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The role of nitric oxide in the mechanical repression of RANKL in bone stromal cells

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Abstract

Both mechanical loading and nitric oxide (NO) have positive influences on bone mass. NO production is induced by mechanical strain via upregulation of eNOS mRNA and protein, the predominant NOS in adult bone. At the same time, strain causes decreased expression of RANKL, a factor critical for osteoclastogenesis. In this study, we harvested primary stromal cells from wild-type (WT) and eNOS^(-/-) mice to test whether induction of NO by mechanical strain was necessary for transducing mechanical inhibition of RANKL. We found that strain inhibition of RANKL expression was prevented by NOS inhibitors (L-NAME and L-NMMA) in WT stromal cells. Surprisingly, stromal cells from eNOS^(-/-) mice showed significant mechanical repression of RANKL expression (p<0.05). Mechanical strain still increased NO production in the absence of eNOS, and was abolished by SMTC, a specific nNOS inhibitor. nNOS mRNA and protein expression were increased by strain in eNOS^(-/-) but not in WT cells, revealing that nNOS was mechanically sensitive. When NO synthesis was blocked with either SMTC or siRNA targeting nNOS in eNOS^(-/-) cells however, strain still was able to suppress RANKL expression by 34%. This indicated that strain suppression of RANKL can also occur through non-NO dependent pathways. While our results confirm the importance of NO in the mechanical control of skeletal remodeling, they also suggest alternative signaling pathways by which mechanical force can produce anti-catabolic effects on the skeleton.

Keywords

nNOS; eNOS; mechanical strain; RANKL; bone remodeling

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Introduction

Mechanical loading influences skeletal structure, directing remodeling through alterations in osteoblast and osteoclast functions. Many laboratories have shown that loading *in vitro* and *in vivo* leads to positive skeletal balance [1–3]. Anti-catabolic effects of loading involve inhibition of the expression of Receptor for Activated NF- κ B Ligand (RANKL) [4–6] which decreases the potential for osteoclast formation [7,8]. Mechanical load also increases nitric oxide generation in bone cells: mechanical strain induces eNOS [9] and fluid shear both activates and induces this NOS isoform [10]. Nitric oxide has potent effects on osteoclasts in bone, both decreasing their formation through repression of RANKL [5,11] as well as through decreasing resorptive activity [12]. In an effort to understand mechanical effects on the skeleton, it is important to clarify whether nitric oxide is required for the anti-catabolic effects of mechanical loading.

Both endothelial (eNOS) and neuronal (nNOS) NOS isoforms are found in bone cells, but eNOS is thought to be the predominant isoform in adult bone [13,14]. nNOS expression is upregulated in fetal bone development and during fracture repair [15,16]. In contrast, inducible NOS (iNOS), which generates a comparatively large burst of NO in response to inflammatory mediators [17], may enhance bone resorption, but is not highly expressed in bone cells [13, 15]. With the dominance of the eNOS isoform in bone, it is not surprising that eNOS deficient transgenic mice (eNOS^(-/-)) have significant abnormalities in bone formation [18–20] as well as increased blood pressure consistent with the role of nitric oxide in vasodilation [21–23]. Reductions in bone volume and bone formation rates in young eNOS^(-/-) mice are related to osteoblast dysfunction: fewer osteoblasts are seen in trabecular bone and have a decreased mineralizing capacity [19]. The detrimental effect of decreased nitric oxide on anabolic processes was not unexpected since NO promotes proliferation, differentiation and activity of osteoblasts *in vitro* [24]. Finally however, despite the delay in bone maturation with respect to bone mineral density the eNOS^(-/-) animal achieves a skeleton at least equivalent to its wild-type counterpart by 12 weeks [18,25]. Thus, while nitric oxide is clearly implicated in skeletal modeling, a deficiency in eNOS can be compensated through unclear adaptive mechanisms, possibly by compensatory increases in other NO synthases [26–28].

The skeletal abnormalities in the eNOS deficient animal confirm the importance of nitric oxide for normal modeling and remodeling. It is possible as well that nitric oxide may mediate many of the downstream effects of mechanical loading generated by strain and shear forces. As mentioned, both strain [29,30] and shear [31] induce NO production in endothelial and muscle tissue. The source of mechanically induced NO in bone cells has been traced to eNOS [9,10, 14] and pharmacological inhibition of NOS by L-NAME *in vivo* abolishes the strain-induced increase in NO production [32]. However, while mechanical strain and NO are linked, a direct connection between the strain-induced increase in nitric oxide and the decrease in RANKL has not been corroborated. In this study we investigate a requirement for NO signaling in mechanical strain regulation of RANKL expression utilizing bone stromal cells from wild type and eNOS^(-/-) animals.

Materials and Methods

Reagents

Fetal Bovine Serum (FBS) was obtained from Hyclone (Logan, UT). α -MEM, Opti-MEM, reverse Transcriptase and Taq Polymerase were purchased from Invitrogen (Carlsbad, CA). The RNeasy Mini kit and DNase I were purchased from QIAGEN (Valencia, CA). RANKL, nNOS and 18S primers were synthesized by Invitrogen. SMTC (S-methyl-L-thiocitrulline), L-NAME (N^G-Nitro-L-arginine methyl ester hydrochloride), L-NMMA (N^G-monomethyl-L-arginine) and D-NAME (N^G-Nitro-D-arginine methyl ester hydrochloride) were from Sigma

(St. Louis, MO). iNOS inhibitor 1400W (N-(3-(Aminomethyl)benzyl)-acetamidine) was purchased from A.G. Scientific, Inc. (San Diego, CA).

