Thiazide Diuretics Directly Induce Osteoblast Differentiation and Mineralized Nodule Formation by Interacting with a Sodium Chloride Co-Transporter in Bone

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ABSTRACT

Thiazide diuretics are used worldwide as a first-choice drug for patients with uncomplicated hypertension. In addition to their antihypertensive effect, thiazides increase bone mineral density and reduce the prevalence of fractures. Traditionally, these effects have been attributed to increased renal calcium reabsorption that occurs secondary to the inhibition of the thiazide-sensitive sodium chloride cotransporter (NCC) in the distal tubule. The aim of the current study was to determine whether thiazides exert a direct bone-forming effect independent of their renal action. We found that the osteoblasts of human and rat bone also express NCC, suggesting that these bone-forming cells may be an additional target for thiazides. In vitro, NCC protein was virtually absent in proliferating human and fetal rat osteoblasts, whereas its expression dramatically increased during differentiation. Thiazides did not affect osteoblast proliferation, but directly stimulated the production of the osteoblast differentiation markers runt-related transcription factor 2 (runx2) and osteopontin. Using overexpression/knockdown studies in fetal rat calvarial cells, we show that thiazides increase the formation of mineralized nodules, but loop diuretics do not. Overall, our study demonstrates that thiazides directly stimulate osteoblast differentiation and bone mineral formation independent of their effects in the kidney. Therefore, in addition to their use as antihypertensive drugs, our results suggest that thiazides may find a role in the prevention and treatment of osteoporosis.


It has been known for several decades that treatment of hypertension with thiazides has the beneficial side effect of strengthening bone.1–5 To date, this bone-protective effect has been attributed to thiazides’ acting at the distal nephron to inhibit the Na+/K+ -Cl− co-transporter (NCC).6 In favor of this hypothesis are two observations. First, patients with Gitelman syndrome and the equivalent murine model, in which NCC is nonfunctional as a result of a mutation in the SLC12A3 gene, exhibit an increased bone mineral density.7,8 Second, patients with pseudohypoaldosteronism type II, who present with an increased NCC activity, exhibit re-
duced bone mineral density. The mechanism by which genetic (Gitelman syndrome) or pharmacologic (thiazide treatment) inhibition of NCC results in enhanced bone mineral density has been hypothesized to be due to increased circulating serum calcium levels. This model proposes that NCC inhibition in the distal tubule evokes hyperpolarization, increased electrical driving force for calcium reabsorption, and a subsequent decrease in urinary calcium loss. However, in patients with Gitelman syndrome, as in patients undergoing thiazide therapy, the expected increase in circulating parathyroid hormone levels that should accompany the increase in free ionized plasma calcium concentration is not seen. In fact, plasma parathyroid hormone levels are either decreased or unchanged, and serum calcium levels remain essentially normal. These observations suggest that the increase in bone mineral density is probably not directly related to the enhanced renal tubular calcium transport but to a direct action of thiazides on bone. Potential mechanisms by which thiazides may exert their effect on bone are via an inhibition of osteoclast-mediated bone resorption and/or by an increase in osteoblastic bone formation.

Although in vitro studies indicate that thiazides are capable of reducing osteoclastic activity independent of NCC, ana.

bolic effects of thiazides have not been demonstrated. Because NCC mRNA has been previously reported in a bone-derived cell line, we hypothesized that thiazides increase bone mineral density by interacting directly with an osteoblast NCC protein. Given the potential therapeutic importance of long-term thiazide treatment in the prevention of age-related osteoporosis, it was the objective of this study to investigate the effects of thiazides by establishing NCC expression in human bone and to determine the effects of thiazides on osteoblast proliferation, differentiation, and mineralized nodule formation in osteoblast models.

RESULTS

For the immunologic detection of NCC expression in skeletal tissue, we used freshly frozen rat femur; freshly frozen human mandible; and EDTA-decalcified, wax-embedded rat femur. At low magnification, we observed NCC immunoreactivity in human and rat cortical and trabecular bone, in both undecalcified, frozen (Figure 1, A and B) and decalcified, paraffin wax-embedded human (data not shown) and rat bone (Figure 1C). No nonspecific immunoreactivity was detected when the primary antibodies were omitted (Figure 1D). NCC immunoreactivity was also observed in snap-frozen sections of rat femur (Figure 1, E through I). With the use of two different antibodies raised against two different epitopes of either human or rat proteins, NCC-specific immunoreactivity was localized to cells of the osteoblastic lineage (particularly osteoblasts) in both human and rat bone (Figure 1, A, G, and top of K). NCC protein was also present in some but not all osteocytes (Figure 1, A, C, and H), perhaps indicating that cells at different stages of differentiation display differential expression of this protein.

