A New Superoxide-generating Oxidase in Murine Osteoclasts*

Su Yang‡, Prema Madyastha, Sarah Bingel, William Ries, and Lyndon Key

From the Division of Endocrinology, Department of Pediatrics, Medical University of South Carolina, Charleston, South Carolina 29425

Superoxide production contributes to osteoclastic bone resorption. Evidence strongly indicates that NADPH oxidase is an enzyme system responsible for superoxide generation in osteoclasts. A membrane-bound subunit, p91, is the catalytic domain of NADPH oxidase. However, osteoclasts from p91 knockout mice still produce superoxide at a rate similar to that observed in wild type mice. This unexpected phenomenon prompted us to examine the osteoclasts for an alternative to the p91-containing oxidase. In this study, the cloning of a NADPH oxidase subunit (Nox 4) with 578 amino acids is reported. Nox 4 has 58% similarity in amino acids with the known p91 subunit of NADPH oxidase. Nox 4 is present and active in osteoclasts. Antisense oligonucleotides of Nox 4 reduced osteoclastic superoxide generation as well as resorption pit formation by osteoclasts. This new oxidase complex was present and functional in osteoclasts from p91 knockout mice, explaining the normal resorptive activity seen in the osteoclasts where no p91 is present.

Osteoclast-generated superoxide directly contributes to bone degradation. The presence of superoxide production at the osteoclast-bone interface suggests a direct effect of superoxide in osteoclastic bone resorption (1, 2). In addition, inhibition of osteoclastic superoxide availability results in a reduction in bone resorption (3, 4). Treatment with interferon γ, a stimulator of NADPH oxidase activity, corrects defective osteoclastic function in osteopetrotic, microphthalmic mice in vivo and in calvaria cultured from these animals (5). In patients with osteopetrosis, increased bone resorption was documented by a decrease in medullary bone (6). Therefore, superoxide generation at the osteoclast-bone interface is necessary for optimal levels of bone resorption.

NADPH oxidase, a common enzyme system that produces superoxide in white cell phagocytes, is also present and active in osteoclasts. Several studies suggest that NADPH oxidase is responsible for osteoclastic superoxide production (3, 7, 8). However, in studies of p91 knockout mice, despite the absence of the p91 catalytic subunit of NADPH oxidase, osteoclasts generate normal amounts of superoxide. X-ray bone density analysis demonstrates that the p91 knockout mice are not osteopetrotic, as would be expected if there were a severe defect in osteoclastic bone resorption (data not shown). In keeping with the normal superoxide production found in the osteoclasts from the p91 knockout mutants, a similar finding has been reported in the lung endothelium. Kubo et al. (9) found that lung endothelial cells from p91 knockout animals generate normal amounts of superoxide. In these endothelial cells, the xanthine oxidase enzyme complex was found to be responsible for generating superoxide. In a study of human fibroblasts, a “second” NADPH oxidase was demonstrated in cells from a patient afflicted with chronic granulomatous disease, who had a mutation in the p91 gene. This fibroblastic oxidase differed immunologically and functionally from NADPH oxidase, substantiating the presence of an alternative oxidase (10). Thus, in at least two cell types, superoxide generation by oxidases other than NADPH oxidase has been observed.

In this study, we have searched for an alternative oxidase responsible for superoxide generation in the osteoclasts from p91 knockout mice. To accomplish this, a mouse EST1 has been identified by searching a gene bank. This EST contains 46% amino acid identity with the C-terminal portion of mouse p91. A complete sequence was obtained by 5′-RACE. Expression of a new oxidase subunit, Nox 4, in the osteoclasts from p91 knockout mice was demonstrated. Finally, using antisense oligonucleotide disruption of the production of Nox 4, we have demonstrated that expression of Nox 4 is related to osteoclastic superoxide generation and bone resorption.

EXPERIMENTAL PROCEDURES

Materials—Anti-Nox 4 antibody was generated at the Medical University of South Carolina facility using a unique peptide (SKTLHSL-SRNNNSYGTKFHEY). The rabbit anti-Nox 4 antibodies were further purified by a peptide affinity column. Preincubation of the serum with Nox 4 peptide (1 μg/ml) resulted in undetectable signals, suggesting the specificity of the antibody. NTB2 liquid photographic emulsion was purchased from Eastman Kodak Co. ECL was obtained from Amersham Pharmacia Biotech. All other reagents were purchased from Sigma.

