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Regulation of Bone Resorption and Osteoclast Survival by Nitric Oxide: Possible Involvement of NMDA-Receptor

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Abstract Nitric oxide has been shown to play an important role in regulation of bone resorption. However, the role of endogenous nitric oxide on osteoclast activity remains still controversial. In this work, using RT-PCR amplification, we demonstrated that rabbit mature osteoclasts express mRNA encoding for neuronal nitric oxide synthase suggesting that this enzyme could be involved in basal nitric oxide production in these cells. Then we assessed the effect of carboxy-PTIO, a nitric oxide scavenger, on in vitro bone resorption and osteoclast survival. Carboxy-PTIO (10–100 μM) inhibited osteoclastic bone resorption in a dose dependent manner and induced osteoclast apoptosis by a mechanism involving caspase 3 activation. These results suggest that basal concentration of endogenous nitric oxide may be essential for normal bone resorption by supporting osteoclast survival. Because osteoclasts express *N*-methyl-D-aspartate-receptor (NMDA-R), we hypothesized that in osteoclasts NMDA-R may be involved in nitric oxide production as in neuronal cells. We confirmed that blockade of NMDA-R with specific non-competitive antagonists, MK801 and DEP, strongly inhibited bone resorption. As for carboxy-PTIO, we showed that blockade of NMDA-R by both antagonists induced osteoclast apoptosis in a dose dependent manner by a mechanism dependent on caspase 3 activation. Intracellular calcium concentration in osteoclasts decreased within minutes in the presence of both antagonists. Finally, MK801-induced osteoclast apoptosis was partially reversed in the presence of small amount of SNAP (100 nM), a nitric oxide donor, suggesting that the effect of NMDA-R on osteoclast apoptotic cell death could be due to a decrease in nitric oxide production. Taken together, our results are consistent with the hypothesis that NMDA-R on osteoclasts could have a similar function as those in neuronal cells, i.e., to allow a calcium influx, which in turn activates a constitutive neuronal nitric oxide synthase. Nitric oxide generated by this pathway may be essential for osteoclast survival and hence for normal bone resorption. J. Cell. Biochem. 88: 1145–1156, 2003.

Key words: osteoclastic cell death; bone resorption inhibition; intracellular calcium; NMDA glutamate receptor; nitric oxide biosynthesis

Throughout life, bone is remodeled in a dynamic process and a balance between osteoblastic bone formation and osteoclastic bone resorption is required for maintenance of skeletal integrity. Regulation of bone resorption consists in two major processes: one is the recruitment of new osteoclasts from hematopoietic precursors, and the other one is the activation and survival of mature osteoclasts. Both processes are under the control of circulating hormones as well as local growth factors and cytokines [Roodman, 1999]. Among the agents which participate in regulation of bone resorption, nitric oxide (NO) has been shown to exert a biphasic effect: while low concentrations seems to be necessary for normal osteoclast functions [Brandi et al., 1995; Evans and Ralston, 1996], higher NO concentrations inhibit osteoclast resorptive activity [Macintyre et al., 1991; Kasten et al., 1994; Kanaoka et al., 2000].

NO is a short-lived free radical which is synthesized from L-arginine by three distinct NO synthase (NOS) isoenzymes: endothelial

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NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). Constitutively expressed in numerous tissues, eNOS and nNOS activities are mainly regulated by changes in free intracellular Ca^{2+} and produce low NO amount over several minutes. The iNOS pathway is, in contrast, mainly regulated at the transcriptional level, and produces large amounts of NO upon exposure to various stimuli such as proinflammatory cytokines [Ralston, 1997].

In osteoclasts, NO derived from the iNOS pathway acts as a mediator of cytokine-induced effects on bone resorption and may be involved in several pathological situations associated with cytokine activation, such as postmenopausal osteoporosis, rheumatoid arthritis, and tumor-associated osteolysis [Evans and Ralston, 1996]. Constitutive production of small amount of NO has been recently demonstrated in osteoclasts. However, so far, little is known about the biological function of low NO endogenous productions and about the NOS isoenzyme responsible for its biosynthesis in osteoclast. Recently, it has been reported that osteoclasts express *N*-methyl-D-aspartate-type glutamate receptors (NMDA-R) [Chenu et al., 1998; Patton et al., 1998; Espinosa et al., 1999], and that dizocilpine maleate (MK801), a non-competitive antagonist of NMDA-R, can block in vitro bone resorption [Peet et al., 1999; Itzstein et al., 2000], suggesting that glutamate may participate in the local control of bone remodeling. However, the exact mechanism of the MK801-induced inhibition of bone resorption and the biological significance of NMDA-R on osteoclasts are not yet clearly understood. Essentially studied in nervous system, these receptors play important roles in many physiological functions of neuronal cells including development, synaptic plasticity, and survival [Garthwaite, 1991]. In neuronal cells, NMDA-R performs its action in concert with the receptor-gated cation channel, which is permeable to calcium (Ca^{2+}) ions. Ca^{2+} influx through NMDA receptors efficiently stimulates nNOS. This stimulation is rendered possible thanks to a co-localization of nNOS to NMDA-R via the post-synaptic density protein, PSD-95, which ensures that calcium entry through the NMDA-R is selectively coupled to nNOS activity in neuronal cells [Brenman et al., 1996; Aoki et al., 1997].

In this study, we hypothesized that a similar mechanism exists in osteoclasts, to specifically couple NMDA receptor-mediated calcium influx to constitutive NO production by nNOS. So, we first demonstrated, by using RT-PCR amplification, that mature osteoclasts express mRNA encoding for nNOS suggesting that this enzyme could be involved in basal production of NO in osteoclast. We then investigated the role of endogenous NO on osteoclast activities by using carboxy-PTIO, an NO scavenger. We showed that depletion of intracellular NO dramatically inhibits bone resorption and induces osteoclast apoptosis, suggesting that a basal NO concentration may be essential for normal osteoclast function by supporting the survival of osteoclasts. At the same time, we reported that MK801 and (±)-1-(1,2-Diphenylethyl)piperidine (DEP), another non-competitive antagonist of NMDA-R, inhibited bone resorption, induced osteoclast apoptosis in a similar extent than carboxy-PTIO, and decreased intracellular calcium concentration ($[Ca^{2+}]_i$). Finally, we demonstrated that MK801-induced osteoclast apoptosis could be partially reversed when osteoclasts are treated with small amount of SNAP (100 nM), an NO donor, suggesting that MK801 effect on osteoclasts apoptosis could be due to a decrease in NO biosynthesis.

