\alpha_v\beta_3$), with or without cell permeabilization. A ring of integrin attachment during bone degradation delimits an extracellular acid compartment, while $\alpha_v\beta_3$ forms focal attachments on non-resorbable substrates. On resorbable substrate but not non-resorbable substrate, $\alpha_v\beta_3$ labeling required cell permeabilization, in keeping with the membrane-matrix apposition that excludes large molecules and allows extracellular acidification. Acid secretion was labeled with the fluorescent weak base indicator lysotracker. NO donors, *S*-nitroso-*N*-acetyl penicillamine (SNAP) or sodium nitroprusside (SNP), downmodulated acid secretion simultaneously with cytoskeletal rearrangement, with $\alpha_v\beta_3$ redistributed to a discontinuous pattern that labeled, on bone substrate, without membrane permeabilization. These effects were reversible, and an inhibitor of NO synthesis, N^G-monomethyl-L-arginine (L-NMMA), increased acid secretion and decreased heterogeneity of attachment structures, showing that NO is an autocrine regulator of attachment. A hydrolysis-resistant activating cGMP analog 8-(4-chlorophenylthio)guanosine-3′,5′-cyclic monophosphate replicated effects of NO donors, while an inhibiting analog, 8-(4-chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate, Rp-isomer, opposed them. On tissue culture or mineralized substrates, NO or cGMP analogs directly regulated motility; after washout cells reattached and survived for days. We conclude that NO is produced by human osteoclasts and regulates acid secretion and cellular motility, in keeping with autocrine and paracrine NO regulation of the resorption cycle. J. Cell. Biochem. 91: 962–972, 2004.

Key words: podosome; integrin; osteoporosis; cytoskeletal disassembly; lacunar resorption

Osteoclasts are multinucleated cells of the monocytic lineage. The physiological function of the osteoclast is to perform lacunar resorption of mineralized tissue, including bone, a function that is retained in human osteoclasts differentiating from circulating monocytes or marrow cells [Quinn et al., 1998]. In this study, we examined the effect of nitric oxide (NO) on human osteoclasts produced in vitro from CD14 selected peripheral mononuclear cells in serum-containing media supplemented with the key osteoclast differentiation cytokines, CSF-1 and RANKL. Although cells formed in tissue may theoretically have some differences with cells forming in bone marrow, this in vitro model has the advantage of being absolutely devoid of mesenchymal cells, which produce NO and other cytokines. In addition, major osteoclast phenotype markers were examined on the cells

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made in vitro, including cathepsin K expression, tartrate-resistant acid phosphatase (TRAP) activity, extracellular acid secretion, expression of the vitronectin receptor, and ability to produce resorption lacunae on mineralized substrate.

During their functional cycle, osteoclasts show dramatic phenotypic changes. They form a sealing zone, a specialized cell-extracellular matrix adhesion structure, that is dynamically regulated with resorption [Faccio et al., 2002]. Within the sealing zone, the osteoclast produces an organelle, the ruffled border, that secretes HCl to dissolve the bone mineral. This HCl secretion is driven by a vacuolar H^+-ATPase [Blair et al., 1989]; its expression in the osteoclast is guided by special membrane subunit of the pump [Li et al., 1999]. It is not known how the specialized sealing zone is targeted by the osteoclastic H^+-ATPase, although it is clear that acid secretion depends on the cell attachment, and that the acid vacuoles appear in the cytoplasm of osteoclasts that are not attached to mineralized substrate. The attachment zone contains a dense cytoplasmic actin ring and this region of the cell is attached to the matrix by transmembrane proteins, central to which is the integrin $\alpha_v\beta_3$ [Ross et al., 1993; Nakamura et al., 1999]. When adhering to plastic or glass, osteoclasts attach via punctate podosomes to which actin filaments are bound [Teti et al., 1989], containing the same proteins found in the bone attachment, including $\alpha_v\beta_3$. The osteoclast is capable of attachment and detachment to bone for many cycles of degradation and it is quite motile when not degrading a pit in the bone. Osteoclast precursors bear different integrins than osteoclasts; these are lost during differentiation. Thus, $\alpha_v\beta_3$ is also used as a marker of cell maturation for in vitro differentiation.

NO is a key factor in progression of inflammatory bone diseases and is produced by bone cells including osteoblasts [Armour et al., 2001]. Thus, paracrine regulation of bone turnover by NO is believed to be important. NO causes osteoclast detachment and terminates bone degrading activity [Brandi et al., 1995]. These effects are mediated by the cGMP-dependent protein kinase via the NO-dependent soluble guanosyl cyclase [Dong et al., 1999]. Effects of NO on cell survival are seen in pre-osteoclasts and osteoclasts [van't Hof and Ralston, 1997]. Further, NO is reported an autocrine product of osteoclasts in the presence of pro-inflammatory cytokines [Sunyer et al., 1996]. However, whether NO plays a functional role in the osteoclast's resorption cycle under normal circumstances is uncertain. It is clear that continuous exposure to high concentrations of NO causes osteoclastic cell death [van't Hof and Ralston, 1997; Mentaverri et al., 2003], but it is unclear that what are the effects of NO on osteoclastic cellular physiology at lower, reversible concentrations. Uncertainties have undoubtedly resulted from variations in uncontrolled variables between cell culture, organ explant, and in vivo studies. Many studies have examined osteoclasts on tissue culture matrix rather than on bone. Some crucial results, such as whether NO is produced by osteoclasts, have varied between species [Sunyer et al., 1996; Dong et al., 1999].

