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ORIGINAL ARTICLE

Fluoride inhibits the response of bone cells to mechanical loading

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Abstract The response of bone cells to mechanical loading is mediated by the cytoskeleton. Since the bone anabolic agent fluoride disrupts the cytoskeleton, we investigated whether fluoride affects the response of bone cells to mechanical loading, and whether this is cytoskeleton mediated. The mechano-response of osteoblasts was assessed in vitro by measuring pulsating fluid flow-induced nitric oxide (NO) production. Osteocyte shape was determined in hamster mandibles in vivo as parameter of osteocyte mechanosensitivity. Pulsating fluid flow (0.7 ± 0.3 Pa, 5 Hz) stimulated NO production by 8-fold within 5 min. NaF (10–50 μM) inhibited pulsating fluid flow-stimulated NO production after 10 min, and decreased F-actin content by ~3-fold. Fluid flow-induced NO response was also inhibited after F-actin disruption by cytochalasin B. NaF treatment resulted in more elongated, smaller osteocytes in interdental bone in vivo. Our results suggest that fluoride inhibits the mechano-response of bone cells, which might occur via cytoskeletal changes. Since decreased mechanosensitivity reduces bone mass, the reported anabolic effect of fluoride on bone mass in vivo is likely mediated by other factors than changed bone cell mechanosensitivity.

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Introduction

Bone is a living tissue capable of adapting its mass and structure to the demands of mechanical usage [1]. Mechanical adaptation is orchestrated by the osteocytes [2]. When bones are loaded, the resulting deformation of the bone matrix will drive the thin layer of interstitial fluid surrounding the network of osteocytes to flow from regions under high pressure to regions under low pressure [3, 4]. This fluid flow is sensed by the osteocytes, which subsequently produce signaling molecules, e.g. nitric oxide (NO) [5–7] which affect osteoblasts and osteoclasts to change their bone remodeling activities leading to changes in bone mass [8–10]. The response of bone cells to mechanical loading is mediated by the cytoskeleton [11]. Moreover, cell shape affects the NO response to mechanical loading, i.e. osteocytes with a round morphology require lower force stimulation in order to increase NO production than osteocytes with a flat morphology [12]. Thus, any factor changing bone cell cytoskeleton or shape may affect bone mass.

Fluoride has been used for decades to enhance bone mass in osteoporosis [13]. Fluoride treatment enhances bone mass by stimulation of bone matrix deposition. Serum concentrations of fluoride in osteoporotic patients treated with fluoride are only 5–15 μM [14]. Fluoride at these concentrations stimulates osteoblast proliferation, differentiation, and matrix production [15–18]. Higher fluoride concentrations increase fracture risk and peripheral bone loss in humans, and therefore fluoride has not become routine therapy for osteoporosis [19]. Fluoride is present in

varying amounts in air, water, and food, and is an essential trace element in the skeletal system and teeth [20]. Fluoride disrupts the cytoskeleton of protozoa [21] and F-actin in ameloblasts [22]. Since fluoride affects bone mass and cytoskeleton, we hypothesized that fluoride inhibits the response of mechano-sensitive bone cells to mechanical loading.

The aim of our study was to determine whether fluoride affects the mechano-response of bone cells in vitro, and whether this effect is modulated via the actin cytoskeleton. Mechano-sensitive MC3T3-E1 osteoblastic cells were treated with or without sodium fluoride (NaF) for 24 h. Subsequently cells were subjected to pulsating fluid flow for 30 min. We used NO production as a parameter for bone cell activation. We also quantified cellular F-actin after fluoride treatment. In addition we determined the effect of fluoride on osteocyte shape in developing hamster mandibles in vivo. Osteocyte shape was determined as parameter for osteocyte mechanosensitivity.