Cloning—Using the mouse p91 sequence as a query to search the murine EST data base, a homologous sequence (accession no. AI746441) was found, which contained 46% identity in amino acid sequence to the C-terminal portion of mouse p91. To obtain a complete sequence, 5′-RACE was carried out using Marathon-ready cDNA (CLONTECH, Palo Alto, CA). PCR was performed using primer AP 1 and the gene-specific primer (5′-CTGCACACCCAGATAAAGTACAGTCTT-3′). A 1.5-kilobase pair PCR product was purified and cloned into a TA vector (Invitrogen, San Diego, CA). The clones containing the insert were sequenced to reveal the complete sequence for the Nox 4 mRNA.

Osteoclast Culture—Osteoclasts were generated by the culture of mouse bone marrow cells for 1–2 weeks in α-minimal essential medium, 10% FBS, and 1% penicillin containing 1 × 10−6 m 1,25-dihydroxyvitamin

*This work was supported by GCRC in Medical University of South Carolina and National Institutes of Health Grant R01-AR141463. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: 316 CSB, Pediatric Endocrinology, Medical University of South Carolina, 171 Ashley Ave., Charleston, SC 29425. Tel.: 843-792-1346, Fax: 843-792-0548; E-mail: yangs@musc.edu.

1 The abbreviations used are: EST, expressed sequence tag; DPI, dihydrolipoamide dehydrogenase; FAD, flavin adenine dinucleotide; 5′-RACE, 5′-rapid amplification of cDNA ends; NBT, nitro blue tetrazolium; Nox 4, NADPH oxidase 4 (mouse); p91, a 91-kDa subunit of NADPH oxidase; PTH, parathyroid hormone; PCR, polymerase chain reaction; TRAP, tartrate-resistant acid phosphatase.

Received for publication, February 7, 2000, and in revised form, November 17, 2000. Published, JBC Papers in Press, November 29, 2000, DOI 10.1074/jbc.M001004200
from each treatment were analyzed. The NBT staining intensity of clear giant cells. Twenty randomly selected NBT-stained osteoclasts were fixed in 10% formalin. Stained osteoclasts were identified as multinucleated. RPMI 1640 medium for 1 h at 37°C. After incubation, osteoclasts were removed nonadherent by vigorous washings. Additional contaminating cells were removed by a brief digestion (5–10 min) with lipase and collagenase (1 mg/ml). We generated 10,000–100,000 osteoclasts from 10 mice. This cell population was 90% pure osteoclasts. These osteoclasts stained positively with TRAP and were able to make resorptive pits on bovine bone slices (Fig. 8).

Anti sense Oligonucleotides—Anti sense oligonucleotides were designed near the ATG start codon of mouse native p91 (5'-CTTCATTGACCCGATTCTC-3') and Nox 4 (5'-AGCTCCTCCAGGACACCGC-3'). Anti sense and the corresponding sense oligonucleotides were synthesized as phosphorothioated oligonucleotides and purified by high performance liquid chromatography (Integrated DNA Technology Inc., Coralville, IA). All oligonucleotides were designed with the help of the Oligo program to minimize self-complementing and dimer formation.

Table I, osteoclasts from p91(-/-) mice were stained by TRAP and the number of osteoclasts. The transfection efficiency of 32P-labeled oligonucleotide was determined by exclusion of trypan blue from the cytoplasm. To demonstrate antisense oligonucleotide incorporation into osteoclasts, the 32P-labeled oligonucleotide was used. Briefly, mouse osteoclasts were cultured on chamber slides. After removing nonadherent cells, osteoclasts were incubated with 32P-labeled antisense oligonucleotide (50,000 cpm/ml, ~0.14 nM). A slide incubated with 0.14 nM unlabeled oligonucleotide in addition to 32P-labeled antisense oligonucleotide was the negative control. At the end of incubation, osteoclasts were fixed with 10% formalin and stained with TRAP to identify multinucleated osteoclasts. Thereafter, slides were air-dried and dipped in NTB2 liquid photographic emulsion. After exposure within a light-tight box at 4°C for 2–3 weeks, slides were then developed. The autoradiograms of osteoclasts incubated with the 32P-labeled oligonucleotide were examined under a microscope.