MATERIALS AND METHODS

Animals and Reagents

Ten-day-old New-Zealand rabbits were purchased from Elevage Scientifique des Dombes (Chatillon/Chalaronne, France). All reagents were obtained from Sigma (St. Louis, MO) except pronase, and fetal calf serum (FCS) which were obtained from France-Biochem and Dominique Dutscher sa, Brumath (France), respectively. (+)-MK801, (±)-1-(1,2-Diphenylethyl)piperidine (DEP), carboxy-PTIO, and S-nitroso-acetyl penicillamine (SNAP) were obtained from Fischer bioblock (France) and Benzyloxycarbonyl-Asp-Glu-Val-Asp (Ome)-fluoromethylketone (Z-DEVD-FMK) was obtained from Calbiochem (France). All culture dishes and plates were purchased from Corning (NY).

Cortical bone was obtained from bovine femora, prepared as cylindrical sticks (diameter 6 mm, length 10–12 cm) and stored into ethanol at 70°C until cutting into slices (thickness 0.2 mm) by a low speed saw (Buehler, France). The slices were washed by treatment with ultrasounds (7 min) and rinsed twice during 15 min at 37°C in α-MEM before cell culture.

Cell Culture

Osteoclasts were isolated from 10-day-old rabbits according to the procedure described by Foged et al. [1996] and modified by Lorget et al. [2000a]. Briefly, for each experiment, long bones were minced with scissors in αMEM containing 10% heat-inactivated FCS and penicillin/streptomycin, then cells were dissociated from bone fragments by vigorous vortexing and collected by centrifugation (4 min, 45 g). This preparation yielded a population of mixed cells constituted by osteoblasts, stromal cells, and osteoclasts. To obtain a population of pure osteoclasts, the pellet of unfractionated bone cells was resuspended in medium and seeded into 6- or 24-well plates or into 100-mm tissue culture dishes. After overnight incubation and several washes, the purified osteoclasts were prepared by removing stromal cells with a solution of 0.001%-pronase in 0.02%-ethylene diamine tetra acetic acid solution (EDTA). Purified osteoclasts were then incubated in medium for 2 h. Next, cells were cultured in αMEM supplemented with 1% FCS containing various amounts of MK-801, DEP, or carboxy-PTIO. Cell purity was assessed using tartrate-resistant acid phosphatase (TRAP) staining and was close to 99%.

Measurement of Osteoclast Bone Resorbing Activity

The unfractionated bone cell preparation was seeded on bovine bone slices. After sedimentation for 45 min, medium and non-adherent cells were removed. Remaining cells were incubated for 48 h with or without the various agents tested. At the end of the culture, bone resorbing activity was evaluated using three different methods: one which measured pit area produced by culture of osteoclasts on bovine bone slices and two others which measured in culture medium the bone collagen degradation products in bone cell culture supernatants harvested after the 48 h of culture period. For pit area measurement, bone cells were removed from bone slices and after a double staining for 1 min with acid hematoxylin solution and for 30 s with a solution of 1% toluidine blue-1%-borate, pit area was quantified by an image analysis system (Biocom les Ulis, France) linked to light microscope (Olympus BH-2, France). For bone collagen degradation products two assessments were used. C-telopeptide type I collagen fragments (CTX) were measured using an enzyme-linked immunosorbent assay (CrossLaps^{TM} for culture developed by Osteometer Bioteck A/S, Herlev, Denmark) and hydroxylysylpyridinoline (HP), a collagen cross-link, were measured by HPLC as previously described [Kamel et al., 1995; Lorget et al., 2000b]. Briefly, supernatants were hydrolyzed for 20 h at 105°C. Then HP was extracted in a CF-1 cellulose column and final eluate was freeze-dried. HP was quantified by fluorescence of the eluate peak using a Jasco FP 1520 spectrofluorimeter with excitation at 297 nm and emission at 395 nm. All results were expressed as percent of control.

Detection of Osteoclast Apoptosis

As previously described by Kameda et al. [1995], after treatment with reagents, cells were fixed with 3.7% formaldehyde for 5 min and stained with 0.2 mM Hoechst 33258 for 10 min. Cells were examined under a fluorescence microscope for determination of morphological changes of chromatin. At least 100 TRAP multinucleated cells were scored for the incidence of apoptosis. The rate of apoptosis was calculated as the ratio of apoptotic osteoclast number per total counted osteoclast number.

Caspase 3 Activity

Purified osteoclasts were washed with phosphate buffered saline solution and cytosolic extracts were obtained using a caspase buffer containing 10 mM HEPES, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, and 5 μg/ml antiproteases (pH 7.4). After 60 min of shaking at 4°C, lysates were obtained by centrifugation (10 min, 12,000*g*). For each sample, 50 μl of clear lysate were incubated with 40 μM enzyme substrate (Ac-DEVD-AMC) for 60 min at 37°C and levels of fluorescence were measured using spectrofluorimeter (Shimadzu RF1501), excitation 380 nm and emission 460 nm. Caspase 3 activity, which plays a key role in apoptosis [Porter and Janicke, 1999], was expressed as arbitrary units of fluorescence per microgram of proteins.

RT-PCR

Total RNA was isolated from MG-63 cells and from rabbit brain, eyes, and purified osteoclasts using Tri Reagent (Sigma). After a treatment during 30 min with a solution of DNase-RNase free (Promega), cDNA was synthesized from 1 μg of total RNA using oligo dT primers and

enhanced avian myeloblastosis virus reverse transcriptase (eAMV-RT) purchased from Sigma. Polymerase chain reaction (PCR) was performed using RedTaq polymerase (Sigma). All PCR were realised by amplification of 4 μl of the 20 μl resuspended reverse transcription product to 40 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 1 min) and then to one cycle of 10 min to 72°C for final extension. The sense oligonucleotide 5′-TCCGTCTCTTTAAGCGCAAG-3′ and the antisense oligonucleotide 5′-TTGACGGCGAGAATGATGTC-3′, which amplified a 153 bp PCR product, were both designed from highly conserved sequences (PDZ domain) of submitted *Oryctolagus cuniculus* neuronal nitric oxide synthase mRNA (accession number: U91584). The amplified products were always electrophoresed with negative controls in 1.4% agarose gel with 1 μg/ml ethidium bromide. PCR product was sequenced to confirm its identity (ABI Prism 310 Gentetic Analyser, Applied biosystems).

