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Direct Microsensor Measurement of Nitric Oxide Production by the Osteoclast

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Nitric oxide (NO) triggers marked osteoclast retraction which closely resembles that due to Ca^{2+}. The effect of Ca^{2+} has been attributed to a stimulated release of NO. Here, we show for the first time, by direct measurement with a microsensor, that osteoclasts do indeed produce NO and that this production is enhanced by a high Ca^{2+}. We also show that the Ca^{2+} ionophore, A23187, mimics the latter. Furthermore, osteoclasts on dentine produce more NO than osteoclasts on glass and NO release from dentine-plated osteoclasts is much less sensitive to stimulation by Ca^{2+}. Finally, the microsomal Ca^{2+} store-depleting agent, thapsigargin, attenuates NO release only from osteoclasts on glass, suggesting that stored Ca^{2+} has the dominant effect in modulating NO release from non-resorbing cells. NO is a powerful inhibitor of bone resorption: a direct demonstration of its production is therefore strong evidence for a role in modulating osteoclast function.

Nitric oxide (NO) mediates functions as critical as vascular homeostasis, inflammation, neurotransmission and bone metabolism (1–9). It is a powerful inhibitor of osteoclastic bone resorption both *in vivo* and *in vitro* (1, 3, 8–10). This effect resembles that of a high extracellular Ca^{2+} that we believe is exerted through a Ca^{2+}-sensing receptor. The osteoclast is also a major producer of NO (6). It expresses both constitutive (e) and inducible (i) NOS isoforms (1, 4). Of these, eNOS is a Ca^{2+}-sensitive enzyme (8, 11); hence Ca^{2+} modulates its catalytic activity. In contrast, the expression rather than catalytic activity of iNOS is regulated by Ca^{2+} (8, 11, 12). Potentially, therefore, NO production from the osteoclast can be triggered by Ca^{2+} changes, through changes in eNOS activity and/or iNOS expression (13).

To explore the relative contributions of the two NOS isoforms to resorption inhibition, it is critical that we separate an early effect of Ca^{2+} on eNOS catalysis from a delayed effect on iNOS expression. This is not possible with current NO detection methods that depend on the slow accumulation of NO biproducts, such as nitrite and citrulline, over prolonged times. We have therefore made real-time NO measurements from single osteoclasts using a microsensor (8). The microsensor allows us to sample NO as close as possible to the osteoclast. This retards NO degradation and prevents the masking of its osteoclastic release by its release from contaminating cells (14).

MATERIALS AND METHODS

Osteoclast isolation and culture. Osteoclasts were isolated from the tibiae of 4 week-old chicks that had been fed on a low-calcium diet for 2.5 weeks (15). Briefly, the tibiae were harvested in cold (4°C) Moscona's low-Ca^{2+} medium ($[Ca^{2+}] < 1\ \mu M$) containing heparin (200 U/l) (Elkins-Sinn Inc., Cherry Hill, NJ). The bones were treated with collagenase (125 kU/l) (Sigma, St. Louis, MO) for 15 minutes (37°C), and then incubated with trypsin (1% w/v) (Gibco, Grand Island, NY) for a further 15 minutes (37°C). This treatment, followed by manual shaking for 4 minutes, dislodged a heterogeneous population of bone cells from the partially digested matrix. The resulting crude cell suspension was filtered through Nytex filters (Tetko, Inc., New York, NY, USA) in ice. The residue was then resuspended in Moscona's low-calcium medium at 0°C and layered for 1 hour at 4°C on 6% Percoll (Sigma). This resulted in the rapid sedimentation of between 12 and 15 million, mainly multinucleated, cells (from 25 chicks). The cells were collected from the Percoll, pelleted at 1500g for 15 minutes at 4°C, and resuspended in Dulbecco's Minimum Essential Medium containing fetal calf serum (FCS, 10% v/v) (Cell Center, University of Pennsylvania, Philadelphia, PA) (DMEM/FCS). The Percoll cell fraction was finally plated at a density of 10^6 cells per 1 ml-well; each well contained both a dentin disc (area ~20 mm^2) and a round glass coverslip (area ~360 mm^2). The respective substrates containing this enriched cell population were further incubated in DMEM/FCS for 24 hours at 37°C in humidified CO_2 (5%). The cells were then fixed in citrate acetone buffer (pH 7.4) and stained for tartrate-resistant acid phosphatase (TRAP), a surrogate osteoclast marker, using a Leukocyte Acid Phosphatase Kit (Sigma). Previous studies have shown that dispersion of Percoll cell fractions on dentin yields ~200 multinucleated cells per 10 mm^2; 90% of these are TRAP-positive (16). In contrast, glass coverslips have a somewhat lower TRAP-