To resolve the question of whether NO regulates normal human bone degradation, we studied effects of NO donors, NO synthesis blockers, and downstream effector including cGMP analogs on attachment and motility of human osteoclasts. We compared results using osteoclasts cultured on mineralized substrate to effects in cells on tissue culture substrate. Studies were performed under conditions permitting long-term cell survival. Use of osteoclasts produced in vitro from CD 14-selected peripheral monocytes allowed determination of the effects of NO on cell attachment and acid secretion without interference from stromal cell or osteoblastic signals. Data using antagonists of NO synthesis suggest that human osteoclasts produce NO in relation to cell motility. Effects of regulators of NO synthesis and of cGMP-dependent protein kinase on motility on tissue culture substrate and mineralized matrix produced similar data, validating previous studies that used tissue culture substrates, although the cell attachment pattern differed. Our studies indicate that human osteoclast attachment, either on bone or tissue culture substrate, is reversibly affected by moderate concentrations of NO and suggest that autocrine NO production is important in regulating attachment and motility of human osteoclasts in vivo.

MATERIALS AND METHODS

Reagents and Antibodies

Cytokines were from Research Diagnostics, Inc. (Flanders, NJ) unless otherwise indicated, and were recombinant human protein ligand

domains, produced in bacteria, used at 10 ng/ml, unless specified. Anti-vitronectin receptor CD51/CD61 ($\alpha_v\beta_3$) was the monoclonal anti-human antibody 23C6 from Santa Cruz (Santa Cruz, CA), used at 1:50. Al-488-conjugated goat anti-mouse (Molecular Probes, Eugene, OR) was used at 1:500. Rhodamine phalloidin and lysotracker were from Molecular Probes and were used at 1:250 and 5 μM, respectively. *S*-nitroso-*N*-acetyl penicillamine (SNAP) and SNP were from Sigma (St Louis, MO). 8-(4-chlorophenylthio)guanosine-3′,5′-cyclic monophosphate (8-pCPT-cGMP) and 8-(4-chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-cGMPS; this abbreviation is used to distinguish the clorophenylthio activator from the phosphorothioate inhibitor, the inhibitor is also often abbreviated Rp-8-pCPT-cGMPS) were from Biolog-Life Science Institute (Bremen, Germany); 6-anilinoquinoline-5,8-guinone (Ly83583) and N^G-monomethyl-L-arginine acetate (L-NMMA) were from Biomol (Plymouth Meeting, PA).

Cell Culture

Leukocytes were taken with IRB approval from discarded buffy coats of leukoreduced red cells as described [Bu et al., 2003]. Leukocytes were enriched by density gradient centrifugation on Ficoll-Hypaque (Amersham Pharmacia, Piscataway, NJ). CD14 cells were selected by suspending 2×10^9 leukocytes in 3 ml of PBS with 0.5% BSA and 2 mM EDTA with 1 ml of magnetic beads conjugated with mouse anti-human CD14 antibody (MACS, Miltenyi Biotec USA, Auburn, CA). After 30 min at 4°C, cells were held in a column with a magnet, washed, and eluted after removing the magnet. Flow cytometry as described [Bu et al., 2003], showed that 95–97% of eluted cells expressed CD14 and CD11b (not illustrated; for similar examples see Bu et al., 2003). For osteoclast differentiation 1×10^5 per cm^2 of these CD14 cells are cultured 2–4 weeks in DMEM medium, supplemented with 10% heat-inactivated new born calf serum, 10 ng/ml each of recombinant human TGFβ and CSF-1 (M-CSF), and 30 ng/ml of RANKL, 10^{-8} M each of 1,25 dihydroxyvitamin D and dexamethasone [Hirayama et al., 2002] on 8 mm diameter bone slices or other conditions as specified. Enzyme makers for osteoclast differentiation and osteoclast pit assays were performed as described [Blair et al., 2000; Hirayama et al., 2002].

Immune and Affinity Labeling

Cells were washed in PBS with 2% fetal calf serum and 0.1% sodium azide, and fixed in 1% paraformaldehyde on ice for 25 min followed by washing and addition of 75% cold ethanol. Cells were stored at −20°C. Where specified, permeabilization used 0.25% polyoxyethylated octyl phenol (Triton X-100) and 40 μg/ml digitonin for 5 min. Prior to antibody reaction, preparations were incubated in 1% bovine serum albumin and 5% goat serum for 30 min to reduce non-specific reaction. Cells were then incubated with primary antibody, washed, and incubated with specified conjugated anti-antibody for 1 h. In some experiments, two antibody labeling procedures were done using mouse and rabbit antibodies in subsequent reaction, or the cells were labeled with rhodamine phalloidin to show filamentous actin or Hoechst dye to show nuclei using the appropriate affinity reactions following the immune labeling procedures. Osteoclasts were labeled without fixation and without permeabilization in some experiments, for expression of $\alpha_V\beta_3$ where fluorescent acid transport indicators were also used, and for expression of the surface antigen CD14. In these experiments, cells were washed in PBS, non-specific reactivity was blocked by incubation with 1% bovine serum albumin and 5% goat serum for 15 min, followed by incubation with primary and secondary antibody.

Motility

For motility assays, tissue culture dishes were sealed, and time-lapse video microscopy was performed by photographing cells in phase contrast for 2 h at 2 min intervals, after pre-treatments indicated in results. The maximal linear deviation was measured from deviation in sequential images after the method of Bear et al. [2002], by overlaying cell outlines and measuring the linear deviation in microns, and dividing this by the time for each cell. To estimate the motility of cells on bone, where the substrate interfered with microscopy, cells were grown on bone and after pre-treatment, as indicated in results, bone slices were vigorously washed twice in PBS and transferred upside-down onto cover-glass chambers that had been pre-coated with 10 μM fibronectin, and incubated in DMEM–HEPES at 37°C for 22 h. At the end of this culture period, cultures were stained for TRAP activity as described [Blair et al.,

2000]; labeled cells on bone and fibronectin-coated glass were then counted.