Fluorimetric Monitoring of Intracellular Free Calcium Ions

Cells seeded on glass coverslips were loaded with 10 μM of Fura2-AM (Molecular Probes) in αMEM containing 10% of FCS for 60 min at 37°C. The coverslip was then inserted into a coverslip chamber and cells were superfused with saline solution containing 20 mM HEPES, 116 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 5 mM $NaCO_3$, 1.8 mM $CaCl_2$, and 1 g/L glucose (pH 7.4). After 5 min of equilibration, dual wavelength measurement was performed (excitation 340/380 nm, emission 505 nm) using a Zeiss Axiovert 35 microscope set up for epifluorescence microscopy. Fluorescence images were collected by a low-light-level ISIT camera (Lhesa, France), digitized and integrated in real time by an image processor (Metafluor, Princeton, NJ). Control and test solutions were superfused by six inlet tubes converging on the coverslip chamber. The perfusion rate was 1.5–2 ml/min, the volume of coverslip chamber was 0.2 ml, and the medium was continuously renewed by aspiration. Data are expressed as the ratio of fluorescence elicited by excitation at 340 and 380 nm.

Statistical Analysis

Results were expressed as the mean ± SEM. The statistical differences among groups were evaluated using Kruskal–Wallis test. Mann–Whitney U-test was then used to identify differences between the groups when Kruskal–Wallis test indicated a significant difference ($P < 0.01$).

RESULTS

Analysis by RT-PCR of the Presence of nNOS in Osteoclast Cells

To determine whether osteoclasts express nNOS mRNA, we designed oligonucleotide primers based on rabbit sequences of nNOS. As shown in Figure 1, RT-PCR analysis with these specific primers allowed identification of a product of predicted size for nNOS (153 bp) in total RNA extracted from rabbit brain (lane 1) and rabbit eyes (lane 2) two tissues known to express nNOS and used as positive controls. Osteoclasts isolated and purified from rabbit long bones also expressed nNOS mRNA (lane 3), while MG-63 osteoblastic cells did not (lane 4). The sequence of the fragment amplified was 100% identical to the published sequence.

Effects of Carboxy-PTIO on Osteoclastic Bone Resorption and Osteoclast Apoptosis

As shown in Figure 2, carboxy-PTIO inhibited bone resorption in a dose-dependent manner, at 100 μM the decrease of bone resorption was around 60%. For investigating the role of

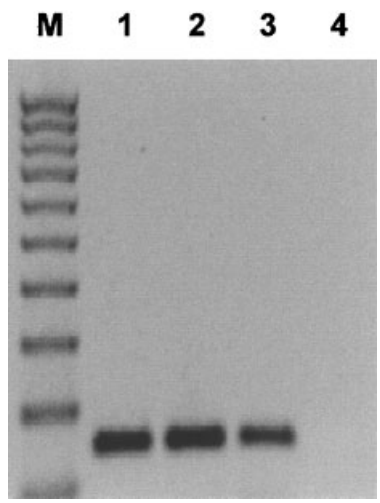

Fig. 1. RT-PCR analysis of 153 bp transcripts corresponding to nNOS. cDNA was synthesized from RNA extracted from rabbit brain (**lane 1**), rabbit eyes (**lane 2**) used as positive controls. **Lanes 3** and **4** correspond to cDNA synthesized with RNA extracted from rabbit purified osteoclasts and MG-63 osteoblastic cells, respectively. **Lane M**, size marker of PCR 100 bp low ladder.

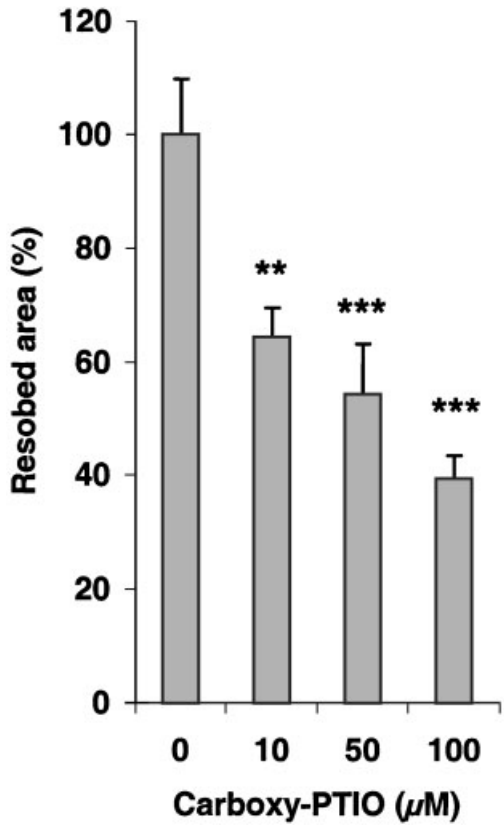

Fig. 2. Effects of carboxy-PTIO on osteoclastic bone resorption assessed by pit area measurement. Rabbit bone cells were cultured for 48 h in αMEM with various amounts of carboxy-PTIO from 10 to 100 μM. Results are expressed as percent of control and represent the mean ± SD of two independent experiments (n = 10). ***$P < 0.001$ compared with controls.

carboxy-PTIO on apoptosis, rabbit osteoclasts were purified from the unfractionated bone cell preparation using a method, which allowed us to obtain a population of very high cell purity (approximately 99% of osteoclast cells). Hoechst staining was performed to detect nuclear fragmentation and chromatin condensation, the morphological characteristics of apoptosis (Fig. 3). Numerous osteoclasts are committed to apoptosis and quantification of this finding showed that carboxy-PTIO induced osteoclast apoptosis in a dose dependent manner (Fig. 4A). After 48 h of culture the rate of apoptotic cells was fourfold higher in osteoclasts treated with 100 μM of carboxy-PTIO as compared to untreated cells. Using a fluorescent peptide substrate, we assessed the involvement of caspase 3 in carboxy-PTIO induced osteoclast apoptosis (Fig. 4B).