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positive cell density (20% of 400 settled cells per 10 mm^2) (Silverton, unpublished). That most mutinucleated and some mononucleated cells display TRAP-positivity and can resorb dentin confirms their identity as osteoclasts (16, 17).

Porphyrinic microsensor measurements. NO production from single osteoclasts was measured electrochemically using a porphyrinic microsensor (18). The latter was prepared by depositing a film of polymeric porphyrinic [Ni(II) tetrakis (3-methoxy-4-hydroxyphenyl) porphyrin] on a thermally sharpened carbon fiber electrode which was then coated with Nafion (14). NO detection by the microsensor depends upon the fast and selective oxidation of NO to NO^+ followed by rapid NO^+ removal by the Nafion ion-exchange layer (sensor current density, stabilized by differential pulse voltamography, 1.5-1.8 mA/cm^2). Current changes were found to be linearly proportional to NO concentration changes at the microsensor surface (16) (detection limit 10^{-9} M) (14). Before each experiment, we prepared a standard saturated solution of NO (2 mM) in an O_2-depleted buffer (200 mM-NaH_2PO_4/Na_2HPO_4) (pH 7.4) under helium. The microsensor was placed in 2 ml-Hank's Balanced Salt Solution (HBSS) (Gibco) or Moscona's low-calcium solution in the well of a microscope (Diaphot, Nikon, Tokyo, Japan). The reference electrode was placed in the same well and the rectifying signal stabilized. Several 2 μl-aliquots of the standard NO solution were drawn up in a Hamilton gas-tight syringe and added to the well sequentially. The peak height for each observed current signal was averaged within every experiment and for every electrode used. Following a 16 hour-incubation in humidified CO_2 (5%) at 37°C, the osteoclast-dentin or osteoclast-glass cultures (n = 4 to 8) were placed in a 2 ml-well containing either HBSS or Moscona's low-calcium buffer (pH 7.4). A micromanipulator was then used to position the microsensor in close apposition to the dorsal surface of one or more osteoclasts. NO measurements were made after the addition of NADPH (1 to 10 μM), A23187 (1 to 4 μM), thapsigargin (1 to 4 μM), L-arginine (1 mM), and superoxide dismutase (1 KU/l).

RESULTS

Validation. There was a linear relationship between the peak microsensor current and the added NO concentration. We could thus calculate the NO concentration of a given sample by plotting its peak current value against that of the NO standard (Fig. 1a). In each experiment, we used one or more electrode and calibrated each electrode against standard. This allowed us to make comparisons of NO concentrations across experiments. The current amplitude of the saturated NO standard, in nAmps/μM-NO (median–13.7; range–2.4 to 17.8, n = 5), was markedly lower than that obtained with NO in O_2-depleted buffer (median–137; range–25 to 254, n = 6). Taken together, these results reflect NO degradation by O_2 and other buffer constituents in our open-well system. We next examined the effect of increasing the distance between the point of NO application and the microsensor. When the application distance was increased from 2 mm to 4 mm, the sharp monophasic peaks were converted to significantly attenuated biphasic signals that followed a ~60 second lag (Fig. 1b and 1c).