Cell Culture

Bone marrow cells were collected from tibiae and femurs of 4–6 week old male wild type (C57BL/6) or eNOS null mice (C57BL/6 background; Jackson Lab, ME). Bone marrow cells were plated in α -MEM/10% FBS at a density of 15 million cells per well on 6-well plates then transferred to Bioflex Collagen I-coated plates (FlexCell, Hillsborough, NC). The next day non-adherent cells were removed as previously described [33]. The work was performed with the approval of the Institutional Care and Use of Animal Committee at Atlanta VA medical center.

Strain application

After 5 days of culture, stromal cells were treated with 10nM 1,25(OH)₂D₃ as indicated for 2 days and strained during the last 24 hrs using a BioFlex strain unit (FlexCell). Strain was applied uniformly and biaxially at 2% elongation, 10 cycles/min. Control plates (unstrained) were kept in the same incubator.

Nitric oxide measurement

Primary stromal cells were pre-treated for 1 hour with or without 100 μ M 1400W, a highly selective iNOS inhibitor, or 10 μ M SMTC, a nNOS inhibitor, before applying strain for the final 24 hours of culture. For NO measurement, a fluorometric assay was used to read nitrite in samples and standards (NaNO₂, 0–10 μ M) as previously described [34]. Briefly, 100 μ l of standards and samples were added to Microtiter 96-well plates (DYNEX Technologies, Chantilly, VA) and mixed with 10 μ l fresh 2,3-diaminonaphthalene (DAN) (prepared in 0.62 M HCl) for 10 minutes at room temperature. The reactions were terminated with 5 μ l of 2.8 N NaOH. Formation of the 2,3-diaminonaphthotriazole was measured using LB 50 Plate Reader (Perkin-Elmer, Boston MA) with excitation at 360 nm and emission at 440 nm. All standards and samples were measured in triplicate.

RNA Isolation and Real-Time RT-PCR

Total RNA was extracted using the RNeasy Mini kit and treated with DNase I to remove contaminating genomic DNA. Reverse transcription was performed with 1 μ g of total RNA in a total volume of 20 μ l per reaction. Real-time PCR was performed using the Bio-Rad iCycler (Hercules, CA). Amplification reactions were performed in 25 μ l containing primers at 0.5 μ M (0.3 μ M for nNOS) and dNTPs (0.2 mM each) in PCR buffer and 0.03 U Taq polymerase along with SYBR-green (Molecular Probes, Eugene, OR) at 1:150,000. Aliquots of cDNA were diluted 10–10000 fold for 18S, RANKL and 5–625 folds for nNOS to generate relative standard curves to which sample cDNA was compared. Standards and samples were run in triplicate. RANKL and 18S primers were as used previously [9,35]. For nNOS, forward and reverse primers were 5'-GGG CAA ACA GTC TCC TAC CA-3' and 5'-AGG GTG TCA GTG AGG ACC AC-3' respectively, creating an amplicon of 99 bp from Exon #3 (GenBank: NM 008712 nt 947-1046). PCR products from all species were normalized for amount of 18S in the same RT sample, which was also standardized on a dilution curve from the RT sample.

Western blots for NOS isoforms

Cells were rinsed with cold PBS twice and resuspended in boiling lysis buffer (1% SDS, 10mM sodium orthovanadate, 10mM Tris pH 7.4). For western blots, 80–150 μ g of total protein was loaded on 7.5% SDS-polyacrylamide gels for electrophoresis and transferred to PVDF membranes. The blots were incubated with anti-NOS isoform antibodies (Transduction Labs, San Diego CA) as the primary antibody overnight. HRP-conjugated second antibody was added

prior to ECL plus substrate (Amersham Bioscience, Piscataway, NJ). The same blot was stripped and re-probed with pan-ERK42/44 or actin (Santa Cruz biotechnology, Santa Cruz, CA) as protein loading control.

SiRNA transfection

In the last 48 hrs of culture, bone stromal cells were transfected with nNOS siRNA, a mixture of two nNOS siRNA (si-nNOS) at 60 nM (Ambion, Austin, TX) or the same concentration of negative control siRNA (Ambion) using 5 μ l of oligofectamine (Invitrogen, Carlsbad, CA). Transfected cells were strained during the final 24 hrs prior to analysis.

Statistics

All values in the figures are expressed as mean \pm SEM. The results were analyzed by Prism (GraphPad, San Diego, CA) using Tukey one way-ANOVA or Student T-tests.

Results

Strain repression of RANKL requires nitric oxide generation in WT stromal cells

We have previously shown that prolonged mechanical strain increases nitric oxide generation and eNOS mRNA expression while inhibiting RANKL expression in stromal cells harvested from C57BL/6 WT mice [9]. Since nitric oxide donors potentially inhibit RANKL expression [11] strain induced nitric oxide production likely contributes to mechanorepression of this gene. As shown in Figure 1A, stromal cells were pretreated with the NOS inhibitor, L-NMMA, which blocks all three NOS isoforms. L-NMMA at both 100 and 200 μ M prevented mechanorepression (2% elongation, 10 cycles/min, 18 h) of RANKL. We next examined whether the highly selective eNOS inhibitor, L-NAME, could prevent strain suppressed RANKL expression. Stromal cultures were treated with 500 μ M of either L-NAME or the inactive enantiomer D-NAME and subjected to overnight strain. L-NAME blocked mechanical repression of RANKL without causing any change in basal levels of expression (Figure 1B). The data indicated that eNOS generated NO was important for strain effects on RANKL expression.