Materials and methods

MC3T3-E1 osteoblast culture

MC3T3-E1 osteoblast-like cells were cultured in α-MEM medium (Gibco, Paisly, UK) supplemented with 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), 50 μg/ml streptomycin (Sigma-Aldrich), 1.4 mM L-glutamine (Sigma), 9 mM β-glycerophosphate (Sigma-Aldrich), 1.9 mM ascorbic acid (vitamin C; Merck, Darmstadt, Germany), and 10% fetal bovine serum (FBS; Gibco) at 37°C, in a humidified atmosphere of 5% CO_2 in air. Medium was changed every 3–4 days. Upon confluence, cells were harvested using 0.25% trypsin and 0.1% EDTA in phosphate buffered saline (PBS), seeded at 2×10^5 cells per 75 cm^2 culture flask (Greiner Bio-One, Kremsmuenster, Austria), and cultured until the cell layer reached confluency again. Then cells were used for pulsating fluid flow experiments as described below.

Disruption of the actin cytoskeleton

Prior to fluid flow stimulation, plated cells were incubated for 1 h in 13 ml of the appropriate medium with the total serum content reduced to 2%. Cells were then divided at random into two groups: (1) untreated control medium; (2) actin-disrupted: medium supplemented with 1 μM cytochalasin B (Sigma-Aldrich), a toxin that prevents actin polymerization [11]. We observed that this concentration of cytoskeletal-disrupting agent had a significant non-toxic effect on cell morphology within 1 h.

Pulsatile fluid flow

Two days before pulsatile fluid flow (PFF) treatment, cells were harvested from the culture flasks and seeded onto polylysine-coated (50 μg/ml; poly-L-lysine hydrobromide, mol wt $15–30 \times 10^4$; Sigma) glass slides (size 2.5×6.5 cm) at 2×10^5 cells/glass slide, and cultured overnight in Petri dishes with 13 ml culture medium containing 10% fetal bovine serum (FBS). Then the culture medium was replaced by α-MEM medium containing 0.2% bovine serum albumin (BSA), antibiotics, ascorbate (Merck), and either or not 10, 25, or 50 μM NaF. Cells were incubated for 24 h, as well as during 30 min of PFF treatment or static control conditions.

PFF was applied as described earlier [5]. Briefly, PFF was generated by pumping 13 ml of culture medium through a parallel-plate flow chamber containing MC3T3-E1 osteoblast-like cells [23]. Cells were subjected to a 5 Hz pulse with a mean shear stress of 0.7 Pa, pulse amplitude of 0.3 Pa and a peak shear stress rate of 8.4 Pa/s [12]. Stationary control cultures were kept in a Petri dish under similar conditions, i.e. at 37°C in a humidified atmosphere of 5% CO_2 in air. After 5, 10, 15, and 30 min of PFF or static control culture, medium was collected and assayed for NO concentrations.

Nitric oxide

NO was measured as nitrite (NO_2^-) accumulation in conditioned medium using Griess reagent containing 1% sulfanylamide, 0.1% naphthylethylene-diamine-dihydrochloride, and 2.5 M H_3PO_4. Serial dilutions of $NaNO_2$ in non-conditioned medium were used as a standard curve. Absorbance was measured at 540 nm with a microplate reader (BioRad Laboratories Inc.).

DNA content

DNA was isolated directly after PFF from bone cell cultures using TRIzol reagent (Gibco) according to the manufacturer's instructions. DNA in the cell lysate was determined by CyQUANT Cell Proliferation assay (Molecular Probes Inc., Eugene, OR, USA).

Staining of filamentous actin (F-actin)

MC3T3-E1 osteoblasts were seeded in 24 well plates at 8.0×10^3 cells/cm^2 (CELLSTAR®, Greiner Bio-one, Alphen a/d Rijn, The Netherlands), incubated overnight, followed by an additional 24 h in α-MEM with 0.2% BSA and with or without 10, 25, or 50 μM NaF. Osteoblasts were washed with PBS, fixed with 4.0% formaldehyde, washed again with PBS, and incubated with 1:250 dilution

of Alexa Fluor 488-phalloidin (Invitrogen) for 1 h in PBS. F-actin was extracted using methanol and quantified as described [24].