Osteoclastic NO release. NO transients in isolated osteoclasts were elicited through the application of NADPH (1 μM to 10 μM) (19) (Fig. 1d). Each transient consisted of a rapid rise to a peak value followed by an exponential decay to near-basal levels (Fig. 1e). NADPH serves as a cofactor for NOS, and at submaximal catalytic rates, the peak height is expected to be a function of the NADPH concentration. Thus, we found a limited, but highly significant, concentration-dependent correlation (r = 0.625; n = 21, p = 0.0024) between released [NO] and applied [NADPH] (Insert to Fig. 1e). We calculated NO release by first deriving the rate of [NO] change, in nM/minute, and then converting this to a release rate, in nmoles/minute. For this, we first defined high and low estimates for NO detection using volumes around the osteoclast (6×10^{-13} m^3) and microsensor electrode (2×10^{-12} m^3), respectively. We then used these estimates to calculate NO release rates yielding values of 1728 and 578 pmol min^{-1} 10^7 $cell^{-1}$ for the cell and electrode, respectively.

To confirm that the NO signal resulted from NADPH action on NOS, we used N-ω-nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor. We found that 300 μM-L-NAME inhibited the NO signal significantly (p = 0.05). Furthermore, we know that most other organic and inorganic species (14) do not interfere with NO detection, as the NO oxidation rate by the microsensor is several orders of magnitude higher. An exception is aminoguanidine, another non-selective NOS inhibitor. The latter, at 250 μM, triggered a large irreversible current surge, possibly due to poor diffusion away from or poor adsorption onto, Nafion. Finally, L-arginine (1 mM), superoxide dismutase (1 kU/l) and thapsigargin (2 μM) did not themselves affect microsensor current (not shown), further attesting to the NO-specificity of the microsensor.

Effects of substrate and Ca^{2+} on osteoclastic NO release. NADPH-induced NO release was then measured in osteoclasts plated on glass coverslips or dentin discs. The magnitude of NO release was in the following order: glass (0-Ca^{2+}) < glass (1.25 mM-Ca^{2+}) < dentine (0-Ca^{2+}) < dentine (1.25 mM-Ca^{2+}) (Table 1). Notably, NO release from dentin (1.25 mM-Ca^{2+}) was significantly greater than that from cells on glass (1.25 mM-Ca^{2+}) (p = 0.037) or those on glass (0-Ca^{2+}) (p = 0.042).

We next looked at changes in NO release in response to changes in cytosolic Ca^{2+} using the Ca^{2+} ionophore, A23187. A23187 elicited rapid NO signals in osteoclasts plated on either glass (585 nM μg^{-1}; ± 193; n = 5) or dentine (663 nM μg^{-1}; ± 214; n = 5). The rapidity of NO release suggests that elevated cytosolic Ca^{2+} possibly triggered eNOS activation.

The NADPH-induced NO signal was however attenuated markedly (or abolished) when osteoclasts plated on glass were pretreated with thapsigargin (2 μM), a microsomal Ca^{2+}-ATPase inhibitor that depletes Ca^{2+} from intracellular stores (20) (Fig. 2a). Curiously, this attenuation was not fully reversed upon the subsequent application of A23187 (1 μM) (Fig. 2b). Note that