The transfection efficiency of 32P-labeled oligonucleotide was determined by the percentage of the 32P-labeled oligonucleotide incorporated into osteoclasts. After being incubated with 32P-labeled oligonucleotide (200,000 cpm/ml), osteoclasts were stained with TRAP and the number of osteoclasts in each dish was determined. The radioactivity in cell lysates and supernatant removed from these cells was measured using a scintillation counter. The transfection efficiency is expressed as a percentage of 32P-labeled oligonucleotide incorporated per 1 × 10⁶ osteoclasts.

Western Blot Analysis—Osteoclasts were cultured in 10-cm plastic culture dishes. After vigorously washing with 1× phosphate-buffered saline, nonosteoclastic cells were removed after a brief digestion (5–10 min) with lipase and collagenase (1 mg/ml). We generated 10,000–100,000 osteoclasts from 10 mice. This cell population was 90% pure osteoclasts. These osteoclasts stained positively with TRAP and were able to make resorptive pits on bovine bone slices (Fig. 8).

RESULTS

The Presence of an Alternative Enzyme Responsible for Superoxide Generation in p91(-/-) Mutants—As shown by Table I, osteoclasts from p91(-/-) mutants produced an amount of superoxide similar to that produced by osteoclasts from wild type mice. In addition, no significant difference in bone resorption was found by comparing cultures in p91(-/-) and C57/Bl6 mice. The hypothesis that an alternative oxidase is present in osteoclasts from the p91(-/-) mutant was supported by a Southern blot (Fig. 1). In this experiment, a 32P-labeled cDNA fragment of the mouse p91 gene, encoding a FAD binding region, was hybridized to genomic DNA digested by a series of restriction endonucleases. Multiple bands were observed when the membrane was washed under conditions of low stringency. Moreover, some bands, identified under conditions of low stringency, disappeared when high stringency was performed (lanes 1, 3, 4, 5). The disappearance of the bands
under high stringency conditions suggested the presence of a homologous, but nonidentical sequence(s) to the mouse p91.

A Complete Sequence of Nox 4—Using the mouse p91 sequence as a query to search the murine EST data base, a homologous sequence (accession no. AI746441) was found that had 46% identity with the C-terminal portion of mouse p91. A complete mRNA sequence (Nox 4, GenBank accession no. AF218723) was obtained by 5' RACE. The predicted amino acid sequence is shown in Fig. 2. Nox 4 has a predicted sequence containing 578 amino acids compared with 570 residues in murine p91. The two sequences have 58% similarity. Conserved regions containing FAD (13, 14) and a NADPH binding site (14, 15) are observed in Nox 4 (Fig. 2). Four histidine residues, which represent the conserved amino acid for a heme binding site (16), are also present in Nox 4.

Expression of Nox 4—The expression of Nox 4 in osteoclasts from both p91(−/−) and C57bl/6 mice was determined by reverse transcription-PCR. As shown in Fig. 3, Nox 4 was expressed in osteoclasts from both animals. Densitometric analysis showed that osteoclasts from p91 knockout mutants contained 2.5 times as much of Nox4 as osteoclasts from the wild type mice. The expression of Nox 4 in leukocytes was also observed in both p91(−/−) and C57bl/6 mice (data not shown). In addition to its expression in osteoclasts and leukocytes, Nox 4 mRNA was expressed in the mouse kidney, liver, and lung (Fig. 4). In contrast, p91 mRNA was highly expressed in the mouse spleen (data not shown), suggesting that Nox 4 may have functions that are different from those of p91.

Effect of Antisense Oligonucleotide on Osteoclasts—When cultured osteoclasts were incubated with antisense oligonucleotide for 2 days, no significant difference in osteoclast viability was observed. As shown in Fig. 5, the majority of cells consist of TRAP-positive osteoclasts (panel B) and uptake of 32P-labeled oligonucleotides is observed in osteoclasts (panel A). The efficiency of osteoclastic uptake of 32P-labeled oligonucleotide is ~10%. Expression of Nox 4 protein in osteoclasts was undetectable after incubation with Nox 4 antisense oligonucleotide, compared with the control (Fig. 6).
Effect of Nox 4 Antisense Oligonucleotides on Superoxide Generation and Bone Resorption—Osteoclastic superoxide generation was determined using an NBT assay. The addition of Nox 4 antisense oligonucleotide resulted in an 80% reduction of superoxide production for both C57Bl/6 and p91 \((\frac{2}{2})\) osteoclasts (Fig. 7). A similar inhibition of superoxide production was observed in earlier studies, using the superoxide scavenger desferal manganese complex (4). Moreover, calcitonin, an inhibitor of osteoclastic function, blocks over 90% of superoxide production.