Strain decreases RANKL mRNA expression in stromal cells from eNOS^(-/-) stromal cells

To further confirm the role of eNOS in strain-induced nitric oxide's relevance to mechanorepression of RANKL, we utilized stromal cells from the eNOS^(-/-) deficient mouse, which has delayed but ultimately adapted skeletal remodeling [25]. First, we examined RANKL expression in stromal cells from eNOS^(-/-) mice. As shown in Figure 2A, 1,25 (OH)₂D₃ induced RANKL expression was largely equivalent in primary stromal cells in both genotypes. In the absence of vitamin D, we were not able to measure differences in strain's significant inhibition of basal RANKL expression comparing between WT and eNOS^(-/-) cells: RANKL in strained cells was reduced to 46.3 \pm 4.1% and 48.3 \pm 7.7% that of unstrained cells in the respective genotypes. Osteoprotegerin (OPG), a decoy receptor for RANKL, showed no significant change in either the eNOS^(-/-) or WT stromal cell cultures measured 18 hours after initiating strain (data not shown).

Surprisingly, mechanical strain continued to cause significant decreases in RANKL mRNA levels in primary stromal cells from mice lacking eNOS: RANKL in eNOS^(-/-) cells was suppressed to 63 \pm 3.8% the level in unstrained cells, i.e., a decrease of 37% (Figure 2B). The degree of strain inhibition in eNOS^(-/-) stromal cells was somewhat less than that caused by mechanical strain in WT mice with RANKL suppressed to 52.7 \pm 4.3% that of unstrained cells, i.e., a decrease of 47% ($p < 0.05$). This suggested that either mechanical control of RANKL

can occur in the absence of NO generation or that there can be another mechanically sensitive source of nitric oxide in bone stromal cells.

Mechanical strain increases NO production via nNOS in eNOS^(-/-) stromal cells

Results showing that L-NAME inhibited strain effects on RANKL in WT cells were therefore challenged by our data showing a persistence of strain induced RANKL repression in eNOS^(-/-) cells, where absence of this dominant nitric oxide synthase isoform should interfere with strain induced nitric oxide generation. To evaluate this, nitric oxide was measured after 24 hours of strain application and normalized to cells/dish. The cell numbers were not significantly different between genotypes (data not shown). In a series of 7 compiled experiments, the baseline concentrations of NO from WT cells was $1.97 \pm 0.3 \mu\text{M}/\text{well}$ while in the eNOS^(-/-) stromal cells the level averaged $2.05 \pm 0.3 \mu\text{M}/\text{well}$, a non-significant difference. As shown in Figure 3, strain was able to significantly increase NO production from eNOS^(-/-) stromal cells, although levels were lower than those generated in WT cell cultures (increases of $281 \pm 21\%$ and $345 \pm 28\%$ respectively, $p=0.03$).

To identify the source of NO generated by strain in eNOS^(-/-) stromal cell cultures, inhibitors of iNOS and nNOS were used. 1400W, a highly selective inhibitor of iNOS [36], did not inhibit strain-induced NO production in either genotype (Figure 4A, B). This indicated that strain induced NO in eNOS^(-/-) stromal cells was not due to contaminating macrophages, where iNOS is a prevalent isoform. SMTC, a potent inhibitor of nNOS at low concentrations ($EC_{50} = 0.31 \mu\text{M}$), also inhibits eNOS ($EC_{50} = 5.4 \mu\text{M}$) and iNOS ($EC_{50} = 34 \mu\text{M}$) at higher concentrations [37]. We found that $10 \mu\text{M}$ SMTC impaired mechanically induced NO production in WT stromal cells ($320 \pm 30\%$ for no treatment and $230 \pm 26\%$ with SMTC). In eNOS^(-/-) cells, SMTC completely blocked the mechanical stimulation of NO production (Figure 4B). The partial inhibition of NO production seen in WT cells, also shown in Figure 4A, may be due to both a partial inhibitory effect of SMTC on eNOS as well as a contribution of nNOS.

We also used an siRNA approach where siRNA targeting nNOS (si-nNOS) decreased nNOS expression by 50% in eNOS^(-/-) cells (see figure 6B). In Figure 4C, si-nNOS prevented a significant mechanical induction of NO in eNOS^(-/-) cells. These data show that strain can induce NO generation via the neuronal isoform of nitric oxide synthase.

Mechanical strain induces nNOS in eNOS^(-/-) stromal cells

To investigate the mechanism whereby mechanical strain increased NO in eNOS^(-/-) cells, we considered up-regulation of nNOS gene expression, similar to the upregulation of eNOS mRNA and protein in WT primary cells [9]. nNOS mRNA levels were measured by real-time RT-PCR. In eNOS^(-/-) stromal cells, nNOS mRNA responded to strain with a significant increase to levels 40% above those measured in unstrained (control) cultures ($p<0.05$) (Figure 5A). In WT stromal cells, strain caused a slight decrease in nNOS expression ($100 \pm 15\%$ for control and $66 \pm 4\%$ with strain), shown on the left of the figure.