RESULTS

Human Osteoclasts on Dentine and Glass: Acid Secreting and Non-Acid Secreting Cells

Osteoclasts are frequently studied using tissue-culture substrates and few comparisons to osteoclast response on resorbable substrates are available. Therefore, we characterized osteoclasts on whale dentine and compared the results to cells on glass. Differentiation in RANKL and CSF-1 is always variable, but at 3–4 weeks many of the cells on bone were multinucleated, typically with 2–3 nuclei (Fig. 1A). Two distinct types of cells were seen, globular polarized cells that made acid lakes seen in lysotracker (Fig. 1B, arrow) which contrasted with the lysosomal pattern of macrophage-like cells (Fig. 1B, arrowheads). On glass, in keeping with earlier reports, only intracellular acid vacuoles were seen (not illustrated); extracellular acid lakes were observed only on bone. The vitronectin receptor $\alpha_v\beta_3$ was strongly expressed and closely associated with an actin ring (Fig. 1C), but actin broadened at levels farther from the attachment into a filamentous ring, the actin-rich zone known from transmission electron microscopy as the clear zone (Fig. 1D). Acid secreting cells also produced lacunae, demonstrated by scanning electron microscopy (Fig. 1E) and labeled for TRAP (Fig. 1F) and cathepsin K (not illustrated), key characteristics of this cell type.

Integrin Attachment Patterns in Osteoclasts on Bone and Glass

In osteoclasts on resorbable substrates, podosomes were usually not seen. Instead, the integrin is found in a linear pattern at the bone attachment even at high magnification (Fig. 2A), while podosomes on cells attached to glass were easily detectible at much lower magnification (Fig. 2B). Observation of the linear $\alpha_v\beta_3$ distribution required permeabilization of the osteoclasts. When cells on bone were labeled with lysotracker and antibody to $\alpha_v\beta_3$, the linear integrin was not detected, although podosomes and intracellular acid compartments in non-acid secreting cells were labeled (Fig. 2C). In all conditions, some cells remained as undifferentiated macrophages. These mononuclear cells did not express the vitronectin receptor (Fig. 2C) or TRAP (not illustrated), and, unlike osteoclasts [Quinn et al., 1998] retained the CD14 antigen (Fig. 2E). These cells were identifiable by their single nuclei, smaller size, and lack of spreading, and were excluded from measurements in subsequent assays. The rings of $\alpha_v\beta_3$ labeling in bone-attached cells varied in width, with many cells attached by simple thin rings as in Figure 2A, while others had broader attachment rings. Laser scanning confocal microscopy showed that the more extensive $\alpha_v\beta_3$ at substrate attachment correlated with the most active bone degrading cells, found on unmistakable pits (Fig. 2F). Broad rings of integrin binding surrounded the pits. The requirement for permeabilization to visualize integrin attachment of bone-attached cells is consistent with an attachment that excludes large molecules [Blair et al., 1986]. This is also in keeping with the electron microscopic appearance of the osteoclastic attachment, and with the reported exclusion of molecules smaller than ~2 kD from diffusion out of the osteoclast's lacuna (Discussion).

Effects of NO on Osteoclast Attachment and Acid Secretion

The responses of human osteoclast acid secretion to NO and cGMP analogs are illustrated in Figure 3. The effect on cellular attachment was dramatic. The NO synthesis inhibitor NMMA was used to reduce potential effects on morphology of autocrine NO synthesis; this had the effect of showing more uniform actin ring (Fig. 3A) or vitronectin ring (not shown) labeling than had been seen in untreated controls, but otherwise the cells were as described in Figure 1B,C. NO donors were expected to affect osteoclast attachment, based on studies of animal cells, but it was unknown how this would occur, and with NO donors this initially proved to be a dramatic redistribution of actin and vitronectin receptors (Fig. 3B) into a punctate pattern resembling podosomes followed by dissolution of the filamentous actin associated with a vacuolar change and detachment of the cells.

Effects of NO donors and inhibitors on acid secretion were measured from lysotracker uptake. Cells grown on dentine were labeled with lysotracker for 4 min after short-term exposure to NO-pathway modifying agents (Fig. 4). NO donors or cGMP analogs reduced acid accumu-