The degree of inhibition of bone resorption by antisense oligonucleotides is shown in Tables II and III. The number of bone resorption pits was reduced to \(\frac{1}{2}\) when antisense oligonucleotides of Nox 4 and p91 were included in the incubation medium (Fig. 8). The average resorption area for C57Bl/6 osteoclasts was reduced by 47\% and 46\%, respectively, by Nox 4 antisense \((p<0.01)\) and p91 antisense \((p<0.01)\). It is important to note that a combination of both the p91 and Nox 4 antisense oligonucleotides reduced resorption by 61\% compared with the control and by an additional 27\% compared with the Nox 4 alone. Furthermore, incubation with the antisense oligonucleotides reduced the bone resorptive activity of p91 \((-/-)\) mutants (Table III). These data suggest that both oxidases are active in osteoclast from the wild type mice and contribute to bone resorption. In addition, Nox 4 remains active in the osteoclasts from p91 knockout animals.

Effect of DPI on Superoxide Generation and Bone Resorption—The p91 subunit is a FAD-containing flavoprotein that transfers electrons to oxygen, producing superoxide. A flav-
A New Superoxide-generating Oxidase

TABLE II
Effect of antisense oligonucleotide on C57Bl/6 osteoclastic bone resorption

Data were collected from two separate experiments with a total of eight bone slices. *, p < 0.01 vs. control; †, NS vs. control. AS, antisense strand; SS, sense strand; DMnC, superoxide scavenger, desferal manganese complex; CT, calcitonin.

|                  | Control | Nox 4 | p91 | Nox 4 + p91 | DMnC | CT |
|------------------|---------|-------|-----|-------------|------|----|
|                   | AS      | SS    | AS  | SS          | AS   | SS |
| Number of pits    | 137     | 63    | 123 | 77          | 125  | 53 |
| Total area resorbed (μm²) | 254,173 | 62,559 | 225,336 | 77,007 | 224,875 | 38,743 | 226,395 | 8109 | 7189 |
| Average resorbed area per pit (μm²) | 1855 ± 457 993* ± 308 1832 | 619 1001* ± 287 1799 | Μ ± 638 731* ± 204 1935† ± 366 901* ± 267 1027* ± 252 |

**FIG. 8. Bone resorption pits generated by osteoclasts.** Osteoclasts were isolated from p91(−/−) neonatal mouse pups and placed on the bone slices for 0.5–1 h. After removing nonadherent cells, 25 μM Nox 4 antisense oligonucleotides were added and incubated for 2 days. Resorption pits on bone slices were visualized by bright field reflected light microscopy after hematoxylin staining. Numerous resorption lacunae formed by osteoclasts are readily identifiable in a control slice (A). A few resorption lacunae are observed when bone slices were treated with antisense oligonucleotide (B).

Superoxide, generated as a consequence of binding to the flavoprotein covalently, thus interrupting the electron transferring process (17, 18). The sequence of Nox 4 reveals two conserved regions for FAD binding. To determine whether the Nox 4 complex comprises the activity of the FAD-containing region for the production of superoxide, DPI inhibition was performed. Table IV shows that DPI inhibited not only osteoclast superoxide generation, but also bone resorption in both the wild type and p91(−/−) mice. These data strongly suggest that a FAD-containing protein was responsible for the majority of the osteoclastic superoxide production in both wild type and p91(−/−) mice. The addition of PTH to the culture medium stimulated superoxide production, allowing for sustained and controlled bone resorption.

**DISCUSSION**

These studies demonstrate that Nox 4 is a p91-like protein that produces osteoclastic superoxide in p91(−/−) mice. The presence of this novel oxidase explains the ability of p91(−/−) osteoclasts to resorb bone normally. In wild type mice, both p91 and Nox 4 are expressed in osteoclasts. It is unclear why murine osteoclasts contain two enzymes that are involved in generating superoxide. Both enzymes may be active in the osteoclasts, due to the need for the production of large quantities of specifically targeted superoxide by the osteoclasts. Alternatively, the relatively low concentrations of Nox 4 in normal osteoclasts (Fig. 4) may suggest that Nox 4 is only expressed in large quantities when the NADPH oxidase is absent or nonfunctional (a 2.5-fold greater amount in p91(−/−) than in the wild type). It is also possible that the involvement of two enzyme systems is needed to effectively regulate osteoclastic superoxide production, allowing for sustained and controlled bone resorption.