Tissue harvesting

A mandibular model of 4-day-old hamsters was used. In short, a single injection of 20 mg NaF/kg body weight was used to test whether NaF affects osteocyte size (5 hamsters). This concentration affects enamel development [25]. Controls (4 hamsters) received NaCl. NaF and NaCl were administered as one single intraperitoneal injection between 10.30 and 11.30 AM. Seventy-six hours later, hamsters were sacrificed, mandibles excised, fixed, demineralized, dehydrated in ascending ethyl-alcohol series, and embedded in Historesin (Leica Microsystems, Mannheim, Germany) [25]. Sections of 5 μm thickness were cut with a microtome in a sagittal plane, mounted on glass slides, and stained with hematoxylin according to Mayer, or toluidine blue. Ethical permission was obtained from the VU University Amsterdam.

Histology

Histologic and histomorphometric analysis was performed using a Leica light microscope with electronic stage table (Leica Microsystems, Wetzlar, Germany). Images were captured using a Leica digital camera directly connected to a computer. All measurements were done using the Leica QWin Pro V 3.5.0 quantitative imaging computer program (Leica Microsystems, Heerbrugg, Switzerland). Within each section, three areas of interest were distinguished: (1) mesial of the first molar, (2) proximal of the first and second molar, and (3) distal of the second molar. In every section these areas of interest were located at 100× magnification, and photographed at 400× magnification. Osteocyte morphology was determined by measuring length and width. Osteocyte shape is expressed as length-over-width ratio in >500 osteocytes per hamster, and the mean shape calculated per animal.

Statistical analysis

PFF data were obtained from 4 experiments. NO production data are expressed as PFF-over-static control. All data are expressed as mean ± SEM. Data were analyzed using Wilcoxon signed-rank test for paired observations. The unpaired *t* test was used for analyzing data from individual hamsters. The paired *t* test was used to analyze measurements between subjects in both control and experimental animals. Differences were considered significant if $p < 0.05$.

Results

Application of fluid shear stress for 30 min to MC3T3-E1 osteoblasts in the presence or absence of NaF did not result in visible changes in bone cell shape or alignment of cells in the flow direction (data not shown). NaF treatment did not affect the total amount of DNA (control, 1.6×10^{-7} g; NaF, 1.6×10^{-7} g). No cells were removed by PFF as assessed by microscopic inspection of the cell cultures.

We investigated whether fluoride affects the mechano-response of MC3T3-E1 osteoblasts. Cells were cultured for 24 h in the presence or absence of NaF, and then treated with or without PFF for 30 min. PFF caused a rapid and significant increase in NO production at 5 min (Fig. 1a). Twenty-four-hour incubation with 10 μM NaF significantly inhibited PFF-induced NO production by 8-fold (Fig. 1a). After 10 min PFF, NaF (10, 25, 50 μM) significantly inhibited the PFF-stimulated NO response (Fig. 1b). We also investigated the effect of PFF and cytoskeletal disruption by cytochalasin B on the NO response of MC3T3-E1 osteoblasts, and found that the PFF-induced increase in NO release at 10 min was inhibited by disrupting F-actin (Fig. 1c).

We then questioned whether the decrease in PFF-stimulated NO production by NaF for 24 h in osteoblasts is mediated via the actin cytoskeleton. In untreated cells, actin fibers were aligned throughout the whole cell (Fig. 2a). After 24 h NaF (10, 25, 50 μM) treatment, actin fibers were at the edge of the cells and less abundant (Fig. 2b). NaF (10, 25, 50 μM) also reduced the amount of F-actin per well by 3-fold (Fig. 2c).

We finally tested whether fluoride affects the mechano-sensitivity of bone cells in vivo, and therefore osteocyte shape was determined in developing hamster mandibles. Three areas of interest were defined, i.e. distal, mesial, and proximal area (Fig. 3a), and the osteocyte length and width were measured (Fig. 3b).