Effects of NMDA-R Blockade on Osteoclast Bone Resorption and Osteoclast Apoptosis

Several techniques were used to assess the effect of NMDA-R blockade on osteoclast bone resorbing activity, the first measures the area of lacuna produced by culture of osteoclasts on bone slices. When bone cells were cultured for 48 h in the presence of 100 μM of MK801, a significant decrease of bone resorption reaching a value of approximately 60% was observed (Fig. 5A). The inhibition of bone resorption by blockade of NMDA-R was confirmed by measurement in culture supernatant of bone collagen degradation products. A significant and similar decrease was assessed by immunoassay measurement of the C-telopeptide fragments of type I collagen (CTX) content in culture medium (Fig. 5B). The decrease was still much higher when bone resorption was assessed by HPLC measurement of hydroxylylpyridinoline (HP) content of culture medium (Fig. 5C).

Blockade of NMDA-R by MK801 directly promoted osteoclast apoptosis in a dose-dependent manner. After 48 h of culture in a medium containing 100 μM of MK801, 82 ± 3% of osteoclasts were apoptotic while in control culture the number of apoptotic osteoclasts was only 20 ± 1%. A similar dose-dependent induction of osteoclast apoptosis was obtained when cells were treated with DEP (Fig. 6A). A time-course experiment of apoptotic changes of osteoclasts cultured with or without MK801 showed that significant difference was observed as early as 12 h of culture (Fig. 6B). Caspase 3 activity was strongly increased in MK801 treated cells as compared to untreated cells (Fig. 7A), and Z-DEVD-fmk a selective caspase 3 inhibitor attenuated the apoptotic cell death induced by 100 μM MK801 in a dose dependent manner (Fig. 7B).

NMDA-Receptor Antagonists Decreased $[Ca^{2+}]_i$

Changes of intracellular calcium concentration in osteoclasts treated with NMDA-R antagonists were monitored by using the fluorescent probe Fura2-AM. In cells treated with 100 μM of MK801 $[Ca^{2+}]_i$ decreased within several minutes (Fig. 8). A similar result was obtained when osteoclasts were perfused with 100 μM of DEP (data not shown).

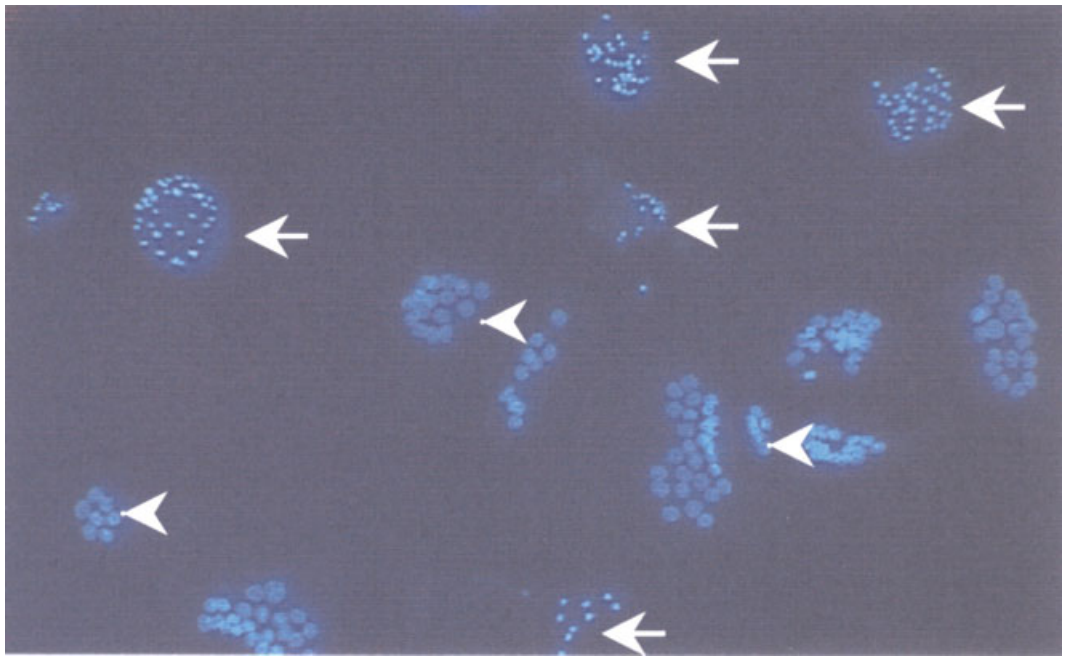

Fig. 3. Carboxy-PTIO-induced osteoclast apoptosis. Apoptosis was detected by staining purified osteoclasts with 0.2 mM Hoechst 33258 to visualize chromatin condensation. The fluorescence micrograph (G × 200) represents normal osteoclasts (arrowheads) and apoptotic osteoclasts with fragmented nuclei (arrows). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