Figure 1. Sodium chloride co-transporter (NCC) is expressed in bone. Using human NCC antibodies, specific immunofluorescence is evident in undecalcified frozen human (A) and rat (B) sections. NCC is expressed in reversal lines (A; arrows), in osteocytes (A; small arrows), and lining osteoblasts (B; arrows). (C) Bright-field photomicrograph of EDTA-decalcified paraffin section of rat femur shows NCC immunoperoxidase activity (brown) in reversal lines (arrows), osteoblasts (arrowheads), and some osteocytes (arrowhead +). A proportion of osteocytes are negative for NCC (arrowhead -). (D) Omission of primary antibodies does not result in staining. (E through I) Photomicrographs of rat femur cryosections show that NCC immunofluorescence is in reversal lines (E and F; arrows), osteoblasts (G; arrows), osteocytes (H; arrows), and some osteoclasts (I; arrows). (J) NCC-specific, distal convoluted tubule staining is present in the rat kidney. Bar = 20 μm throughout. (K, top) NCC immunoreactivity in osteoblast-derived MG63 cells (ob; 30 μg of total homogenate loaded) is comparable to that detected in human kidney cortex sample (kc; 30 μg), with predicted sizes for the NCC monomer (M) and dimer (D) of approximately 160 and 320 kD, respectively. (K, bottom) Expression of the ubiquitously expressed type 1 Na⁺-K⁺-2Cl⁻ co-transporter (NKCC1) is also expressed in MG63 cells using an anti-rat NKCC1 antibody (ob, 30 μg of total homogenate loaded). The figure also shows that, as expected, NKCC1 is low abundant in human kidney cortex and outer medulla. om, kidney outer medulla, 10 μg.

Occasionally, osteoclasts in the rat cryosections (Figure 11) but not those in sections of decalcified bone (data not shown) also
expressed NCC; immunostaining in osteoclasts was absent in human freshly frozen and paraffin sections (data not shown). Furthermore, no NCC immunoreactivity was detectable in either human or rat cartilage (data not shown). Positive control experiments carried out on rat kidney cryosections (Figure 1J) confirmed the expression of NCC exclusively in the distal convoluted tubule, thus demonstrating the specificity of the antibody. To investigate the size of human NCC protein in osteoblasts, we performed Western analysis on crude membrane-enriched fractions from human osteoblast-derived MG63 cells. Human kidney cortex was used as the control tissue. Results showed comparable immunoreactivities of the expected molecular weights for the NCC monomer and dimer (Figure 1K, top). Figure 1K also shows that MG63 cells expressed the type 1 Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter (the ubiquitously expressed form NKCC1; Figure 1K, bottom), whereas they lack the kidney-specific isoform of the type 2 Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter (NKCC2; data not shown). Western analysis was performed on homogenates of established osteoblast cellular models after they had reached confluence. These were rat osteosarcoma UMR-106 cell line, mouse osteoblast-derived T3 clonal cell line, and fetal rat calvaria (FRC). To varying degrees, all of these osteoblast-derived lines expressed immunoreactivity of the expected size for NCC (Figure 2A). After enzymatic deglycosylation with N-glycanase, the molecular weight of the NCC monomeric protein in MG63 cells was reduced from approximately 160 kD to approximately 120 kD, a size close to the unglycosylated core protein (Figure 2B). It is interesting that Western analysis also showed that NCC protein expression levels in FRC cells (Figure 2C) and in MG63 cells (Figure 2D) were virtually undetectable in proliferating cells (up to 10 d in culture). In contrast, in postconfluent, differentiating FRC and MG63 cells, NCC protein expression levels increased up to 15 d after confluence.

It is conceivable that the osteoanabolic effects of metolazone could be ascribed to stimulation of cell proliferation. In line with the absence of NCC expression in preconfluent, proliferating osteoblasts, FRC cell proliferation was not affected by metolazone (1 to 100 μM) for up to 2 wk in culture (Figure 3A, three independent experiments performed in triplicate). Type I collagen is the first marker of osteoblast differentiation. Our data show that neither metolazone nor chlorothiazide had any effect on the production of type I collagen (Figure 3B, three independent experiments performed in triplicate). In contrast, metolazone produced a concentration-dependent increase in the expression of later osteoblast differentiation markers runt-related transcription factor 2 (Runx2; Figure 3, C and D, five independent observations from three independent cell isolations) and osteopontin (Figure 3, E and F, five independent observations from three independent cell isolations) in both FRC (Figure 3, C and E) and MG63 (Figure 3, D and F) cells. In addition, chronic metolazone treatment per se increased NCC expression in a dosage-dependent manner (data not shown).

The effect of thiazides and of loop diuretics on mineralized nodule formation was tested in postconfluent FRC cells kept in culture up to 3 wk, and mineralization was visualized using von Kossa staining. Figure 4 shows that both metolazone and chlorothiazide treatment of FRC cells induced a dramatic, concentration-dependent increase in mineralized nodule formation. Figure 4 also shows that, under the same experimental conditions, the loop diuretic bumetanide did not increase mineralization of FRC cells. These results indicate that thiazides increase mineralization and strongly suggest that this effect is dependent on NCC expression.