In the distal, mesial, and proximal area, NaF did not affect osteocyte length (Fig. 4a). In the proximal area, but not in the other areas, osteocyte width was 9% smaller when treated with NaF compared to control (marginally significant, $p = 0.07$) (Fig. 4b). NaF increased the osteocyte length/width ratio by 3% in the mesial area (marginally significant, $p = 0.08$), but not in the other areas (Fig. 4c).

Discussion

Mechano-sensitive bone cells play a key role in bone remodeling. The cytoskeleton mediates the response of bone cells to mechanical loading, and cytoskeletal changes can therefore affect bone remodeling [11]. Since the bone

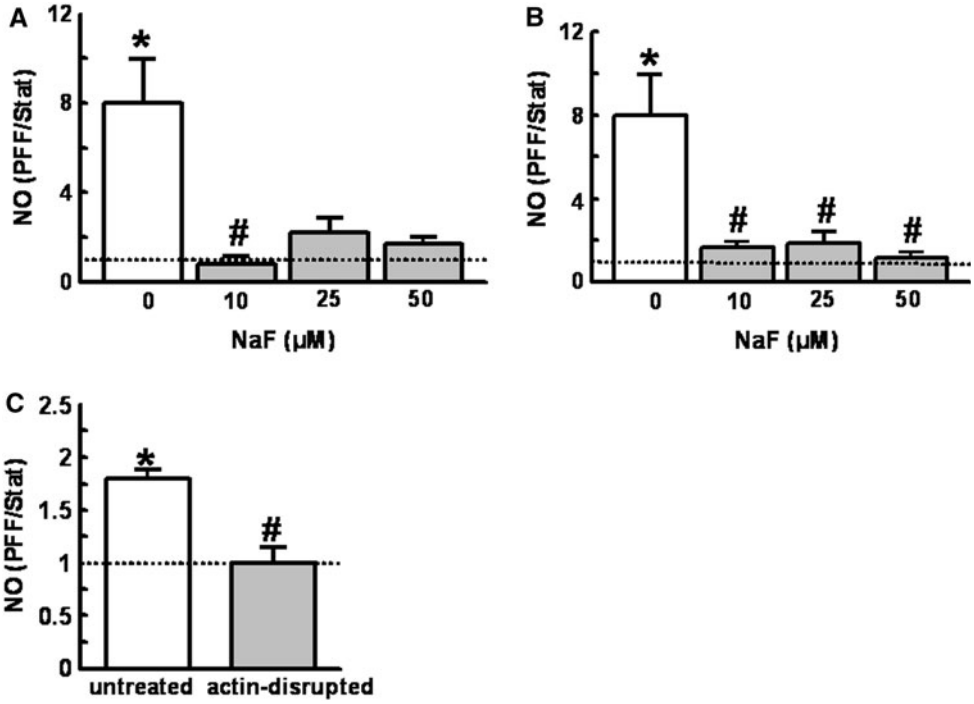

Fig. 1 Effect of 24 h NaF treatment (10, 25 or 50 μM) or actin cytoskeleton disruption on PFF-stimulated NO production by MC3T3-E1 osteoblasts. NaF inhibited the PFF-stimulated NO production by MC3T3-E1 osteoblasts, **a** after 5 min PFF, and **b** after 10 min PFF. **c** F-actin cytoskeleton disruption inhibited PFF-stimulated NO production at 10 min [11]. Values are mean ± SEM of PFF-treated-over-static control ratios (T/C of 5 independent experiments). *Dashed line*, T/C = 1 (no effect). *Stat* stationary control culture, *PFF* pulsating fluid flow. *Significantly different from 1; #Significantly different from untreated, $p < 0.05$