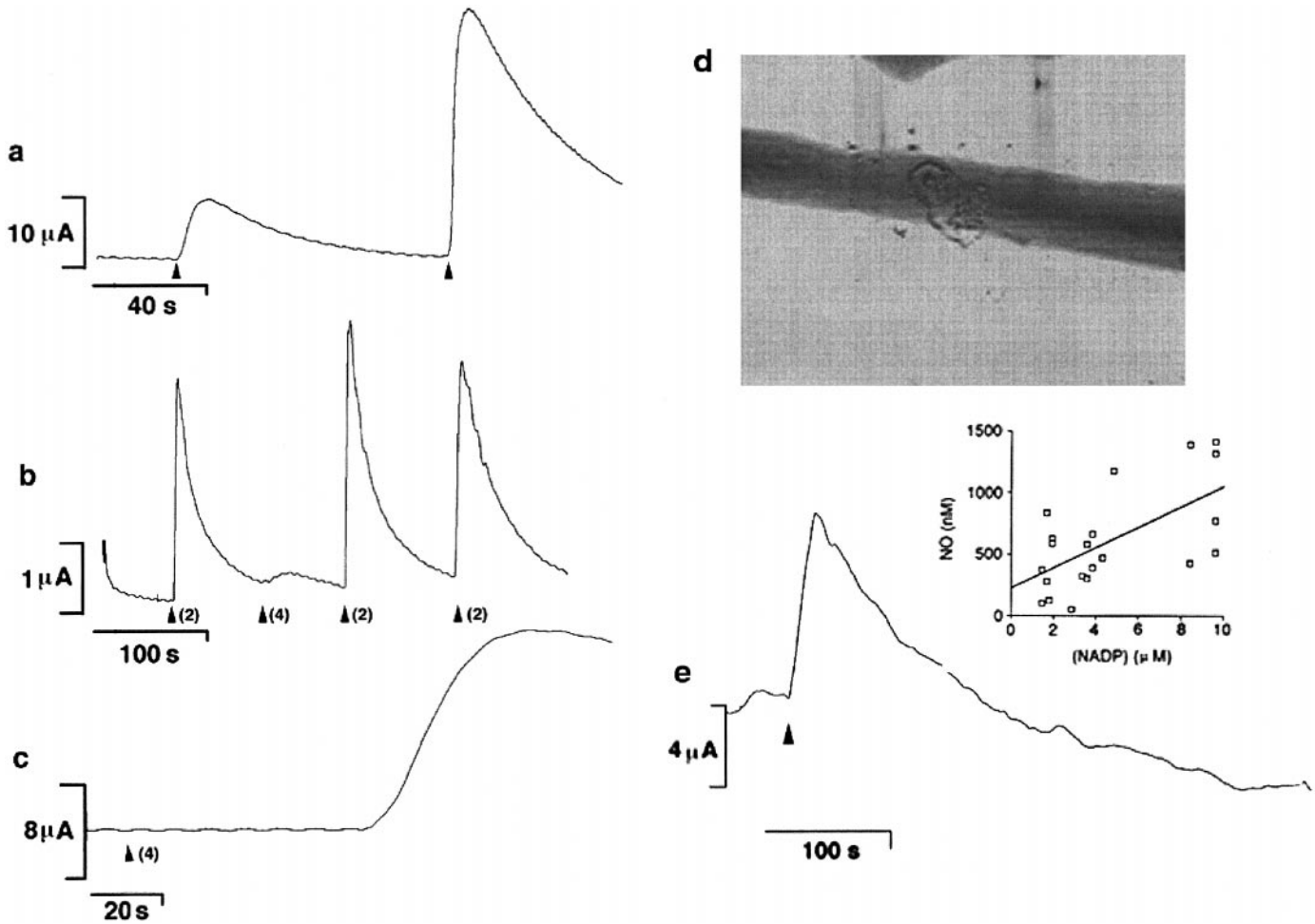

FIG. 1. Panels a to c: Representative microsensor current traces showing the effect of nitric oxide (NO) gas (at ▲) on current waveform and amplitude (μA) (panels a–c) plotted as a function of time (seconds, s). Panels b and c show the addition of NO at a distance of either 2 mm (1) or 4 mm (2) from the microsensor. The microsensor detection limit for NO is ~1 nM. Panel d: NO-selective microsensor placed in close apposition to the dorsal surface of an osteoclast (~50 μ × 30 μ). The central, dark shadow of the electrode image represents the sharpened carbon fiber (diameter ~10 μ). The thick outer coating, seen as a light gray shadow, represents polymeric porphyrin that binds the NO and Nafion. Note that the microsensor image is not precisely in focus in the osteoclast plane and, thus, appears larger. Panel e: Representative microsensor current traces (μA) obtained from the osteoclast surface in response to application (at ↑) of the NO synthase (NOS) cofactor, NADPH. The insert to panel e shows the correlation between released NO (nM) and applied NADPH (μM) ($r = 0.625$, $p = 0.0024$, $n = 21$ measurements).