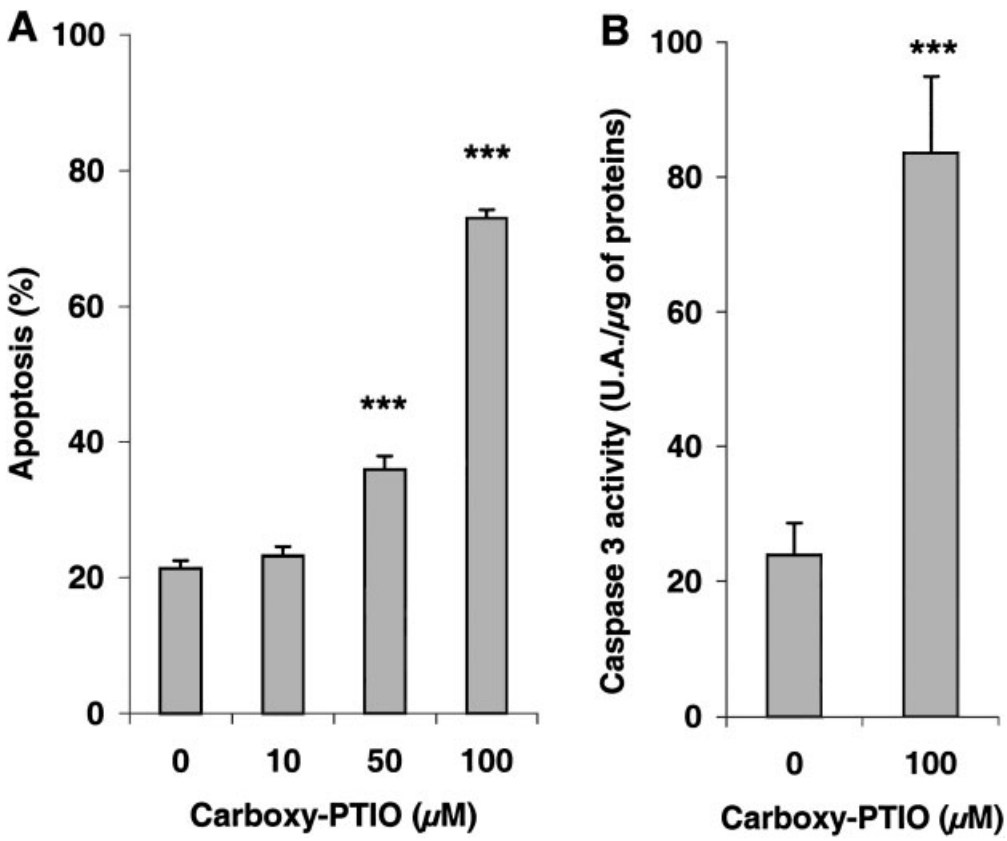

Fig. 4. Effects of carboxy-PTIO on osteoclast apoptosis and on caspase 3 activation. **A**: Purified osteoclasts were cultured for 48 h in the presence of increasing amounts of carboxy-PTIO from 10 to 100 μM. Results are expressed as the mean ± SD of two independent experiments (n = 12). ***$P < 0.001$ compared with controls. **B**: Purified osteoclasts were incubated with or without 100 μM of carboxy-PTIO for 1 day at 37°C. Results are expressed as the mean ± SD of two independent experiments (n = 6). ***$P < 0.001$ compared with untreated control cells.

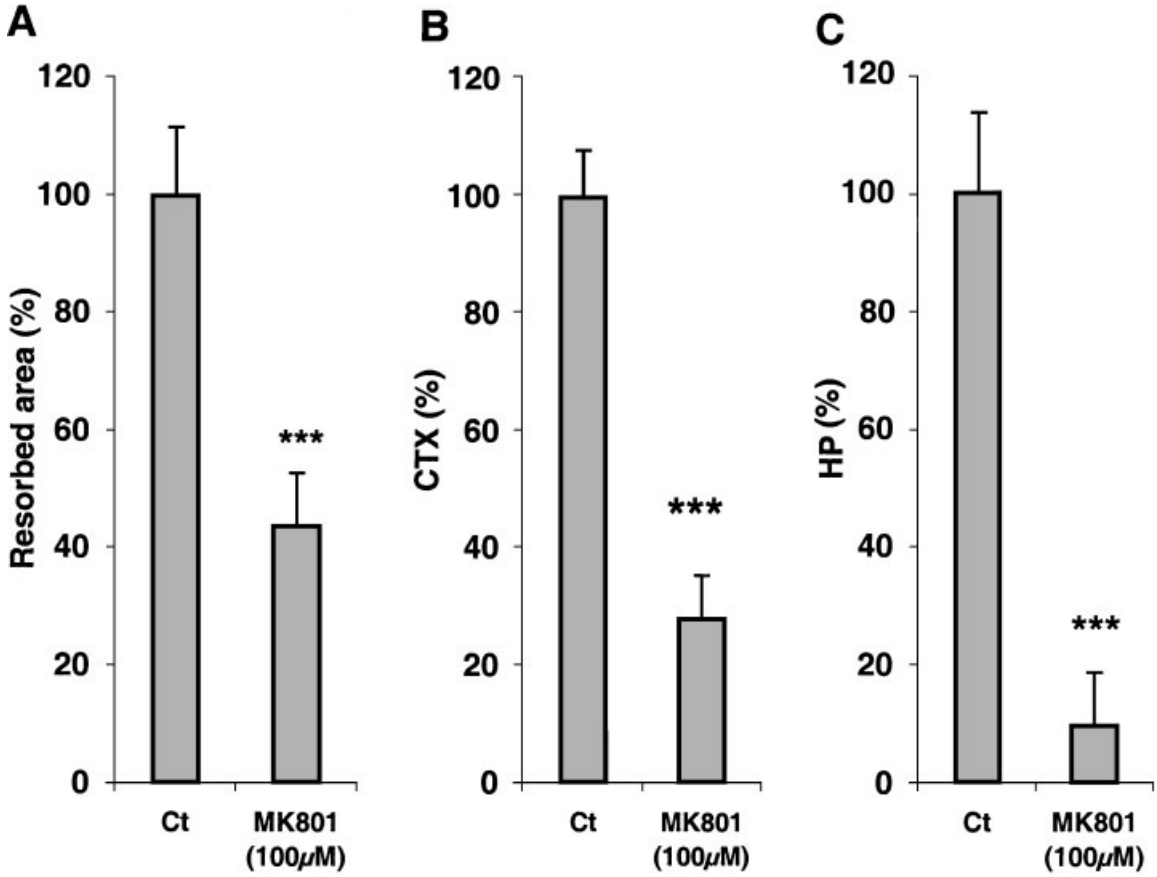

Fig. 5. Effect of MK801 on osteoclastic bone resorption. Rabbit bone cells were cultured for 48 h in αMEM in the absence or the presence of various amounts of MK801 from 10 to 100 μM. The bone resorbing activity was assessed by (**A**) pit area measurement as well as by determination in the culture supernatant of the (**B**) C-telopeptide fragments (CTX) and (**C**) hydroxylysylpyridinoline (HP). Results are expressed as percent of control and represent the mean ± SD of two independent experiments (n = 8). ***$P < 0.001$ compared with controls.