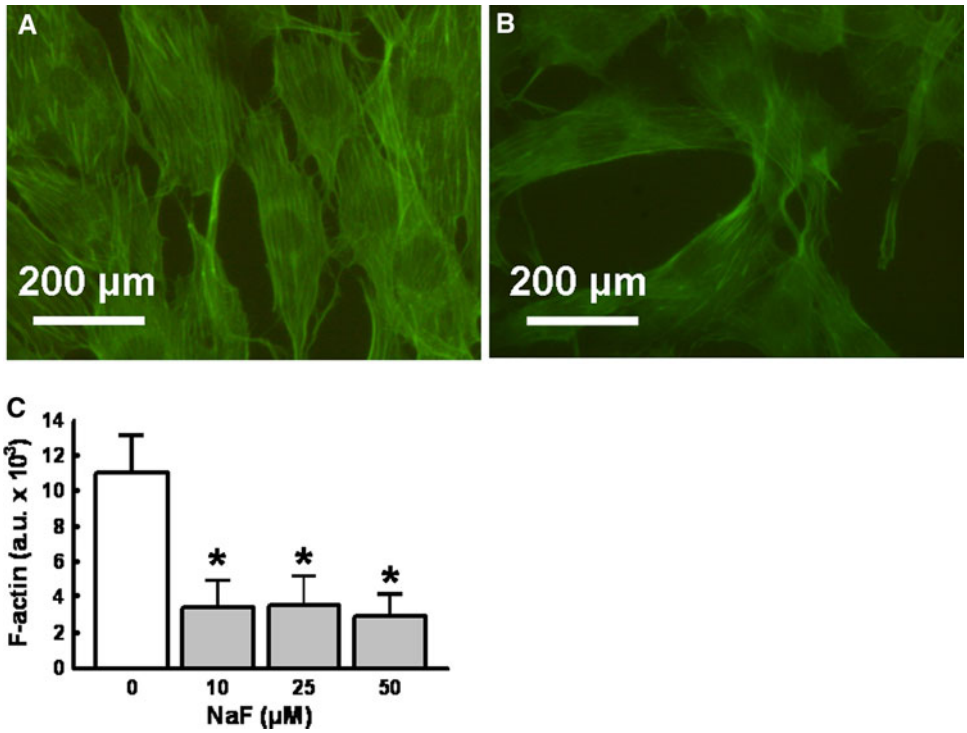

Fig. 2 Immunofluorescence analysis and quantification of actin cytoskeleton in MC3T3-E1 osteoblasts. F-actin (*green*) was visualized with alexa fluor 488 phalloidin. Representative images of **a** control MC3T3-E1 osteoblasts, and **b** F-actin cytoskeleton in MC3T3-E1 osteoblasts after 24 h NaF (10 μM) treatment. **c** Quantification of F-actin in MC3T3-E1 osteoblasts. Values are mean ± SEM of 4 cultures from 2 independent experiments. *Significant effect of NaF on F-actin, $p < 0.05$

anabolic agent fluoride affects the cytoskeleton, we hypothesized that (1) fluoride reduces the mechano-response of osteoblasts to mechanical loading by pulsating fluid flow in vitro, and that this is modulated via the actin cytoskeleton, and (2) that fluoride affects osteocyte mechanosensitivity, the bone mechanosensors par excellence, in hamster mandibles in vivo. In this report, we present compelling evidence that treatment with fluoride at physiological concentrations in vitro inhibits PFF-stimulated NO production by MC3T3-E1 osteoblasts. The same micromolar concentrations of fluoride affected the arrangement and amount of F-actin in these cells. Inhibition of the NO response to PFF was also observed after cytoskeletal disruption by cytochalasin B. The NO