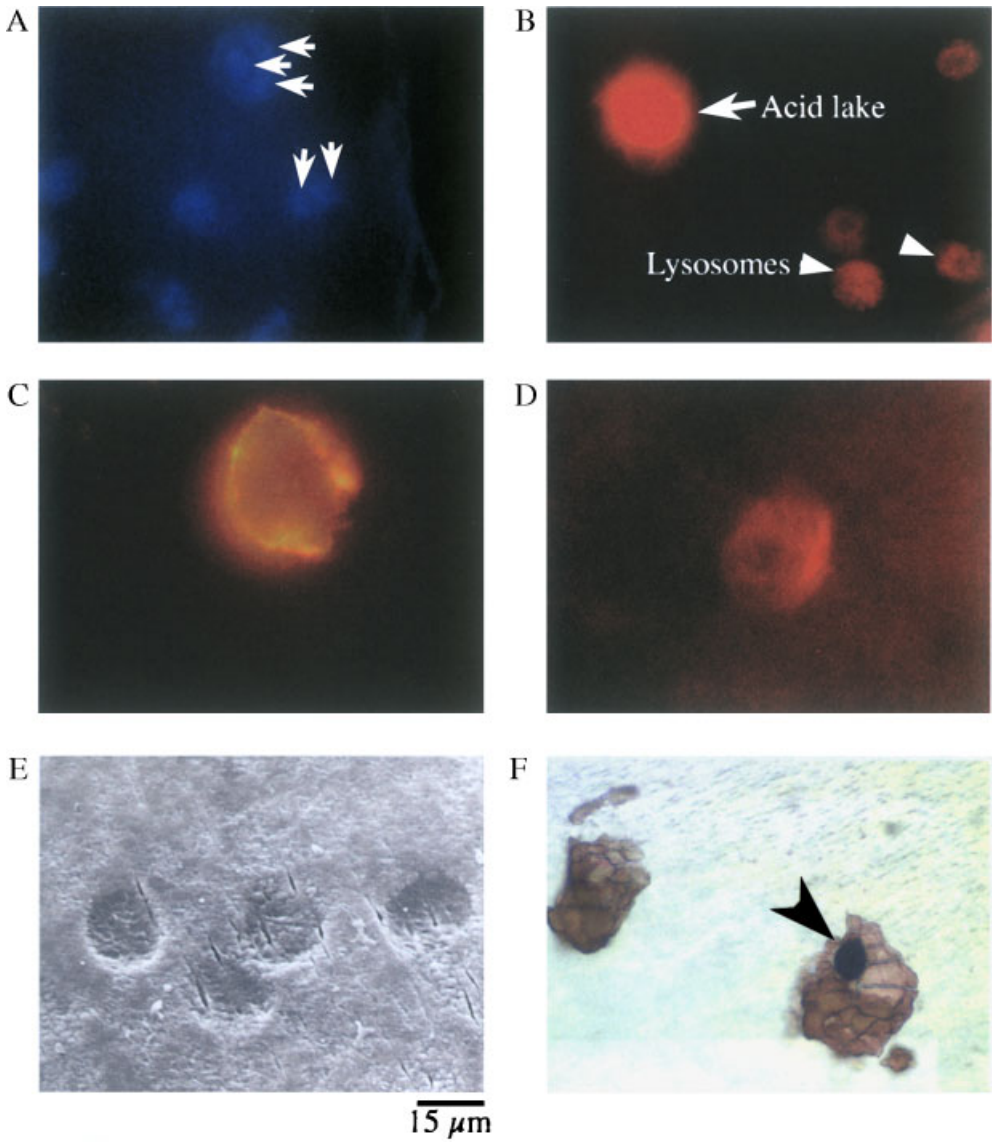

Fig. 1. Human osteoclasts on dentine. Osteoclasts are multinucleated cells, the physiological function of which is lacunar resorption; additional key markers include acid secretion, expression of the vitronectin receptor, and tartrate resistant acid phosphatase (TRAP) activity. **A**: At 3–4 weeks, a significant fraction of cells, typically 30%, of bone attached cells had multiple nuclei (arrows) (400×). **B**: Two distinct phenotypes were seen most clearly by examining acid secretion with lysotracker red. A fraction of bone attached cells made prominent acid lakes (arrow) while other cells retained a macrophage-like lysosomal pattern (arrowheads) (400×). **C**: The linear $\alpha_v\beta_3$ expression in acid-secreting cells was closely associated with an actin ring (integrin $\alpha_v\beta_3$, green, actin red, 400×). **D**: At a plane 3 μm above the attachment, the actin ring was a broad, filamentous ring. **E**: Scanning electron micrograph of a preparation as in (B) showing a pit complex. Pits occurred only with acid lakes and toluidine blue staining showed that superficially attached monocytes (as small cells in (B)) did not produce pits (not illustrated). **F**: Osteoclasts on bone labeled for tartrate resistant osteoclast activity. In this field, a single osteoclast is present which labels strongly (arrowhead). Areas of matrix that have been modified by osteoclasts also label due to residual secreted enzyme. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

lation, while NO synthase inhibition or a blocking cGMP analog increased acid accumulation. The data demonstrate that lysotracker intensity was rapidly reduced ~twofold either in cells treated with the rapidly hydrolyzed NO donor, SNP (full chemical names of agents are given in "Materials and Methods") or 8-pCPT-cGMP, a cGMP analog, when compared to cells treated with the NO-synthesis inhibitor, L-NMMA, or a cGMP analog that blocks activation of cGMP-dependent protein kinase, Rp-cGMPS. The guanosyl cyclase inhibitor