Western blot analysis was used to confirm the response of nNOS mRNA to mechanical strain. Despite absence of eNOS in the eNOS^(-/-) stromal cells, nNOS protein was not differentially expressed between genotypes (Figure 5B). iNOS protein was undetectable, again indicating that macrophages were not a major contaminant in these cultures (data not shown). After strain application, nNOS levels increased in the eNOS^(-/-) stromal cells but slightly decreased in the WT stromal cells (Figure 5C), paralleling the mRNA results of 5A. Thus, mechanical induction of nitric oxide in eNOS^(-/-) stromal cells occurs through increased nNOS expression.

Strain repression of RANKL in eNOS^(-/-) stromal cells is not dependent on nitric oxide

We expected that blocking NO generation by nNOS in eNOS^(-/-) stromal cells should thus prevent mechanorepression of RANKL. We utilized the nNOS specific inhibitor, SMTC, to this end. As shown in Figure 6A, SMTC did not interfere with strain downregulation of RANKL expression. In fact, the inhibition of RANKL in the presence and absence of the nNOS inhibitor SMTC was equivalent ($66.1\% \pm 8.5\%$ and $69.4\% \pm 6.6\%$ respectively). As this did not concur with the findings in figure 1, where blockade of NO production from eNOS in WT cells prevented strain's effect on RANKL, we also used a second approach with siRNA to knock down nNOS in eNOS^(-/-) stromal cells. siRNA targeting nNOS decreased nNOS mRNA by 50% (Figure 6B), an effect consistent with near complete inhibition of mechanically induced NO production in the eNOS deficient cells as was demonstrated in Figure 4C. Western analysis confirmed that nNOS protein level decreased by at least half when nNOS mRNA expression was knocked down by nNOS siRNA (Figure 6C). Suppression of nNOS by nNOS siRNA in unstrained cells caused an unexpected decrease in the vitamin-D stimulated RANKL of nearly 40%, shown in the gray bars of Figure 6D, perhaps suggesting a tonic inhibition of RANKL by osteoblast nNOS. In confirmation of the finding that SMTC did not prevent strain suppression of RANKL, in the presence of nNOS knockdown, RANKL was still significantly decreased by strain (>50% reduction, $p < 0.05$ for both conditions, figure 6D).

Discussion

Nitric oxide may have particular importance in translating biophysical effects from exercise into adaptive responses in bone tissue, as it does in other mechanically responsive tissues. Skeletal loading promotes an anti-catabolic state where osteoclastic bone resorption is prevented [38]; this requires suppression of RANKL signaling by bone cells. Mechanistically, strain produces this catabolic state by decreasing RANKL mRNA expression in bone cells [5,39], while concurrently causing the generation of nitric oxide [9]. This is also true for loading effects due to shear, where fluid flow generates nitric oxide [10,40], and also decreases RANKL [6]. Importantly, nitric oxide has a potent effect, when dosed in culture, to inhibit osteoclast formation by downregulation of RANKL signaling [11]. Indeed, in the work presented here we have shown that nitric oxide was necessary for mechanical strain suppression of RANKL; when nitric oxide synthase was inhibited in WT stromal cultures, RANKL levels were insensitive to strain regulation.

Both eNOS and nNOS have been detected in bone cells [16,41] but eNOS predominates after the fetal period [14,15]. In the *presence* of eNOS, as in the studies of van't Hof, et al [42], nNOS deficient animals have increased bone largely due to decreased osteoclasts and bone turnover through unknown mechanisms. In the *absence* of eNOS, mice have a unique phenotype involving multiple organ systems. With regard to the skeleton, eNOS^(-/-) animals have delayed skeletal maturation [18–20], but finally achieve an overshoot in bone density [25]. We here have shown that nNOS is sensitive to strain up-regulation and NO generation in osteoprogenitor cells – essentially “taking over” for eNOS as the mechanically regulated NO synthase to preserve normal skeletal homeostasis. Finally, in mice deficient in the inflammatory NOS isoform, iNOS, although no bone abnormalities have been described under normal conditions, iNOS deficient mice are partially protected from ovariectomy [43] and inflammatory [44] induced bone loss. Taken together, this evidence reminds us that in the normal, NO synthase replete bone cell, nitric oxide regulation of skeletal remodeling is complex.

Mechanical inhibition of RANKL was only modestly blunted in murine stromal cells lacking a functional eNOS gene. Indeed, strain was able to cause nitric oxide generation in eNOS^(-/-) cultures, indicating another NOS isoform with the ability to respond to mechanical input. Although our primary stromal cultures inevitably contained some macrophages, the low

level of iNOS message suggested macrophage contamination was very low. Pharmacologic inhibition of nNOS, but not iNOS, abolished strain induced NO production in eNOS^(-/-) cells. Mechanical regulation of nNOS has been noted in muscle [45] and, studies have shown that in the vascular system of animals deficient in eNOS, nNOS serves to initiate flow-induced coronary dilatation [26,28]. The ability of nNOS to compensate and ultimately adapt for a lack of eNOS in response to mechanical input suggests that NO is an important, if not critical, determinant of cellular function with the mechanical environment. Furthermore, although, Kim et al [6] have reported that oscillatory fluid flow transiently increased OPG mRNA expression when cells were exposed to fluid flow, in our experiments, neither eNOS replete nor deficient cells responded to prolonged strain application with changes in OPG.