thapsigargin did elicit a rise in cytosolic Ca^{2+}, indicating a depletion of Ca^{2+} stores (20). In contrast, osteoclasts on dentine were insensitive to attenuation by either a single or repeated applications of thapsigargin (2 μM) (Fig. 2d and 2e). Repeated applications of NADPH to osteoclasts on glass or dentine did not attenuate responses to a further application (Fig. 2c and 2f).

It should be noted that thapsigargin and A23187 were used, respectively, to deplete intracellular Ca^{2+} stores and trigger Ca^{2+} influx (20). Their distant effects suggest that constitutive NO release in non-resorbing osteoclasts (on glass), possibly from eNOS catalysis, is modulated predominantly by stored Ca^{2+}. In contrast with cells plated on dentin, whilst A23187 still triggers

TABLE 1

Substrate	$[Ca^{2+}]$	[NO]
Glass	1.25	9.4 ± 3.2^a
	0	3.7 ± 1.3^b
Dentine	1.25	30.5 ± 5.5
	0	18.8 ± 11

Note. Nitric oxide (NO) release (nM/μM-NADPH) triggered by NADPH application to osteoclasts that were incubated on glass or dentine in either Hanks' Balanced Salt Solution ($[Ca^{2+}] = 1.25$ mM) or in Moscona's low Ca^{2+} medium ($[Ca^{2+}] < 1$ μM) (see "Materials and Methods"). Results are represented as mean ± standard error of the mean ($n = 3$ to 6). Statistics by students' t-test with Bonferroni's Correction for Inequality: $^a p = 0.037$ and $^b p = 0.042$, compared with dentine (1.25 mM-Ca^{2+}).