Generation of superoxide is a common function of phagocytes and osteoclasts. Superoxide produced by phagocytes plays an important role in bacterial killing and in normal host protection (19). Osteoclasts generate superoxide that is necessary for bone resorption. Activated osteoclasts generate a ruffled-border space adjacent to bone which serves as the active site for bone resorption. Osteoclasts secrete hydrogen ions, proteinases, and superoxide into the ruffled border space to excavate a resorption pit or lacuna on the bone surface. Although an 80% decrease in superoxide production was observed when antisense oligonucleotides were used to inhibit the production of the oxidase, bone resorption declined by ~50%. Antisense oligonucleotides block superoxide generation, but acid production and protease secretion from osteoclasts are presumably not altered. Thus, osteoclasts continue to degrade bone, but the amount of bone resorption is decreased (2, 4). In previous studies (4), Ries et al. reported that pits obtained in the presence of a superoxide scavenger still contained dangling collagen fibrils, while pits formed by control osteoclasts, in the absence of the scavenger, had a smooth surface. In the study, osteoclasts in the presence of the scavenger formed only one pit. Osteoclasts without the scavenger moved to form multiple pits. Thus, bone resorption activity is not halted in the absence of superoxide, but only reduced. This explains why there is a correlation between reduced superoxide production and bone resorption in the absence of a one to one relationship.

Of great interest, patients with osteopetrosis have reduced, not absent bone resorption as evidenced by N-telopeptide excretion (6). The defects in superoxide generation described in patients with osteopetrosis are related to the mechanisms that regulate oxidase activity, such as the regulatory components of the oxidase (20) or the ability of cells to depolarize, triggering the activation of oxidases (21). Based upon current data, no explanation exists for the absence of reduced bone resorption in the p91 knockout animal or humans with chronic granulomatous disease. Perhaps it is possible to up-regulate Nox 4 (or its human counterpart) when the NADPH oxidase is defective, as suggested in the increased amount of Nox 4 mRNA present in p91 knockout animals (Fig. 3).

In addition to being expressed and active in osteoclasts, the Nox4-containing oxidase does not appear to be stimulated to have the “oxidative burst” phenomenon observed in phagocytes, but rather a steady level of superoxide production (data not shown). Kinetic experiments have suggested that the NADPH oxidase is stimulated in bursts, both in white cells (22) and in osteoclasts (23). Thus, in the wild type animals, the greater bone resorption may result from a burst of superoxide.
production after the initial exposure to PTH, a time when the bulk of calcium release occurs in the calvarial calcium release assay (24). Thus, by 48 h of incubation, a greater calcium release would be seen in the wild type cultures than in the p91\(^{+/−}\) osteoclasts, despite a similar, post-stimulation level of superoxide production. The difference in the kinetics of the two enzymes would explain the difference in the amount of calcium released, but does not detract from the fact that PTH stimulates superoxide production and bone resorption significantly in both sets of cultures. The fact that the superoxide and the bone resorptive activity were decreased in osteoclasts from each animal by DPI, suggested that the stimulation of bone resorption required superoxide production. Since the p91\(^{+/−}\) animals do not contain NADPH oxidase, the only conclusion to be drawn is that there was stimulation of bone resorption by the activity of the alternative oxidase. These observations are in keeping with the inhibition seen during the antisense overexpression, demonstrating that both the p91 and the Nox 4 contribute to osteoclastic superoxide production and bone resorption.

Not only does superoxide directly contribute to bone resorption by facilitating the degradation of bone matrix proteins (4), it is also involved in the activation and formation of osteoclasts (25). A number of reports have suggested that the types of physiological functions for the superoxide generated by different cell types are linked with cellular signaling and activation (26–28). Superoxide has been shown to play a central role in the activation of the transcription factor NF-κB in osteoclasts (30). NF-κB enhances the transcription of genes signaling osteoclast activation. Thus, besides their destructive properties, superoxide and its related free radicals may be involved in mediation of cellular differentiation and activation. Modification of cellular differentiation and activation might explain why many nonphagocytic cells such as lymphocytes, fibroblasts, and endothelial cells produce superoxide.