While in the eNOS sufficient animal, strain required NO generation through eNOS to inhibit RANKL, in eNOS deficient cells where NO generation was blocked, strain repression of RANKL expression persisted. We were not entirely surprised by this finding, since we have been able to show RANKL downregulation in an immortalized osteoblast line which does not express much eNOS or nNOS, and does not respond to strain with NO generation [5]. In the eNOS replete situation, in contrast, part of mechanical strain's effect is to increase eNOS expression, a process that requires earlier activation of ERK1/2 [9]. Strain regulation of cell phenotype thus likely evolves from early processes initiated through ERK1/2 which involve secondary effects due to nitric oxide generation. Adaptation that allows a continued mechanical response in the absence of eNOS occurs partially through upregulation of nNOS, and possibly through other indirect effects of MAPK activation.

The studies performed here have limitations. While the mechanical environment of bone marrow stromal cells is not defined, only one regimen has been tested. Although it is not unlikely that altering frequency, cycle number or magnitude of the mechanical input would result in different results, it is clear that a low magnitude strain applied at a frequency achievable in vivo does cause a response that is similar to other mechanical responses attained with higher frequencies or with, for instance, shear [6]. As well, studies using material from knock-out animals, or even wild-type animals of different genetic background, will have biological variation in response due to the need to adapt.

In summary, despite an important role for nitric oxide in transducing mechanical into cellular effects, bone cells can find a way to respond to load in its absence. This adaptability confirms that biophysical factors activate a redundant set of intracellular signaling pathways to convey mechanical information to bone cells. Appreciation of these alternate signaling pathways will require further investigation.

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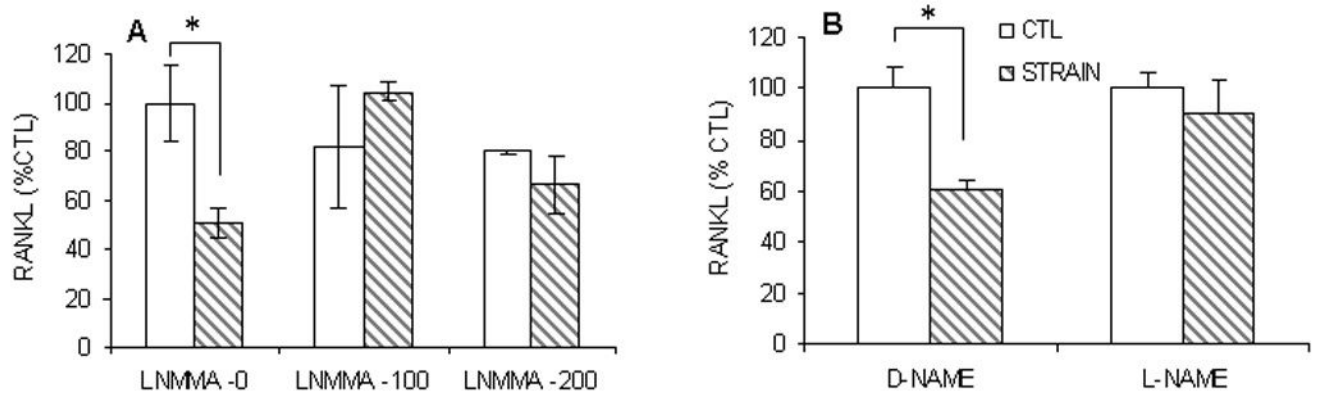


Figure 1. eNOS inhibitors prevent strain inhibition of RANKL expression in wild type stromal cells

A. Primary bone stromal cells from WT mice were treated with 10nM $1,25(\text{OH})_2\text{D}_3$ for 48 h and with the non-specific NOS inhibitor L-NMMA at either 100 or 200 μM as specified. Both doses of L-NMMA prevented mechanorepression of RANKL. Experiments were repeated more than once with similar results. (“*” shows significant effect of strain, $p < 0.01$). **B.** Wild-type stromal cells were treated similarly as in (A), but with eNOS specific inhibitor, L-NAME during the second 24 h prior to application of strain. L-NAME, but not D-NAME (both at 500 μM) prevented the mechanical inhibition of RANKL mRNA (“*” = $p < 0.01$). RANKL, corrected for 18S RNA was analyzed by RT and real time-PCR. The data are expressed as a percentage of RANKL mRNA measured in unstrained cells.