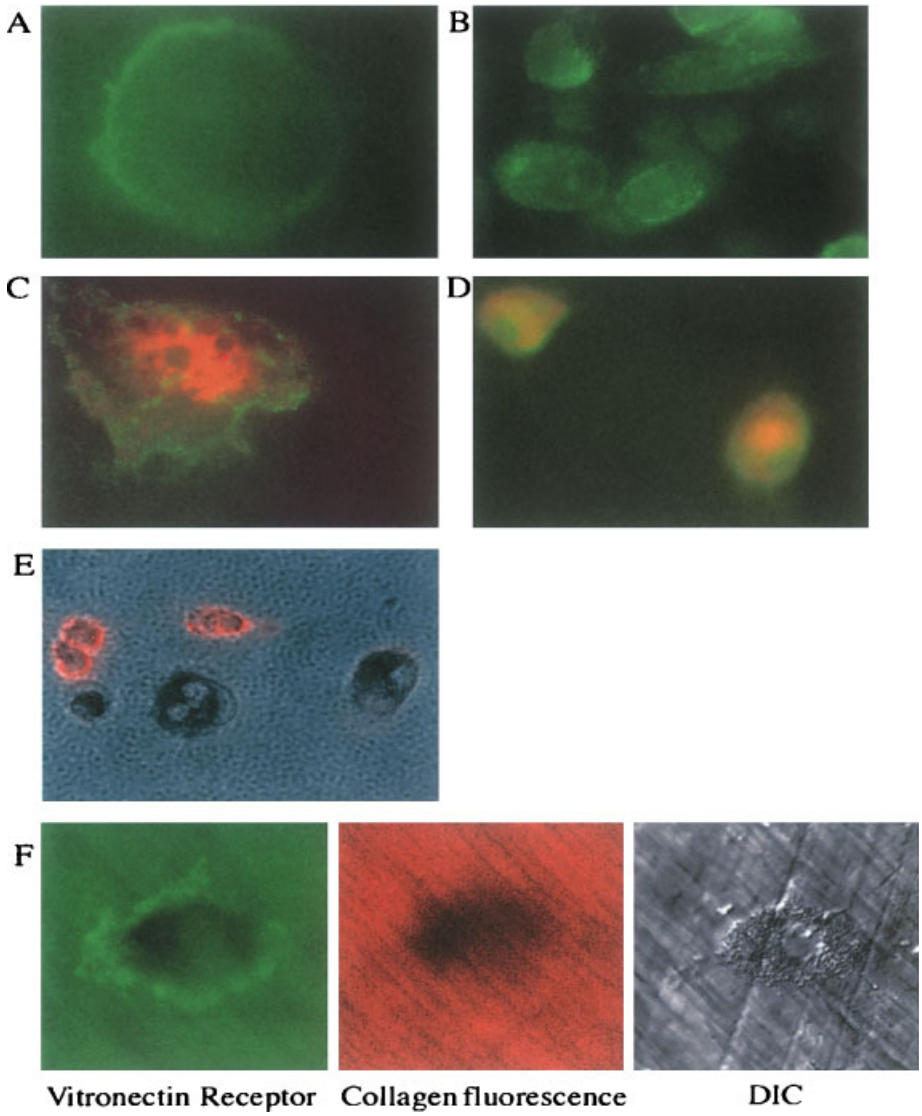

Fig. 2. Vitronectin receptor $\alpha_v\beta_3$ in osteoclasts on bone and glass. **A**: Bone-attached osteoclasts expressed the integrin $\alpha_v\beta_3$ in a linear pattern at the bone attachment, seen after fixation, permeabilization, and labeling (1000×, 115 μm across). **B**: Cells on glass retained the well-described podosomal pattern (integrin $\alpha_v\beta_3$, green). **C–D**: When labeling was done without permeabilization, cells on bone that had intracellular acid compartments showed podosomes (C, lysotracker red and $\alpha_v\beta_3$, green labeling), while in cells with acid lakes the integrin site was inaccessible to the antibody (D, lysotracker red and $\alpha_v\beta_3$, green labeling); the linear $\alpha_v\beta_3$ was only labeled in fixed, permeablized cells as in (A). The same conditions were used for the photographs in C and D, including photographic exposure. Because acid lakes are very bright, these bleed through into the green channel, making the acid site appear yellow in the composite, but no punctate or linear integrin labeling is present. **E**: Large, spread cells lost CD14, which marked smaller undifferentiated cells (red channel). Large cells, which bore vitronectin receptor and expressed TRAP, were used in studies of NO response and motility. Photographs in B–E 400× (300 μm across). **F**: Pattern of $\alpha_v\beta_3$ in an osteoclast on a resorption pit (**left panel**); the antibody labeling is a bright band superimposed on the collagen autofluorescence from the underlying bone. This is a sum of twenty-seven sequential 0.5 μm thick slices from the edge of the bone attachment into the resorption pit, which appears as a dark area at the center. The **middle panel** shows collagen autofluorescence (only) in red; the **right panel** shows a DIC image of the cell on the pit. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Ly83583, 1 μM, had a similar short-term effects to Rp-cGMPS (not illustrated), but cell survival was poor after Ly83583 treatment in long term cultures, and this was not used in subsequent analysis of motility. Importantly, cells without any treatment gave intermediate results, indicating that autocrine NO production may be important in regulation of osteoclastic acid secretion. The effect of L-NMMA relative to control cells was statistically significant ($P < 0.05$).