A group at Emory University (31) reported an alternative oxidase (MOX) in human (AF127763) and rat (AF152963) tissues. The murine Nox 4 protein, reported here, has 56% similarity to MOX found in humans and 54% to that of the rat. The existence of oxidases, other than NADPH oxidase, in different species and tissues suggests that there is a biological role for these oxidases, perhaps in generating the appropriate amount or the targeted amounts of superoxide. The link between osteoclastic superoxide production and bone resorption has been underscored in a variety of studies (1–4, 8, 25). The absence of defective bone resorption by osteoclasts derived from p91 knockout animals would suggest that NADPH oxidase is not necessary for normal bone resorption.

NADPH oxidase has been considered to be the oxidase responsible for all superoxide generation by osteoclasts (3, 7, 8). The data presented here demonstrate that at least one additional oxidase, Nox 4, contributes to superoxide production and bone resorption by both p91 knockout and wild type murine osteoclasts.

Note Added in Proof—The nomenclature for a variety of non-myeloid NADPH oxidases has developed rapidly over the past 2 years. In Trends. Biochem. Sci. (25, 459–461, 2000), Lambeth et al. established a standardized nomenclature for the NADPH oxidases. Lambeth et al. sites our gene sequence (GenBank™ accession number NM_015760, originally registered as AF218723 with the GenBank™ by Yang and Key in December, 1999) as the murine NOX4. Later, the identical gene sequence was registered by Geiszt et al. (GenBank™ AF261444) in April, 2000. The mouse NOX4 sequence was first published by Geiszt et al. in Proc. Natl. Acad. Sci. U. S. A. 97, 8010–8014, 2000 (manuscript first submitted, March, 2000) and is the subject of the current report (Yang et al., manuscript first submitted February, 2000). Additionally, Geiszt et al. described the homologous human NOX4 sequence (GenBank™ AF261943, registered on April, 2000) in their paper in the Proc. Natl. Acad. Sci. U. S. A. 97, 8010–8014, 2000. The same sequence for human NOX4 was registered by Cheng and Lambeth et al. with the GenBank™ (AF254621) on April, 2000 and reported by Lambeth et al. in Trends Biochem. Sci. 25, 459–461, 2000. Shiiose et al. published work describing the same human NOX4 sequence (GenBank™ AB041035, registered in March, 2000) in J. Biol. Chem. 276, 1417–1423, 2001 (manuscript first submitted August, 2000).

REFERENCES
1. Key, L. L., Ries, W. L., Taylor, R. G., Hays, B. D., and Pitzer, B. L. (1990) Bone 11, 115–119
2. Key, L. L., Wolf, W. C., Gundberg, C. M., and Ries, W. L. (1994) Bone 5, 431–436
3. Darden, A. G., Ries, W. L., Wolf, W. C., Rodriguez, R. M., and Key, L. L. (1996) J. Bone Miner. Res. 11, 671–675
4. Ries, W. L., Key, L. L., and Rodriguez, R. M. (1992) J. Bone Miner. Res 7, 931–939
5. Rodriguez, R. M., Key, L. L., and Ries, W. L. (1993) Pediatr. Res. 33, 382–389
6. Key, L. L., Rodriguez, R. M., Willi, S. M., Wright, N. M., Hatcher, H. C., Eyre, D. R., Cure, J. K., Griffin, P. P., and Ries, W. L. (1995) N. Engl. J. Med. 332, 1594–1599
7. Steinbeek, M. J., Appel, W. H., Verhoeven, A. J., and Karnovsky, M. J. (1994) J. Cell Biol. 126, 765–772
8. Yang, S., Ries, W. L., and Key, L. L. (1998) Calcif. Tissue Int. 63, 346–350
9. Kubo, H., Morgenstern, D., Quinlan, W. M., Ward, P. A., Dinauer, M. C., and Doerschuk, C. M. (1996) J. Clin. Invest. 97, 2680–2684
10. Meier, B., Jesaitis, A. J., Emmendorfer, A., Roessler, J., and Quin, M. T.

### TABLE III

| Treatment | Number of pits | Total area resorbed (μm²) | Average resorbed area per pit (μm²) |
|-----------|----------------|---------------------------|-----------------------------------|
| Control   | 111            | 151,204                   | 1362 ± 378                        |
| SS        | 95             | 122,510                   |                                    |
| Nox 4     | 59             | 43,354                    |                                    |
| AS        | 6              | 4922                      |                                    |

Data were collected from two separate experiments with total of eight bone slices. *, p < 0.01 vs. control; †, NS vs. control. SS, sense strand; AS, antisense strand; CT, calcitonin.