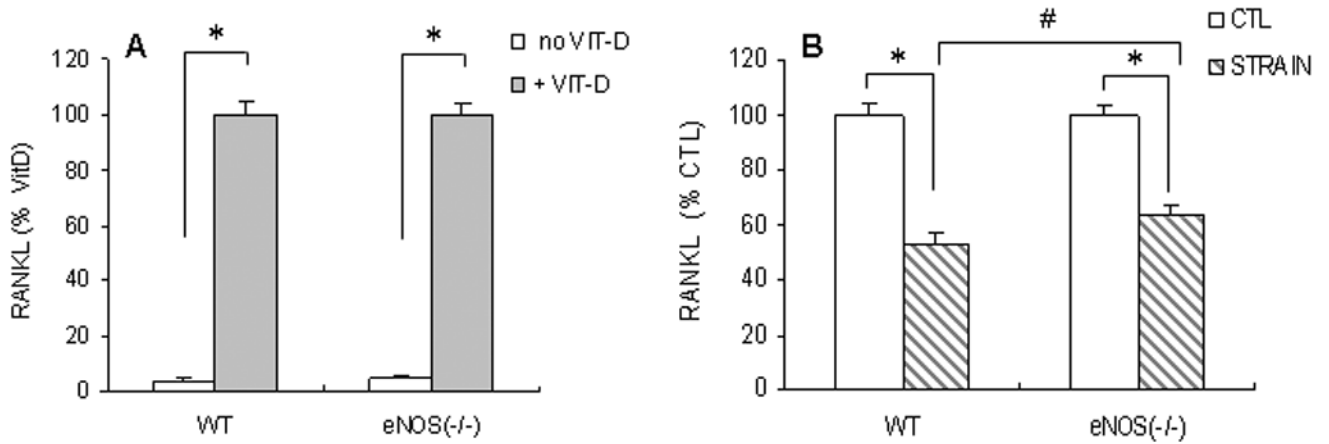


Figure 2. Mechanical strain inhibition of RANKL persists in eNOS^(-/-) stromal cells

A. 1,25(OH)₂D₃ significantly enhanced the RANKL expression in both WT and eNOS^(-/-) stromal cells. Both genotypes had a ~40-fold increase in RANKL expression. **B.** Primary bone stromal cells from WT and eNOS^(-/-) mice were treated with 10nM 1,25(OH)₂D₃ for 48 hours and strained for the last 24 hours of culture prior to analysis of RANKL mRNA (normalized for 18S). The unstrained condition is represented as 100% within each genotype. Strain application decreased RANKL levels to 52.7 ± 4.3 % and 63 ± 3.8 % of unstrained wild-type and eNOS^(-/-) cells respectively. “*” denotes significant decreases of RANKL mRNA levels with strain in WT and eNOS^(-/-) stromal cells compared to unstrained control cells within genotype, and “#” denotes significant difference between the strain effect on WT cells and eNOS^(-/-) cells (p < 0.05). Data were compiled from 5 experiments for both A and B.

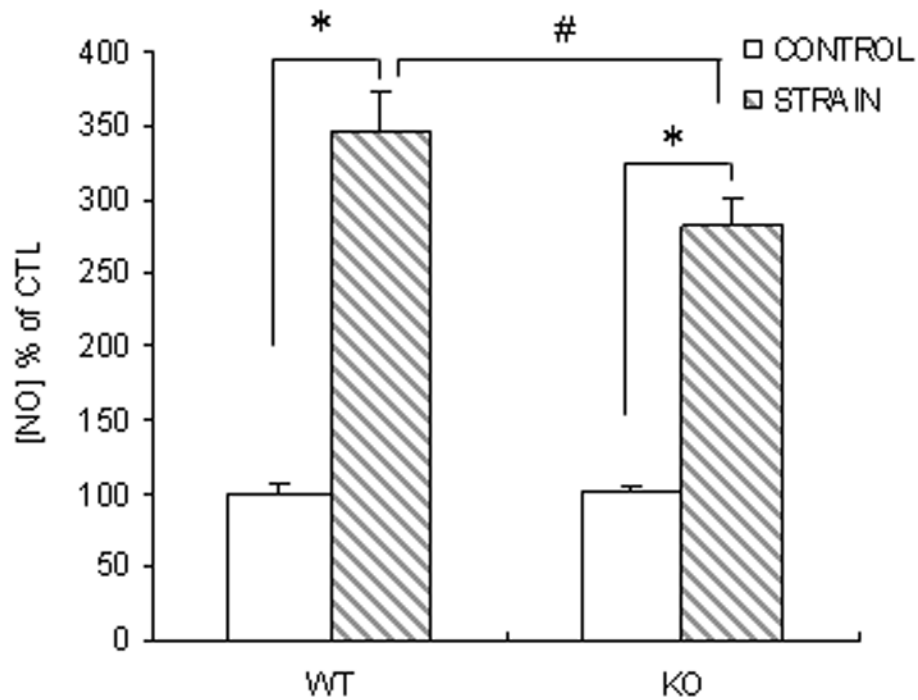


Figure 3. Strain increases NO production in eNOS^(-/-) stromal cells

Nitric oxide production, normalized to cell number, was significantly increased in both cell genotypes after 24 hours of strain. Strain-induced NO production in eNOS^(-/-) cells was lower than in WT cells ($p < 0.05$). “*” represents significant difference between control and strained cells in each group, and “#” denotes significant difference between the level of strain induced NO in WT cells and eNOS^(-/-) cells. Baseline NO production measured in the DAN assay was not different: WT = $1.97 \pm 0.3 \mu\text{M}/\text{well}$, eNOS^(-/-) = $2.05 \pm 0.3 \mu\text{M}/\text{well}$. Data were compiled from 5–7 separate experiments.