Spontaneous NO Donor Partially Abrogate MK801 Induced Osteoclast Apoptosis

We have examined the effects of low amounts of SNAP, a spontaneous NO donor on the MK801-induced osteoclast apoptosis. When SNAP (50 or 100 nM) was introduced in the culture medium in combination with 100 μM of MK801 a significant and dose-dependent decrease of MK801-induced apoptotic cell death was observed (Fig. 9). Addition of SNAP alone at these concentrations did not modulate osteoclast apoptosis (data not shown).

DISCUSSION

There is now strong evidence to suggest that NO plays an important role as a local regulator of bone resorption. NO generated in the bone microenvironment from osteoblasts, bone marrow stromal cells, or macrophages acts as a paracrine signal, which can either inhibit or stimulate osteoclastic bone resorption, depending on the concentration at which it is released. Osteoclasts themselves produce NO, suggesting that NO may also represent an autocrine mediator of osteoclast function. Little is known about the enzymatic system involved in basal NO production. Recently, Silverton et al. [1999] using a microsensor measurement of NO production by the osteoclast, demonstrated that changes in both extracellular and intracellular Ca^{2+} affect the NO release, suggesting that calcium sensitive enzymes are involved in NO production. Among constitutive isoforms of NOS whose activity is regulated by calcium, there are two possible candidates, eNOS and nNOS. The presence of eNOS has been previously identified in isolated osteoclasts [Brandi et al., 1995; Fox and Chow, 1998], and nNOS transcript has been shown to be expressed in

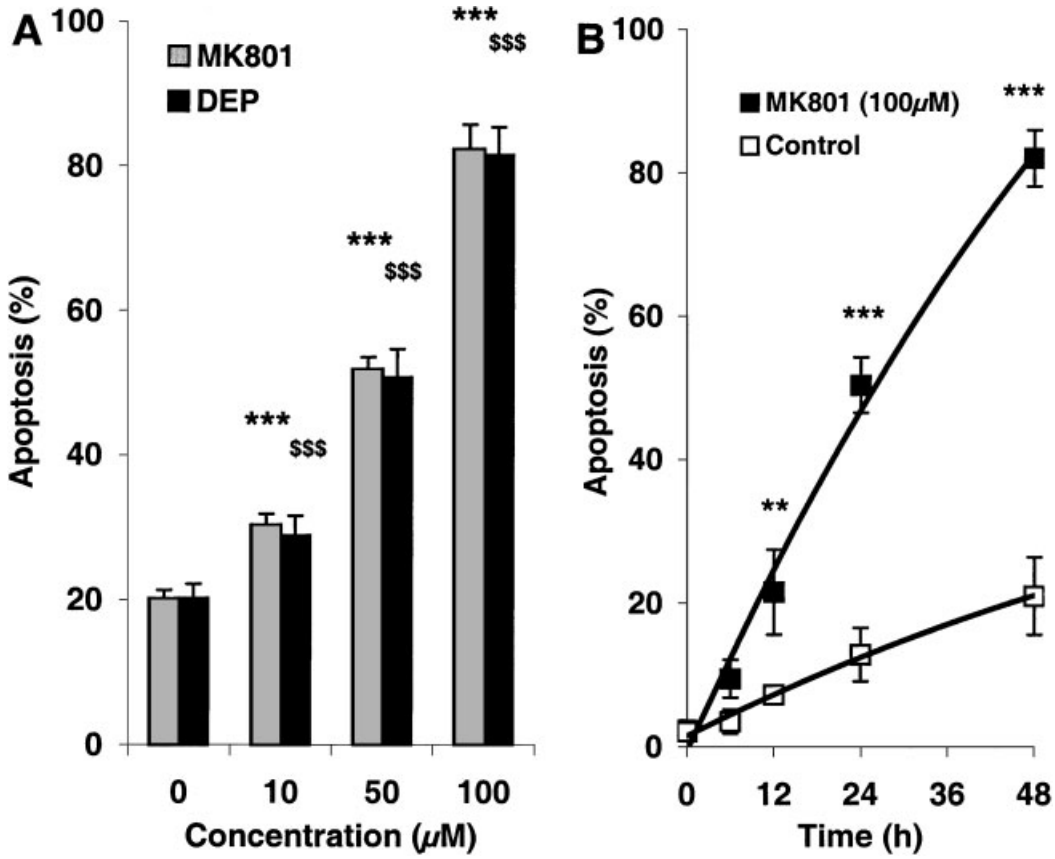

Fig. 6. **A**: Dose dependent effect of MK801 and DEP on osteoclast apoptosis. Rabbit purified osteoclasts were cultured for 48 h in αMEM in the presence of various amounts of MK801 and DEP from 10 to 100 μM. Results are expressed as the mean ± SD of two independent experiments (n = 12). $^{***}P < 0.001$ and $^{\$\$\$}P < 0.001$ compared to respective controls. **B**: Time course of apoptotic cell death of osteoclasts cultured with or without MK801 (n = 6). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with untreated control cells.

extract from femora but not at a cellular level [Helfrich et al., 1997; Hukkanen et al., 1999]. Recently, it has been reported in transgenic mice with targeted inactivation of the eNOS gene [Aguirre et al., 2001; Armour et al., 2001], a reduction in bone mass due to a decrease in osteoblast maturation and in bone formation rather than an increase in osteoclast activity and bone resorption. These data suggest that constitutive NO production by the eNOS pathway may be essential in the modulation of osteoblastic functions while for osteoclasts another NOS isoform pathway may be involved. Studies of bone metabolism in nNOS knock-out (KO) mice may help to define the role of NO generated in osteoclasts by this pathway. Such study has been recently carried out by Van't Hof et al. [2002], who demonstrated that nNOS deficient mice have increased bone mass, indicating that nNOS plays a major role in bone remodeling. In this study, we showed by RT-PCR amplification that rabbit osteoclasts express mRNA encoding for the nNOS. Although it is difficult to draw firm conclusions on the involvement of a protein from RT-PCR studies, one may hypothesize that nNOS could participate in the basal NO production in osteoclast. To be totally compelling, we needed to demonstrate the presence of nNOS at a protein level by using immunofluorescence and Western blot studies. Unfortunately, such studies are difficult to carry out in our model of rabbit mature osteoclasts, due to both the low amount of protein collected from cell lysate and to the lack of specific antibodies directed against rabbit proteins. Further studies should be performed using another cell model of osteoclast. The role of endogenous NO production on osteoclastic bone resorption is still controversial. Kasten et al. [1994] found that blockade of NO production using L-NAME and aminoguanidine stimulated bone resorption by isolated chicken