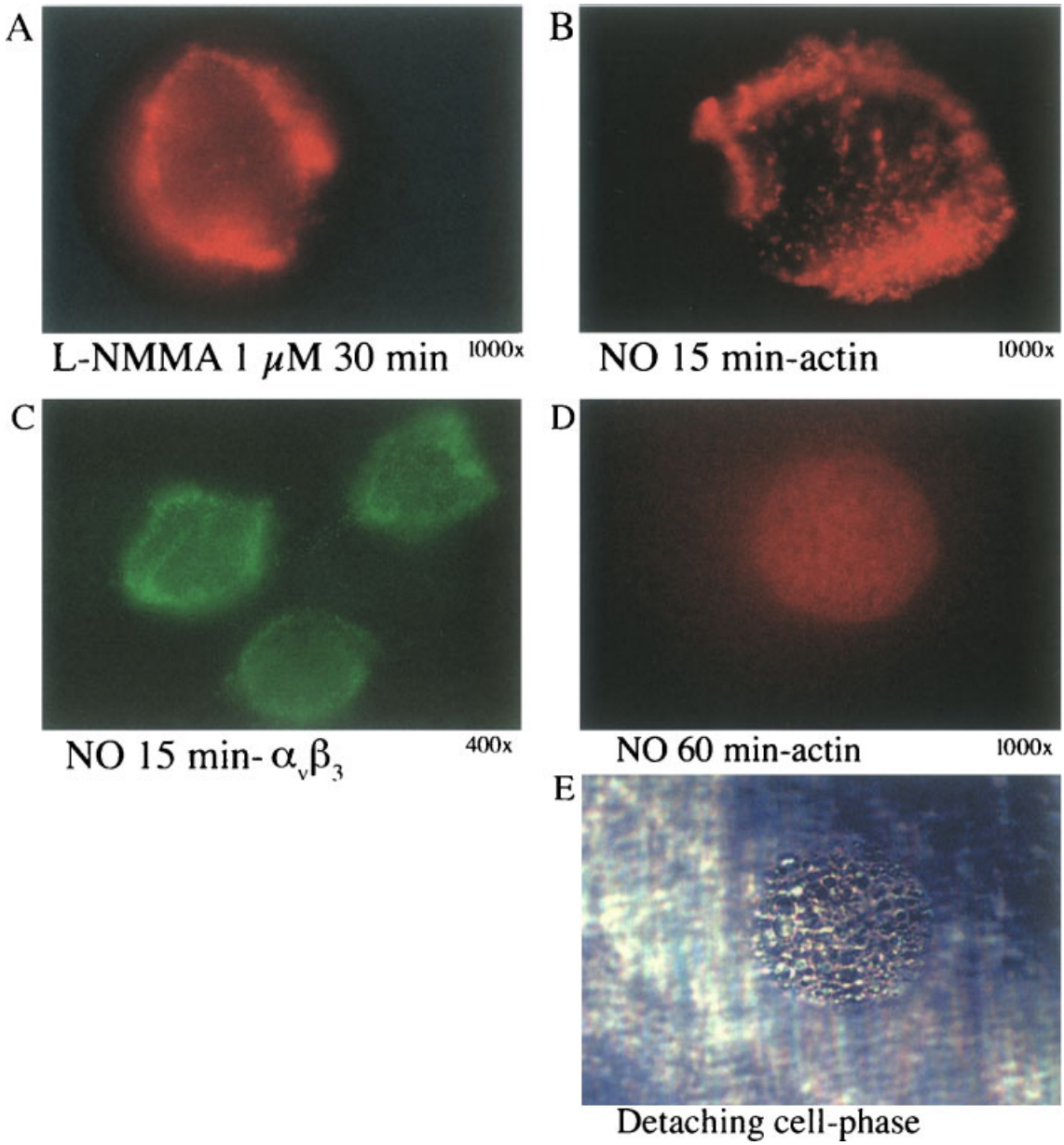

Fig. 3. Effect of NO on bone attachment. **A**: An antagonist of NO synthesis, NG-monomethyl-L-arginine, left the linear pattern of the attachment site and actin ring unchanged. **B**–**C**: Short term treatment using the rapidly hydrolyzed NO donor sodium nitroprusside (SNP, 100 μM, cells 15 min after treatment) caused the attachment to break up into podosome-like punctate areas of the cell, shown in red (actin) and green (integrin $\alpha_v\beta_3$). **D**: After longer treatment using the slowly hydrolyzed NO donor *S*-nitroso-*N*-acetyl penicillamine (SNAP, 20 mM, 1 h), localized integrin labeling was lost (not illustrated) and actin labeling became a diffuse, weak signal. **E**: In coordination with the loss of focal attachment and filamentous actin, the cells detached and assumed a globular shape with a prominent vacuolar pattern. This is the same cell shown in (D) in transmitted light. Field sizes are indicated in Figure 1 for the magnifications shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Effect of Modifying NO Pathways on Cellular Motility on Glass and Dentine

Osteoclasts from CD14 positive cells on glass were incubated with NO pathway effectors and inhibitors as in Figure 4 and then cellular movement was followed by time lapse photography measuring the maximal linear deviation over 80 or 120 min after the method of Bear et al. [2002]. Untreated cells had an intermediate level of motility relative to nitroprusside or 8-CPT-cGMP treatments, which significantly increased motility. The cGMP and NO synthesis antagonists, Rp-cGMPS and L-NMMA,

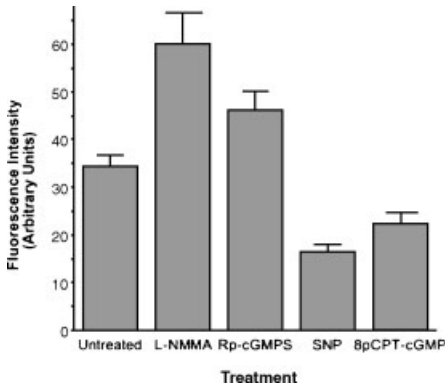

Fig. 4. Effect of modifying NO pathway on acid lakes. Osteoclasts from CD14 positive cells on bone four weeks were incubated with nitroprusside 100 μM, 15 min, a hydrolysis resistant cGMP analog, 8-pCPT-cGMP, 100 μM, 2 h, a blocking cGMP analog, Rp-cGMPS, 50 μM, 30 min, or an arginine analog that inhibits NO synthesis, L-NMMA, 1 μM, 30 min (for full chemical names, see ''Materials and Methods''). This was followed by lysotracker 50 nM, 2 min, and fluorescence intensity was determined after an additional 2 min from 12 bit digital images using Fovea Pro software (Gainesville, FL). Average lysotracker intensity was reduced by an average of ~twofold in cells treated with the rapidly hydrolyzed NO donor nitroprusside or 8-pCPT-cGMP compared to cells pre-treated with either the cGMP analog or L-NMMA. Each bar is the average ± standard error of the mean of fluorescence in a minimum of nine fields. The entire experiment was done using the one preparation of cells on 1 day; similar results were obtained in two additional separate experiments. The differences in lysotracker intensity either Rp-cGMPS or L-NMMA relative to 8-pCPT-cGMP or SNP are significant. $P < 0.01$, by Student's *t*-test. The effect of L-NMMA relative to untreated ($P < 0.05$) suggests that NO may be an autocrine, as well as a paracrine regulator of osteoclast function.

decreased motility (Fig. 5A). The difference between control and L-NMMA was of marginal significance but all other differences were significant relative to the untreated cells, and the effectors versus inhibitor treatments were all significantly different. When NO activating treatments were followed with a washout, cells survived and returned to intermediate levels of motility (Fig. 5B), although with the non-hydrolyzable analog 8-CPT-cGMP, the effect on motility was much more long-lasting than with the short half-life NO donor SNP. Effect on osteoclast motility was confirmed using cells on bone by treating cells attached to bone with the NO donors or inhibitors and then recovering cells that remained attached to bone or were recovered on a vitronectin-coated glass chamber (Fig. 5C), scoring only cells labeling with TRAP to assure that only differentiated cells (Fig. 2B) were assayed. This showed that detachment was directly related to NO activation, although some cells were removed from bone in the absence of exogenous treatment, as expected with results pointing to an active endogenous or autocrine, cGMP-activated system (Figs. 3 and 4).