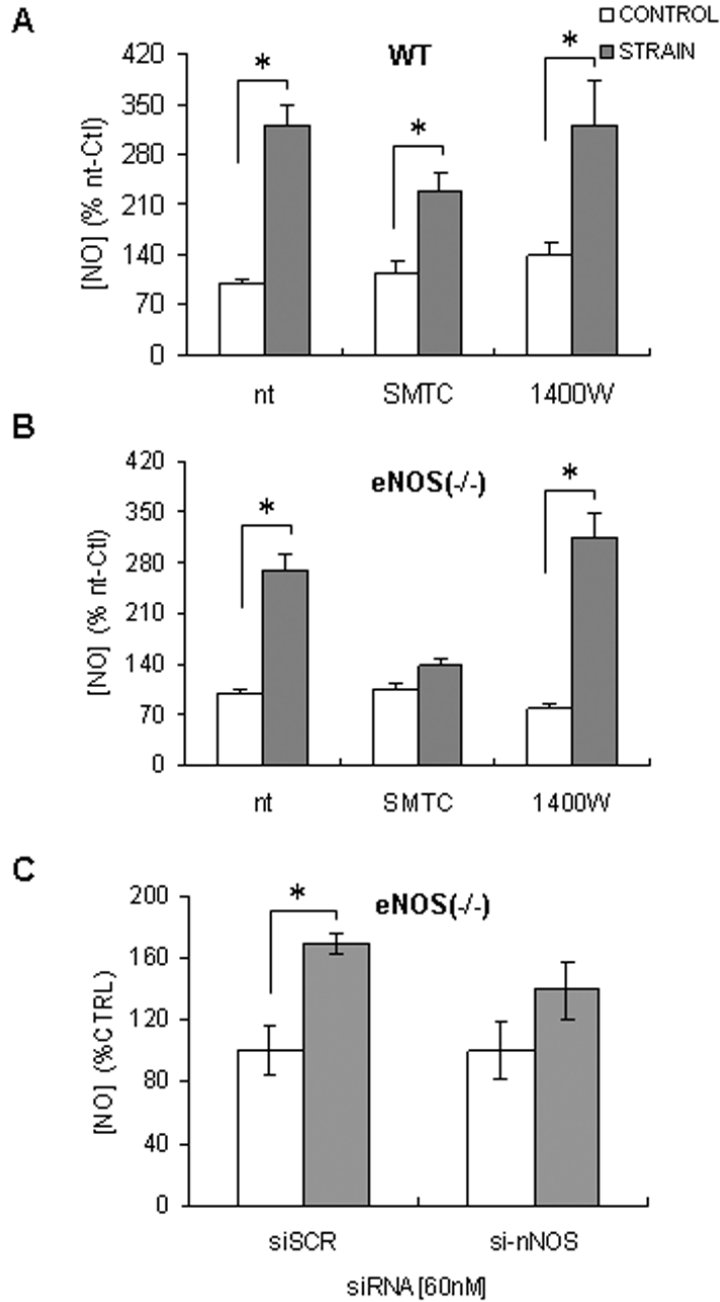


Figure 4. Preventing nNOS activity prevents strain-induced NO production in eNOS^(-/-) stromal cells

A. WT stromal cells were treated with 1400W (100μM) or SMTC (10μM) 1 hour prior to overnight strain. 1400W did not affect strain induced NO, but SMTC partially inhibited the effect. **B.** SMTC entirely blocked strain induced NO production in eNOS^(-/-) cultures. **C.** siRNA targeting nNOS (si-nNOS) prevented mechanically induced increases in NO in eNOS^(-/-) cultures. Data were compiled from 3–6 separate experiments for all figures. * represents significant differences between unstrained control cells and strained cells (p<0.05).

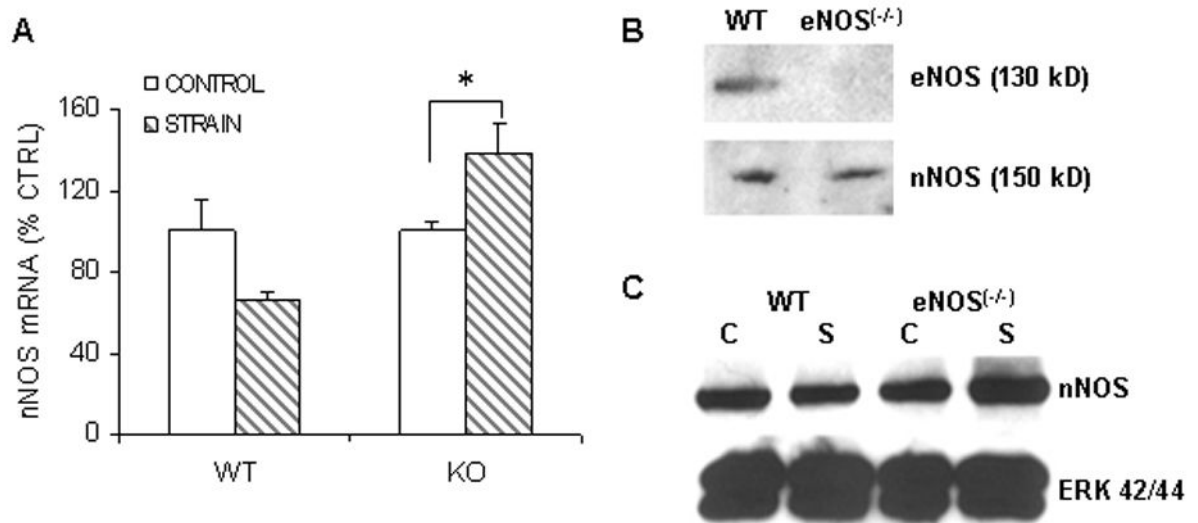


Figure 5. nNOS expression is up-regulated by mechanical strain in eNOS^(-/-) stromal cells
 Stromal cells from both genotypes of mice were cultured for 7 days prior to application of strain during the last 24 hrs. Total RNA was extracted for RT-PCR and total protein was isolated for western analysis. **A.** Real-time PCR showed that strain significantly increased nNOS gene expression in eNOS^(-/-) (KO) cells (normalized to 18S RNA expression). Data were compiled from 3–5 independent experiments. The asterisk reflects a significant difference between control and strained cultures ($p < 0.05$). **B.** Western blotting showed eNOS and nNOS protein expressions in WT and eNOS^(-/-) stromal cells. iNOS was not visible after probing with specific antibody (data not shown). **C.** nNOS protein increased in cell cultures after 24 hours of strain. ERK shows equal loading of protein. The experiment was repeated twice with similar results.