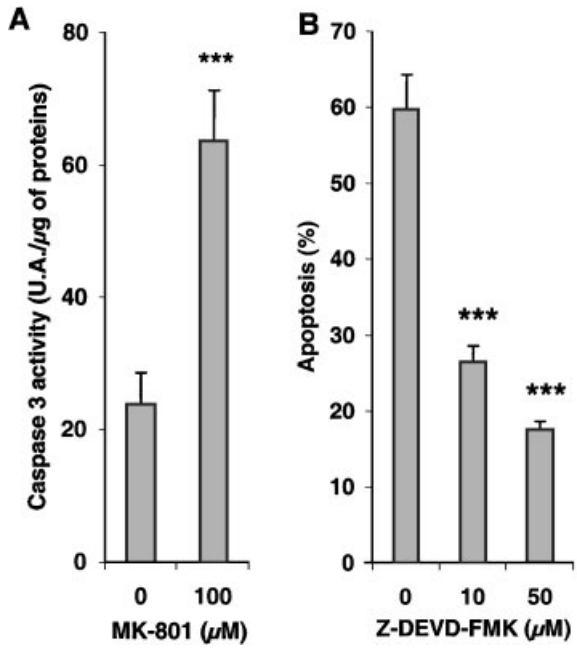

Fig. 7. **A**: Effect of MK801 on caspase 3 activation. Purified osteoclasts were incubated with or without 100 µM of MK801 for 1 day at 37°C. Caspase 3 activity of the lysate was measured as described in Materials and Methods. Results are expressed as the mean ± SD of two independent experiments (n = 6). ***$P < 0.001$ compared with untreated control cells. **B**: Effect of caspase 3 inhibitor (Z-DEVD-fmk) on MK801-induced osteoclast apoptosis. Rabbit purified osteoclasts were cultured for 18 h in αMEM in the presence of Z-DEVD-fmk (10 and 50 µM). Results are expressed as the mean ± SD of two independent experiments (n = 12). ***$P < 0.001$ compared with culture treated with MK801 alone.

osteoclasts, and on the basis of these results proposed that basal production of NO may exert a tonic inhibitory effect on osteoclast activity. Brandi et al. [1995] have reported that L-NMMA, inhibited bone resorption by isolated rat osteoclasts and concluded that basal production of NO might be necessary for normal osteoclast function. Although the reason for this discrepancy is still unclear, it could be attributable to side effects independent of NOS inhibition of the compounds, which have been used as NOS inhibitors. In view of this, we attempted to clarify the role of endogenous NO by using carboxy-PTIO, a compound which is known as an NO scavenger and we investigated the functional consequences of NO depletion on bone resorption and osteoclast activity. Carboxy-PTIO has been used extensively in biophysical studies and reacts very selectively with NO [Amano and Noda, 1995; Wanikiat et al., 1997]. Herein, we demonstrated that carboxy-PTIO inhibits osteoclastic bone resorption in a dose-dependent manner, suggesting that trapping intracellular NO dramatically modulates osteoclast activity. This observation is consistent with previous reports [Brandi et al., 1995; Evans and Ralston, 1996] suggesting that a minimal basal production of NO is required for osteoclast function. It is now clearly established that osteoclasts are cells with a short lifespan and they die rapidly by apoptosis, a process common to many regenerating tissues. Bone resorption process is closely dependent of osteoclast survival [Parfitt et al., 1996; Hughes and

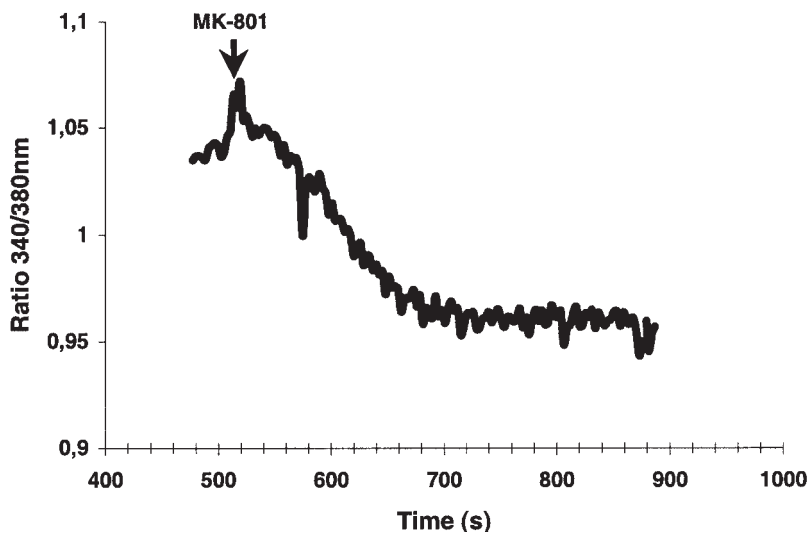

Fig. 8. Decrease in the intraosteoclastic calcium concentration induced by the perfusion of MK801 (100 µM). Data are expressed as the ratio of Fura2-AM fluorescence elicited by excitation at 340 and 380 nm.