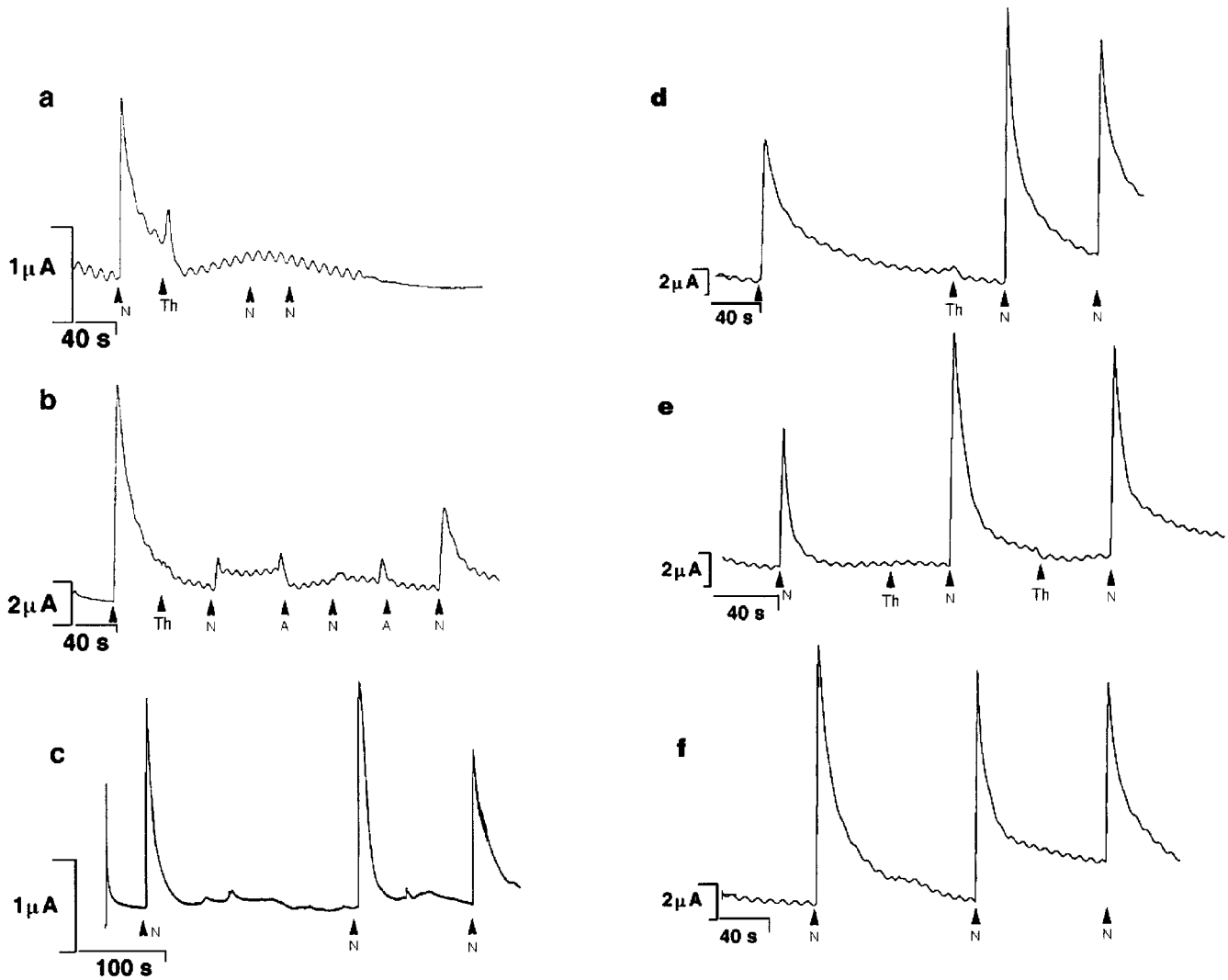

FIG. 2. Representative microsensor current tracings (μA) in response to application of the nitric oxide synthase (NOS) cofactor, NADPH (N) to osteoclasts incubated on glass (panels a to c) or on dentine (panels d to f) in Hanks Balanced Salt Solution (HBSS, $[Ca^{2+}]$ = 1.25 mM) either before or after thapsigargin (2 μM) (Th) or A23187 (A) in various treatment protocols, as indicated. Thapsigargin abolished the NO transient due to NADPH (c) on glass. The NO response on dentine (f) was uninfluenced by thapsigargin (d, e). A23187 (b) produced a modest NO transient and repeated application was followed by a partial restoration of the response to NADPH previously inhibited by thapsigargin.

NO signals, thapsigargin does not attenuate NO release. Taken together, the results suggest that a relatively Ca^{2+}-insensitive NOS, possibly iNOS, was being induced during resorption.

DISCUSSION

A major strength of NO microsensor as opposed to the citrulline and nitrite based measurement system is in its ability to measure the free radical at or near their production site. Labile free radicals, such as NO and $O_2^{-\cdot}$ are known to be destroyed rapidly in the intracellular, extracellular, and plasma membrane compartments. For example, whereas purified neutrophil-derived myeloperoxidase (MP) produces ~30 amol $O_2^{-\cdot}$ $second^{-1}$ $cell^{-1}$ (0.1 unit MP per 2.5 $\times$ 10^7 cells), neutrophils themselves only produce 0.126 amol $O_2^{-\cdot}$ $second^{-1}$ $cell^{-1}$ (21), suggesting that transcellular $O_2^{-\cdot}$ degradation can be high. Although, corresponding data has not yet been obtained for NO, we know that NO signal intensity is attenuated markedly as the application distance from the microsensor is increased. Thus, by applying the microsensor closest to the osteoclast surface, we are, in effect enhancing specificity and taking advantage of the negligible diffusional distance.