### TABLE IV

| Treatment | Superoxide production (OD) | Bone resorption (experimental/control) |
|-----------|-----------------------------|---------------------------------------|
| PTH (3 μg/ml) | 0.67 ± 0.02 | 1.33 ± 0.03 |
| (n = 10)      | (n = 18)      | (n = 8)      |
| DPI (1 μΜ)   | 0.11 ± 0.04* | 0.36 ± 0.04* |
| (n = 9)       | (n = 13)      | (n = 8)      |
| PTH + DPI    | 0.23 ± 0.07  | 0.71 ± 0.02  |
| (n = 10)      | (n = 18)      | (n = 8)      |

* Significant difference compared with control, p < 0.001.
A New Superoxide-generating Oxidase

(1993) Biochem. J. 289, 481–486

11. Takahashi, N., Udagawa, N., Akatsu, T., Tanaka, H., Shionome, M., and Suda, T. (1991) J. Bone Miner. Res. 6, 977–85
12. Madayastha, M., Yang, S., Ries, W., and Key, L. L. (2000) J. Interferon Cytokine Res. 20, 645–652
13. Sumimoto, H., Sakamoto, N., Nozaki, M., Sakaki, Y., Takeshige, K., and Minakami, S. (1992) Biochem. Biophys. Res. Commun. 186, 1368–1375
14. Segal, A. W., West, I., Wiencjes, F., Nügent, J. H., Chavan, A. J., Haley, B., Garcia, R. C., Rosen, H., and Scrace, G. (1992) Biochem. J. 284, 781–788
15. Rotrosen, D., Yeung, C. L., Leto, T. L., Malech, H. L., and Kwang, C. H. (1992) Science 256, 1459–1462
16. Yu, L., Quinn, M. T., Gross, A. R., and Dinuauer, M. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7993–7998
17. O'Donnell, V. B., Tew, D. G., Jones, O. T., and England, P. J. (1993) Biochem. J. 290, 41–47
18. Doussiere, J., and Vignais, P. V. (1992) Eur. J. Biochem. 208, 61–71
19. Jones, O. T. (1994) BioEssays 16, 919–923
20. Yang, S., Ries, W., and Key, L. (1999) Mol. Cell. Biochem. 199, 15–24
21. Beard, C. J., Key, L., Newburger, P. E., Ezekowitz, R. A, Arceci, R., Miller, B., Proto, P., Ryan, T., Anast, C., and Simons, E. R. (1996) J. Lab. Clin. Med. 108, 488–505
22. Chanock, S. J., Benna, J., Smith, R. M., and Babiior, B. M. (1994) J. Biol. Chem. 269, 24519–24522
23. Fallon, M., Silvertton, S., Smith, P., Moskal, T., Constantinescu, C., Feldman, R., Golub, E., and Shapiro, I. (1987) J. Bone Miner. Res., Suppl. I, 1
24. Reynolds, J. J., and Dingle, J. T. (1970) Calcif. Tissue Res. 4, 339–349
25. Garrett, I. R., Boyce, B. F., Orefield, R., Boenwald, L., Pseur, J., and Mundy, G. R. (1990) J. Clin. Invest. 85, 632–639
26. Brumell, J. H., Burkhardt, A. L., Bolan, J. B., and Grinstein, S. (1996) J. Biol. Chem. 271, 1455–1461
27. Fialkow, L., Chan, C. K., Grinstein, S., and Downey, G. P. (1993) J. Biol. Chem. 268, 17131–17137
28. Schreck, R., Albermann, K., and Baeuerle, P. A. (1992) Free Radical Res. Commun. 17, 221–237
29. Suzuki, Y. J., Forman, H. J., and Sevanian, A. (1997) Free Radical Biol. Med. 22, 269–285
30. Hall, T. J., Schaeublin, M., Jeker, H., Fuller, K., and Chambers, T. J. (1995) Biochem. Biophys. Res. Commun. 207, 280–284
31. Suh, Y. A., Arnold, R. S., Lassegue, B., Shi, J., Xu, X., Sorescu, D., Chung, A. B., Griendling, K. K., and Lambeth, J. D. (1999) Nature 401, 79–82