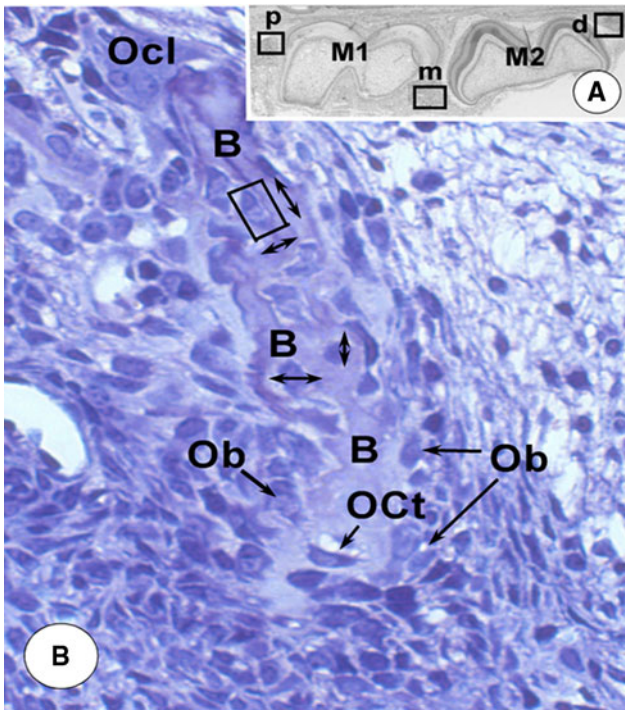

Fig. 3 Histological section of a hamster first and second molar showing the areas of interest for determining osteocyte morphology. **a** Undecalcified, hematoxylin–eosin stained section of a first (*M1*) and second (*M2*) hamster molar at post-natal day 4 from an animal injected with 20 mg NaF/kg body weight, and killed 76 h later, showing the three areas of interest, i.e. proximal area (*p*), mesial area (*m*), and distal area (*d*) (magnification, ×50). **b** Higher magnification of **a** stained with toluidine blue (magnification, ×400). The *square* shows a single osteocyte, and *lines with double arrow heads* indicate length and width of the osteocyte. *Ocl* osteoclast, *Ob* osteoblast, *OCt* osteocyte, *B* bone

production (PFF/Stat) in the control (0 μM) is about 8 in Fig. 1a, b, while in Fig. 1c, NO production (PFF/Stat) in "untreated" is about 1.8. We adopted Fig. 1c from McGarry et al. [11]. In these experiments, a low percentage of serum (2%) was present in the culture medium during the mechanical loading (PFF) experiments. In our experiments (Fig. 1a, b) 0.3% BSA was added to the medium rather than serum. Serum in culture medium will not alter the activation of biological pathways by PFF in osteoblasts, but serum does alter the magnitude of the response of osteoblasts to pulsating fluid flow (unpublished observations within our laboratory). In addition, we found that fluoride marginally changes osteocyte shape in the mesial region in hamster mandibles in vivo. Fluoride treatment resulted in more elongated and smaller osteocytes. Why this occurs only in the mesial region is not clear. Our results suggest that fluoride inhibits the mechano-response of bone cells by cytoskeletal changes.

We applied mechanical loading by PFF of 0.7 ± 0.3 Pa at 5 Hz. This stimulus is based on the currently available knowledge on physiological bone loading stimuli. The frequency spectra after loading of the hip bone in living humans have been calculated to range between 1 and 3 Hz for walking cycles and reaching 8–9 Hz for running cycles [26]. The fluid shear stress amplitude around osteocytes, resulting from daily mechanical loads, have been determined theoretically to range from 0.8 to 3 Pa due to physiological strains in humans [23]. Although the accuracy of these calculations is currently under debate, it is noteworthy that bone cells in vitro seem to be highly sensitive to shear stress in the order of this magnitude.

We showed that fluoride affects the arrangement and amount of F-actin in MC3T3-E1 osteoblasts. These findings agree with a study showing that fluoride affects the activity of several g-proteins involved in the microtubular system and other cytoskeletal compounds in protozoa [21]. Since the response of bone cells to mechanical loading is mediated by the cytoskeleton [11], our findings suggest that fluoride affects the mechano-response of osteoblasts via modulation of cellular F-actin.

Another explanation for the fluoride-induced decrease in NO response to mechanical loading by PFF in MC3T3-E1 osteoblasts could be increased activity of superoxide dismutase (SOD). Fluoride induces oxidative damage in mouse osteoblasts [27]. Oxidative damage in cells by reactive oxygen species is controlled by increasing SOD activity. SOD scavenges free radicals such as NO, and turns these into oxygen and hydrogen peroxide [28]. It is possible that in the presence of NaF, osteoblasts experienced oxidative stress and did respond with increased SOD activity, thereby eliminating the NO produced as a result of PFF treatment.