In this study, the NO microsensor has been applied successfully to (a) demonstrate directly, the release of

NO from resorbing and non-resorbing osteoclasts, and (b) assess the effect of changes in extracellular, cytosolic and stored Ca^{2+} on NO release. Extracellular Ca^{2+} has been implicated in the feedback control of osteoclast function (10). Changes in extracellular [Ca^{2+}] are known to trigger cytosolic Ca^{2+} signals through the activation of the Ca^{2+} sensing receptor (10, 13, 22–24). Cytosolic [Ca^{2+}] changes are, in turn, accompanied by cell retraction, reduced enzyme and acid secretion, and, in the longer term, diminished bone resorption (10, 13, 22–24). It has been suggested that NO plays a critical role in the anti-resorptive effects of Ca^{2+}. Thus, Ca^{2+}-induced osteoclast retraction is reversed promptly by N^G-monomethyl-L-arginine, a known NOS inhibitor (3). In parallel, aminoguanidine, another NOS inhibitor, reverses both the resorption-inhibition and nitrite production induced by the Ca^{2+} ionophore, A23187 (1, 4).

The present study extends these observations through the use of our microsensor. We find that both extracellular Ca^{2+} and dentine enhance NO production from osteoclasts. More directly, an increased intracellular Ca^{2+} level itself triggers NO release in both resorbing (on dentine) and non-resorbing (on glass) cells. This indicates that both extra- and intracellular Ca^{2+} affect the activity of a Ca^{2+}-sensitive NOS, that is most likely an eNOS. It is however, unlikely, that Ca^{2+} affects the expression of iNOS within minutes of A23187 application to non resorbing cells. Furthermore, in non-resorbing cells, NO release is inhibited upon depletion of intracellular Ca^{2+} stores by the microsomal membrane Ca^{2+}-ATPase inhibitor, thapsigargin. In contrast, resorbing cells are thapsigargin-insensitive; this is consistent with possible induction of iNOS during resorption. This is also in line with the observation that cells on dentin release significantly more NO than cells on glass.

Three key conclusions thus emerge from this study: that resorbing osteoclasts release more NO than non-resorbing cells; that eNOS catalysis is modulated by extracellular, cytosolic and stored Ca^{2+}; and that iNOS is possibly induced in osteoclasts upon resorption. The greater NO release from resorbing osteoclasts, we believe, represents a summation of the stimulatory effects of Ca^{2+} on eNOS catalysis and iNOS expression. The mechanism through which Ca^{2+} affects iNOS expression remains unclear, though. Whether the iNOS gene has a Ca^{2+}-response element (CaRE) in its promoter, or whether the effects of Ca^{2+} are exerted indirectly through protein kinase C (4), remains to be determined. We also have no mechanistic information on the importance of stored Ca^{2+} in controlling NO release in non-resorbing cells. One possibility is that eNOS exists in close proximity to Ca^{2+} stores, the fullness of which depends largely upon extracellular Ca^{2+}. These aspects require further investigation.