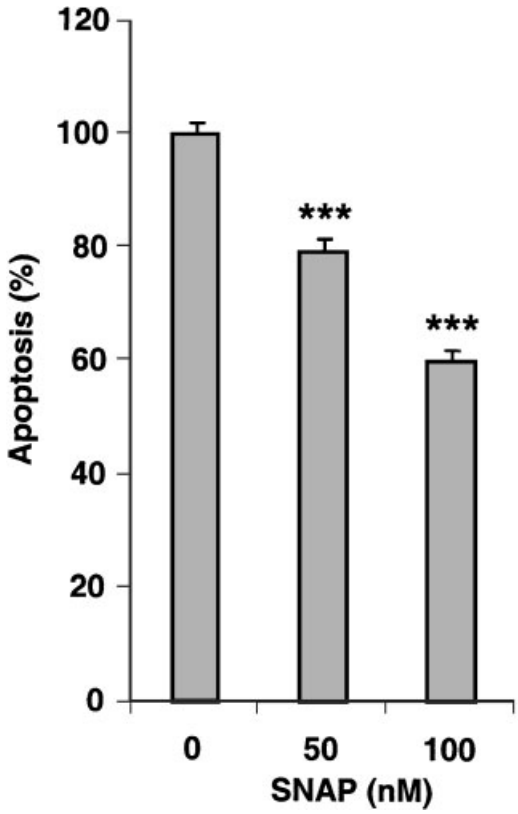

Fig. 9. Effect of SNAP (an NO donor) on MK801-induced osteoclast apoptosis. Rabbit purified osteoclasts were cultured for 18 h in αMEM in the presence of SNAP (50 and 100 nM). Results are expressed as percent of MK801-induced osteoclast apoptotic rate and are representative of three independent experiments (n = 18). $^{***}P < 0.001$ compared with culture treated with MK801 alone.

Boyce, 1997; Jimi et al., 1998; Okahashi et al., 1998] and it has been shown that many agents, which have potent antiresorptive properties act by inducing osteoclast apoptosis. This is the case for estrogen [Kameda et al., 1997] and bisphosphonate [Hughes et al., 1995], which are the most potent inhibitors of bone resorption used currently in the treatment of osteoporosis. Therefore, to identify the mechanism whereby carboxy-PTIO inhibits bone resorption, we tested whether it can exert its effects directly on osteoclast apoptosis. For this purpose, highly purified mature rabbit osteoclasts were obtained from unfractionated bone cells. We demonstrated that depletion of intracellular NO by carboxy-PTIO directly triggers apoptotic cell death in mature osteoclasts by a mechanism involving activation of caspase 3. This suggests that basal NO production may be essential for normal bone resorption by supporting the survival of osteoclasts.

Several recent studies [Chenu et al., 1998; Patton et al., 1998; Laketic-Ljubojevic et al., 1999] have identified the presence of NMDA-R on osteoclasts, however their functional significance has not yet been elucidated. In neuronal cells, NMDA-R are preferentially involved in NO biosynthesis [Dingledine et al., 1999]. In these cells, nNOS is linked to NMDA-R via postsynaptic density-95 protein (PSD-95) using PDZ/PDZ interaction [Brenman et al., 1996]. This association ensures that calcium influxes through NMDA-receptor are selectively coupled to nNOS activity. Due to the presence of nNOS in osteoclasts, as suggested in this study, and the recent demonstration that osteoclasts expressed PSD-95 [Itzstein et al., 2001], one may hypothesize that in osteoclasts a similar mechanism may exist. We first studied the effects of NMDA-R blockade with MK801, a well-known non-competitive antagonist of NMDA-R, on osteoclastic bone resorption. Bone resorption process was assessed by using three different methods. We confirmed and extended previous studies [Itzstein et al., 2000] showing that MK801 (100 μM) inhibits strongly mature osteoclastic bone resorption. Our results are in accordance with those reported by Chenu et al. [1998], but contrast with those of Peet et al. [1999], who reported a lack of effect of MK801 on bone resorption. At that time, this discrepancy between data obtained using a similar model of mature rabbit osteoclasts remains difficult to explain. To investigate the mechanism responsible for the MK801-induced inhibition of bone resorption, we tested the effects of this compound on osteoclast apoptosis. We showed that MK801 directly promotes osteoclast apoptosis in the same dose range required for inhibition of bone resorption. The MK801-induced osteoclast apoptosis was dependent on caspase 3 activation since MK801 was responsible for an increase in caspase 3 activity. Moreover, Z-DEVD, a selective caspase 3 inhibitor, was able to block completely the effect of MK801. Finally, we showed that DEP, another non-competitive antagonist of NMDA-R, also induced osteoclast apoptosis in a dose-dependent manner and to a similar extent as MK801. Taken together, these data suggest that inhibition of bone resorption by NMDA-R blockade could be due, at least in part, to a direct effect on osteoclast apoptosis. Our results differed from those obtained by Itzstein et al. [2000], who reported that osteoclast survival was not

affected by MK801 treatment although they also used purified rabbit osteoclasts. However, they isolated and purified their osteoclasts from 1-day-old rabbit bones while in our study osteoclasts were prepared from 10-day-old rabbit. Recently it has been reported, in cultured cortical neurones and in developing brain, that MK801 effects on apoptotic cell death is dependent of the maturation state of the cells [Ikonomidou et al., 1999].

As MK801 and DEP are two compounds known to block the NMDA receptor-gated cation channel permeable to calcium ions, we then investigated the consequence of NMDA-R blockade on $[Ca^{2+}]_i$ concentration in osteoclasts. We demonstrated that both MK801 and DEP reduced within minutes the $[Ca^{2+}]_i$, suggesting that as in neuronal cells, NMDA-R present in osteoclasts, could be involved in maintenance of $[Ca^{2+}]_i$ homeostasis. In order to establish a link between the NMDA-R, $[Ca^{2+}]_i$ and NO biosynthesis, we attempted to measure the intracellular NO concentration after NMDA-R blockade with MK801. Unfortunately, the methods that we used were not sensitive enough to measure very low NO concentrations. Nevertheless, we showed that small amount of SNAP an NO-releasing compound, which alone has no effects on osteoclast survival, reversed partially the MK801-induced osteoclast apoptosis. This result makes possible the hypothesis that NMDA-R could be involved in NO production in osteoclasts. A stronger link could be established between NMDA-R blockade and NO production by demonstrating in osteoclasts isolated from nNOS KO mice, that MK801 has no effect on osteoclast activity.

In summary, the results presented in this study demonstrate that a basal NO production in osteoclast cells is required for their normal activity. The effects of NMDA-R blockade by non-competitive antagonists on osteoclastic bone resorption and osteoclast survival are closely related to those of carboxy-PTIO, a scavenger of intracellular NO. Taken together, our results are consistent with the hypothesis that NMDA-R on osteoclasts have a similar function as those in neuronal cells, i.e., to allow a calcium influx which in turn activates a constitutive NOS such as nNOS. NO generated by this pathway may be essential for osteoclast survival and hence for normal bone resorption.

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