DISCUSSION

Osteoclasts are cells that resorb bone by producing a tight sealing zone on bone and then secreting acid into that compartment [reviewed in Blair and Athanasou, 2004]. Only osteoclasts on resorbable substrate secrete acid, and osteoclasts are motile cells that may make many lacunae during cycles of activity of a single cell. In this study, we performed some of the first studies of motility and acid resorption using human osteoclasts produced by in vitro differentiation. We focused on the effects of NO related pathways on osteoclast acid secretion and motility. There are species differences in the hormonal response and regulation, and our use of human cells makes the result specific to human osteoclast response, insofar as the in vitro differentiation replicates the phenotype, which appears to be the case by the major markers of the osteoclast and its activity (Fig. 1). Marrow preparations are alternative ways to make osteoclasts; these place the osteoclast in a cellular environment similar to bone but suffer from the difficulty that, since the cell population is mixed, it is difficult to attribute any response to a particular cell. In this case, therefore, we used cells produced from circulating human CD14 cells under the influence of RANKL, although it is debatable whether cells in their normal physiological milieu, that is, in the presence of osteoblasts, may have some differences in differentiation and activity.

NO has long been appreciated as an important signaling molecule in bone. Study on mouse marrow suggested that NO exerts biphasic effects on osteoclast activity: high concentrations of NO inhibit bone resorption, whereas lower concentrations potentiate bone resorption [Ralston et al., 1995]. On the other hand, in avian osteoclasts, cGMP was a negative regulator of osteoclasts activity [Dong et al., 1999] and in this system NO appeared to be produced only by non-osteoclastic cells. There are numerous additional studies, in most cases using animal models and frequently studying effects of cells on tissue culture substrates [reviewed in Blair et al., 2002], which generally support

downregulation of osteoclastic activity at high concentrations of NO but which otherwise do not point to a specific physiological mechanism. In this study, we compared the effects of NO and downstream cGMP-related mechanisms on osteoclast function on bone and on glass, using human osteoclasts produced in vitro.

It should be noted at this point that in murine models it is established that the constitutive eNOS is expressed in osteoblasts and that this is important for response to estrogen [Armour et al., 2001] and NO synthase–I (iNOS), the inducible form attributed to osteoclasts and related cells, when eliminated produces osteoclasts that cannot be activated by the well-characterized stimulus IL-1 [van't Hof et al., 2000; Jung et al., 2003]. Thus, there is strong correlative evidence consistent with the results shown here using biochemical intermediates, although the effect of NO and its downstream effectors on the attachment structure (Fig. 2) has not previously been studied.

In osteoclasts, bone attachment incorporated continuously distributed vitronectin receptors, which were redistributed to a podosome-like arrangement by NO (Fig. 3). These findings have two important implications. First, they reflect how the osteoclastic attachment to bone works; this requires limiting diffusion from the HCl-containing compartment. Cells attached via podosomes have attachment sites so permeable as to permit labeling of integrin receptors