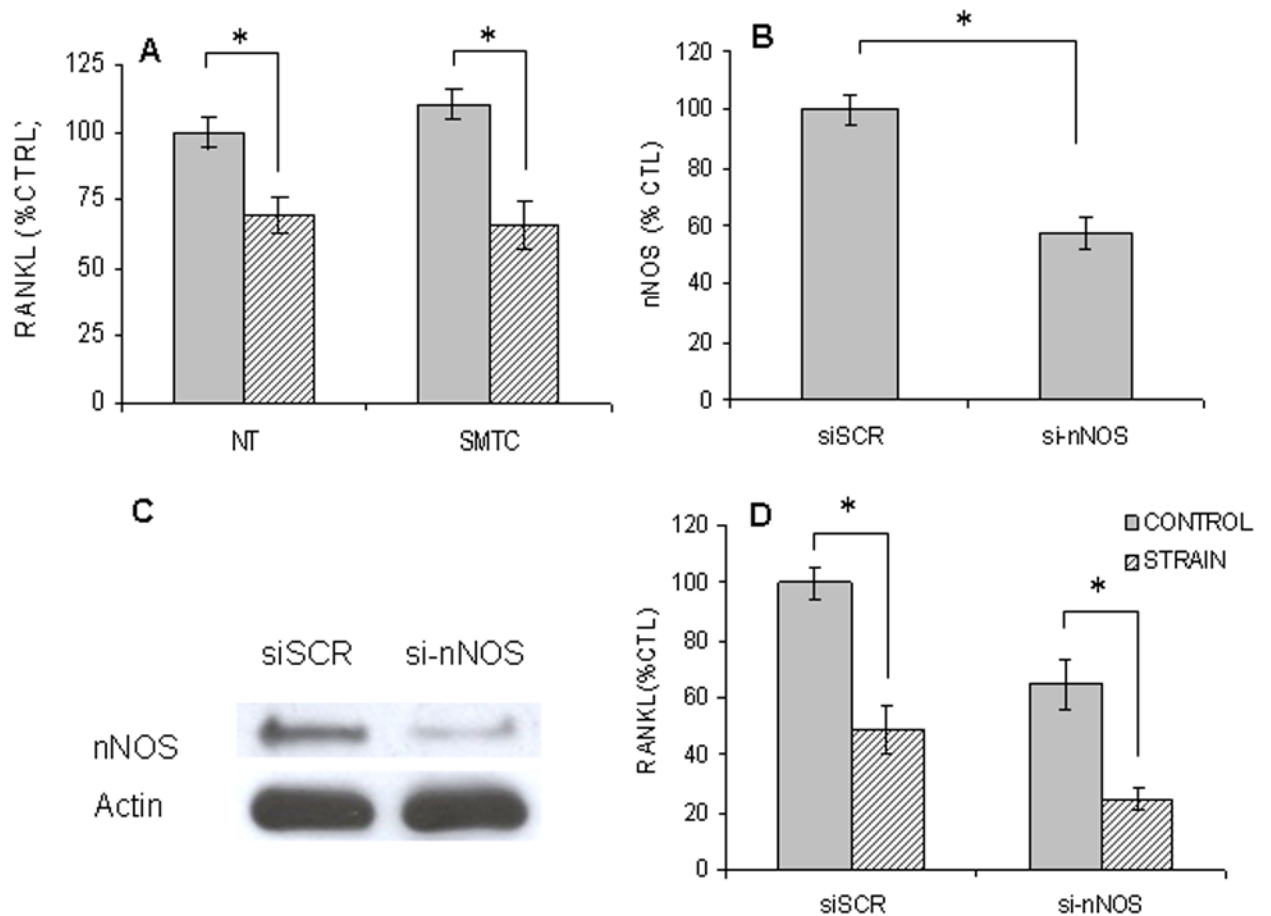


Figure 6. Strain-induced inhibition of RANKL expression in $eNOS^{-/-}$ cells is measurable when nNOS activity is blocked

A. Stromal cells from $eNOS^{-/-}$ mice were treated as in Figure 3A. Total RNA was isolated for RT-PCR. SMTC-treated $eNOS^{-/-}$ cells sustained the ability to respond to mechanical inhibition of RANKL expression. Data were compiled from 7 independent experiments; the asterisk shows difference from control, $p < 0.05$. **B.** si-nNOS decreased nNOS mRNA expression compared to the negative control siRNA (siSCR). These cultures did not generate significantly increased NO in response to mechanical strain, see Fig 4C. **C.** nNOS siRNA reduced nNOS protein expression compared to the negative control siRNA (siSCR) in murine stromal cells. **D.** si-nNOS caused a significant reduction in basal RANKL expression to $64 \pm 9\%$ that measured in $eNOS^{-/-}$ cells treated with a nonsense siRNA (siSCR). However, mechanical strain still significantly repressed RANKL in cultures treated with si-nNOS. Data were compiled from 4 experiments, with *significantly different from control, $p < 0.05$.