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REFERENCES

1. Brandi, M. L., Hukkanen, M., Umeda, T., Moradi-Bidhendi, N., Bianchi, S., Gross, S. S., Polak, J. M., and MacIntyre, I. (1995) *Proc. Natl. Acad. Sci. (USA)* **92,** 2954–2958.
2. Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H., and Fishman, M. C. (1993) *Cell* **75,** 1273–1286.
3. MacIntyre, I., Zaidi, M., Alam, A. S. M. T., Datta, H. K., Moonga, B. S., Lidbury, P. S., Hecker, M., and Vane, J. (1991) *Proc. Natl. Acad. Sci. (USA)* **88,** 2936–2940.
4. Malinski, T., Taha, Z., Grunfeld, S., Patton, S., Kapturczak, M., and Tomboulian, P. (1993) *Biochem. Biophys. Res. Comm.* **193,** 1076–1082.
5. Nathan, C. (1992) *FASEB* **6,** 3051–3064.
6. Silverton, S. F., Mesaros, S., Markham, G. D., and Malinski, T. (1995) *Endocrinology* **136,** 5244–5247.
7. Stamler, J. S., Jia, L., Eu, J. P., McMahon, T. J., Demchenko, I. T., Bonaventura, J., Gernert, K., and Piantadosi, C. A. (1997) *Science* **276,** 2034–2037.
8. Sunyer, T., Rothe, L., Kirsch, D., Jiang, X., Anderson, F., Osdoby, P., and Collin-Osdoby, P. (1997) *Endocrinol.* **136,** 2148–2162.
9. Zaidi, M., Alam, A. S. M. T., Bax, B. E., Shankar, V. S., Bax, C. M. R., Gill, J. S., and Pazianas, M. (1993) *Bone* **14,** 97–102.
10. Zaidi, M., Alam, A. S. M. T., Huang, C. L.-H., Pazianas, M., Bax, C. M. R., Bax, B. E., Moonga, B. S., Bevis, P. J. R., and Shankar, V. S. (1993) *Cell Calcium* **14,** 271–277.
11. Nathan, C., and Xie, Q.-W. (1994) *Cell* **78,** 915–918.
12. Abu-Soud, H. M., and Stuehr, D. J. (1993) *Proc. Natl. Acad. Sci. (USA)* **90,** 10769–10772.
13. Moonga, B. S., Moss, D. W., Patchell, A., and Zaidi, M. (1990) *J. Physiol.* **429,** 29–45.
14. Malinski, T., Mesaros, S., and Tomboulian, P. (1996) *Methods Enzymol.* **268,** 58–69.
15. Oursler, M. J., Collin-Osdoby, P., Anderson, F., Li, L., Webber, D., and Osdoby, P. (1991) *J. Bone Min. Res.* **6,** 375–385.
16. Kang, J., Cornejo, R., Hirokawa, K., DiGiorgio, S. M., Howard, D. F., and Silverton, S. F. (1996) *Cells and Materials* **6,** 21–30.
17. Shapiro, I. M., Howard, D., and Silverton, S. F. (1994) *Cells and Materials* **4,** 371–378.
18. Malinski, T., and Taha, Z. (1992) *Nature* **358,** 676–678.
19. Silverton, S. F., Matsumoto, H., DeBolt, K., Reginato, A., and Shapiro, I. M. (1989) *Bone* **10,** 45–51.
20. Zaidi, M., Shankar, V. S., Bax, C. M. R., Bax, B. E., Bevis, P. J. R., Pazianas, M., Alam, A. S. M. T., and Huang, C. L.-H. (1993) *J. Bone Min. Res.* **8,** 961–967.
21. Steinbeck, M. J., Appel, W. H., Verhoeven, A. J., and Karnovsky, M. J. (1994) *J. Cell Biol.* **126,** 765–772.
22. Malgaroli, A., Meldolesi, J., Zambonin-Zallone, A., and Teti, A. (1989) *J. Biol. Chem.* **264,** 14342–14347.
23. Zaidi, M., Datta, H. K., Patchell, A., Moonga, B. S., and MacIntyre, I. (1989) *Biochem. Biophys. Res. Commun.* **163,** 1461–1465.
24. Zaidi, M., Shankar, V. S., Tunwell, R. E., Adebanjo, O. A., Pazianas, M., Simon, B., Rifkin, B. R., Venkitaraman, A., Huang, C. L.-H., and Lai, F. A. (1995) *J. Clin. Invest.* **96,** 1582–1590.