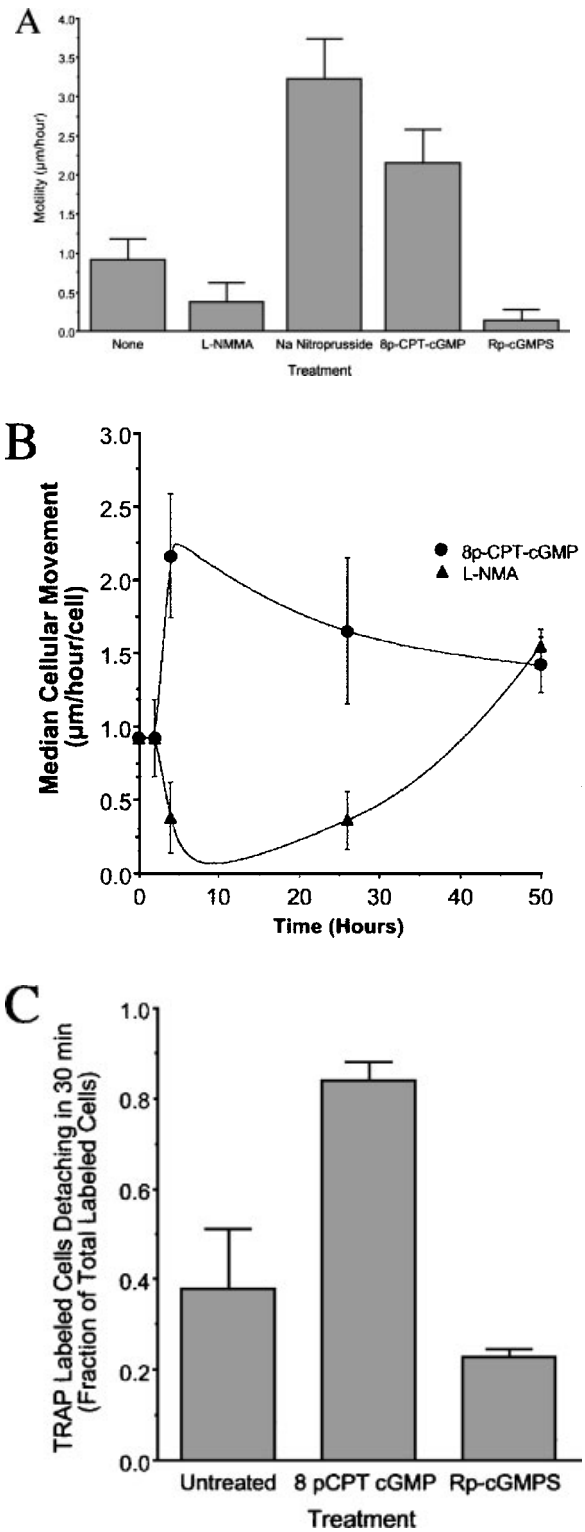

Fig. 5. NO and downstream effectors increase motility on glass and bone. **A**: Osteoclasts from CD14 positive cells on bone four weeks were incubated with effectors as in Figure 4 and then cellular movement was followed by time lapse photography measuring the maximal linear deviation over 80 or 120 min. The results shown are pooled data from two or three determinations with similar results, mean ± SEM with n = 8–12. The difference between control and L-NMMA treatment is of marginal significance ($P \sim 0.1$) but the effects of nitroprusside and the cyclic GMP agonist and antagonist are significant ($P < 0.05$). **B**: Timecourse of L-NMMA and 8-pCPT-cGMP effects on osteoclast motility. Baseline data for motility were determined at 0–2 h, cells treated for 2 h with 1 µM L-NMMA or 50 µM 8-pCPT-cGMP and motility was averaged over an additional 2 h period with washout and two additional determinations of motility 1 and 2 days later. This demonstrated that cells survived and that motility differences were eliminated by 2 days. **C**: Effect on osteoclast motility on bone. NO agonists and antagonists were applied to cells on bone for 30 min and the bone slices were inverted onto clean vitronectin coated glass plates in fresh media without additions. TRAP labeled cells attached to the bone or attached to the glass plate after 22 h were counted, and expressed as proportion ± SD, n = 3. Note that detachment from bone with reattachment to the underlying plate was increased by activation of the non-hydrolyzable cGMP analog 8-pCPT-cGMP, and decreased by the antagonist Rp-cGMPS; the difference between controls and the antagonist is not statistically different but other differences have $P < 0.05$. Total number of cells per determination was 66 ± 32.

in non-permeabilized cells (Fig. 2C). Second, the reversible effects on cell attachment and cytoskeleton (Fig. 3) suggest that a primary role of NO in bone is to mediate detachment and motility in the osteoclast, which permits cells to make multiple lacunae (e.g., Fig. 1E). Indeed, we found that the linear integrin distribution, which excluded antibody labeling in living cells, was associated with lacunar resorption and acid secretion (Fig. 2). This strongly suggests that when a continuous linear pattern of integrin binding surrounds the osteoclast attachment, the cell is closely enough apposed to the substrate that diffusion from the attachment site is limited, so acid can accumulate and dissolve the bone mineral. It remains to be seen what mechanism is responsible for the unique linear distribution of $\alpha_v\beta_3$. In any case, all models of cellular movement would require dissolution of attachment for cell movement to occur, and when NO or cGMP were inhibited, cellular motility was essentially eliminated while average acid secretion activity increased (Figs. 4 and 5). It will be interesting to follow this effect over longer times, in that very deep osteoclast pits might result but individual osteoclasts may become incapable of producing multiple pits when NO and cGMP activity are eliminated.

It is worth noting that the tight association of the osteoclast's membrane to bone during resorption may explain the often-noted difficulty of releasing osteoclasts by trypsinization. Under conditions where the linear expression of $\alpha_v\beta_3$ prevents labeling by the antibody, it is not surprising that trypsin does not attack the binding successfully. Preliminary studies (not illustrated) showed that NO donors increased the yield of trypsin-detached cells, in keeping with the effect of NO on the attachment that permits antibody labeling of $\alpha_v\beta_3$ in unpermeabilized cells.

The reversible effects of NO and cGMP on the osteoclastic attachment and cell movement and autocrine NO synthesis (Fig. 4) suggest that NO production is fundamental to the activity of the human osteoclast, rather than representing a side-pathway that might cause osteoclastic apoptosis or terminate resorption. Recent studies point to the endothelial NO synthase (eNOS) as the major source of NO regulating bone resorption, with this being produced by osteoprogenitor cells and regulated by strain [Rubin et al., 2003]. Our study is completely consistent with this and indeed our earlier study suggested that osteoblasts or mesenchymal cells were the only source of NO in unstimulated avian marrow cells [Dong et al., 1999]. However, in the current study, the presence of NO synthase in osteoclasts, recently indicated by murine iNOS deletion studies of Jung et al. [2003], is also strongly supported. This study follows earlier studies by Sunyer et al. [1996] arguing that NO is an autocrine regulator of avian osteoclasts. Our present study also supports this model in human cells, and points to a specific physiological mechanism for carrying out the physiological NO-regulated function. Recent study in rabbits suggests that NO production by osteoclasts may be regulated by the *N*-methyl-D-aspartate-receptor, which serves this function in neurons [Mentaverri et al., 2003]; our study suggests that this pathway deserves attention in human bone as a possible regulatory target. NO donors have been promoted as treatments for pathological bone loss, with promising results in human and rodent osteoporosis [Jamal et al., 1998; Hukkanen et al., 2003]. The effectiveness of NO donors may relate to its effect on osteoclastic survival and bone resorption at high NO concentrations. However, this is by no means certain. NO also affects osteoblasts, regulating both osteoblast proliferation and bone formation [Armour et al., 2001; Kanamaru et al., 2001] and thus, NO may act as both an anabolic or catabolic stimulus. Indeed, at high NO concentrations, NO inhibits bone formation [van't Hof and Ralston, 2001] and interaction of other signalling pathways regulates osteoclastic apoptosis in the presence of NO [Kanaoka et al., 2000].

In summary, the present study shows NO is produced by human osteoclasts and regulates cycles of lacunar resorption via mediating rearrangement of the cellular attachment, in particular $\alpha_v\beta_3$ integrin attachment. High concentrations of NO essentially eliminated cellular reattachment.

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