The effect of fluoride on the arrangement and amount of F-actin in osteoblasts in vitro is in agreement with our in vivo observations. Fluoride did not affect osteocyte length in the mesial, distal, and proximal regions of the hamster mandible, but it decreased osteocyte width in the proximal region and increased osteocyte length-over-width ratio in the mesial region, indicating that a single high dose of NaF in hamster pups resulted in the formation of more elongated and smaller osteocytes in the growing mandible. Bone around developing molar teeth has a high turnover, and osteoblasts briefly exposed to fluoride turn into osteocytes within 3 days. Our results suggest that fluoride inhibits the mechano-response via cytoskeletal changes resulting in changes in osteocyte morphology, since differences in osteocyte morphology indicate differences in mechanosensitivity [12].

The decreased NO response to mechanical loading by PFF, in combination with the affected cytoskeleton and the smaller osteocytes in vivo, as a result of fluoride treatment suggest that bone cell mechanosensitivity is affected by fluoride. We conclude that fluoride causes rearrangement

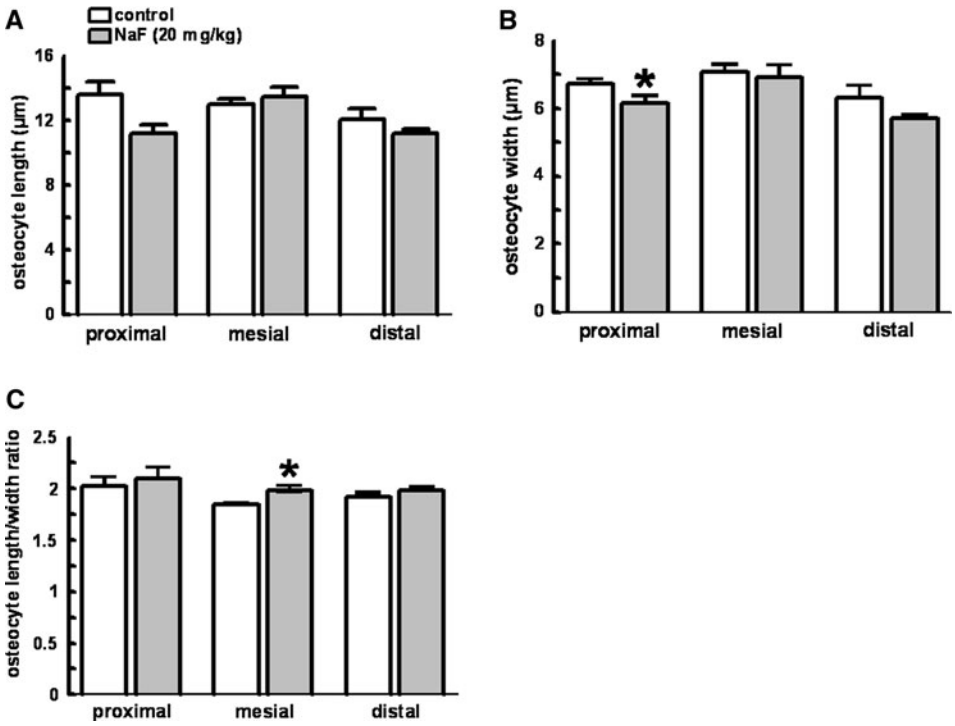

Fig. 4 Effect of a single high dose of NaF (20 mg/kg bodyweight) on osteocyte length and width in hamster mandibles. **a** Osteocyte length, **b** osteocyte width, and **c** osteocyte length/width ratio. Values are mean ± SEM of 5 NaF-treated hamsters and 4 controls. *Marginally significant effect of fluoride, $p = 0.07$ (**b**), $p = 0.08$ (**c**)

of the actin cytoskeleton, thereby changing osteocyte shape and mechanical loading-induced NO production. The known anabolic effect of fluoride on bone mass is therefore likely mediated by other factors than changed bone cell mechanosensitivity.

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