To confirm that NCC is required for the thiazide-evoked mineralization, we genetically manipulated NCC expression in postconfluent FRC cells. First, to rule out nonspecific effects, we transfected postconfluent FRC cells with an empty vector (pcDNA3.1) and measured mineralization in the presence or absence of concentrations of metolazone known to evoke statistically significant effects in nontransfected cells (i.e., 10 μM; see Figure 4). Figure 5A shows that the effects of metolazone...
were maintained even after plasmid delivery. Indeed, 10 μM metolazone increased the number of mineralized nodules per well from 285.9 ± 16.3 to 470.2 ± 18.6 (n = 9 observations from three independent cell isolations; P < 0.05). In the absence of metolazone treatment, neither overexpression nor antisense knockdown evoked significant changes in mineralized nodule formation (Figure 5B). In contrast, overexpression of NCC increased metolazone-dependent mineralized nodule formation by approximately 50% (49.3 ± 13.0%; n = 12 replicates from four independent cell isolations), an effect that was completely prevented by NCC knockdown (−13 ± 8.7%; n = 12 replicates from four independent cell isolations; P < 0.05).

**DISCUSSION**

Long-term thiazide treatment is associated with a reduction in the risk for hip and wrist fractures in postmenopausal women and elderly men.1–5 This bone-sparing effect is thought to occur through blockage of the renal sodium chloride co-transporter NCC and subsequent reduction in urinary calcium excretion. Thiazides stimulate mineralized nodule formation by FRC cells. (A) Continuous treatment of FRC cells with MET (1 to 100 μM) and CTZ (1 and 10 μM) for 10 to 21 d after confluence induces dosage-dependent increases in mineralized nodule formation (MET: n = 3; *P < 0.05, **P < 0.01; CTZ: n = 3; *P < 0.05). The loop diuretic bumetanide (BMT; 10 μM) does not significantly affect nodule formation. (B) Representative von Kossa staining of FRC cells (15 d after confluence) treated with MET (1 to 100 μM) shows a concentration-dependent increase in the number of mineralized nodules (top), whereas no effects on FRC cell mineralization are seen in the presence of BMT (10 μM). (Bottom) The number of mineralized nodules is comparable for MET- and CTZ-treated postconfluent FRC cells (representative of three independent cell isolations).
cretion. Whether thiazides directly effect new bone formation independent of their renal action has never been demonstrated. Here, we show that NCC is expressed in freshly frozen and decalcified sections of human and rat bone; in cells of the osteoblast lineage, particularly osteoblasts; and, to a lesser extent, in the osteocytes, in both rat and human bone.

Immunoblotting performed on crude membrane extracts of freshly isolated (FRC), osteosarcoma-derived (UMR-106) and virally transformed (2T3) cells confirmed that NCC in osteoblasts is of an equivalent molecular mass as its renal counterpart. The renal NCC contains two N-linked glycosylation sites that are important for sensitivity to thiazides. Enzymatic deglycosylation of NCC in MG63 cells demonstrated the presence of carbohydrate residues in the osteoblast protein. Taken together, these observations show that NCC is expressed in bone and suggest that the osteoblast NCC, like the kidney NCC, may also be a target for thiazide diuretics. Thus, the bone-protective effects of thiazides may be due to their direct interaction with this protein in the osteoblasts.

Bone formation is characterized by a distinctive sequence of events beginning with the commitment of mesenchymal cells to osteoblast lineage, followed by osteoblastic proliferation and differentiation. This sequence of events culminates in the formation of mineralized extracellular matrix by terminally differentiated osteoblasts. Previous studies have ruled out an effect of hydrochlorothiazide on human bone marrow stromal cells, suggesting that the thiazide-dependent enhanced bone mineral density is not due to an increase in osteoblast progenitors. However, the effects of thiazides on osteoblast proliferation are controversial, with either an increase or no change in proliferation having been reported, depending on species. Therefore, we investigated the effects of thiazides on osteoblast proliferation, differentiation, and mineralization to ascribe a potential role of NCC in each of these events. To exclude the possibility that the effects of thiazides could be indirect (i.e., a consequence of their renal actions), we carried out these studies in vitro, using both primary and established models of osteoblasts of either rat or human derivation, namely FRC and MG63 cells, respectively. First, we tested the effects of metolazone and of chlorothiazide on FRC cells and found that neither of these compounds affected proliferation rates for up to 10 d in culture. Consistent with a lack of effect of thiazides on osteoblast proliferation, NCC protein expression levels in FRC cells and in MG63 cells were negligible during the proliferative phase (i.e., up to 10 d in culture). At this point, FRC cells had stopped proliferating and begun their differentiation process, evident from increased expression of alkaline phosphatase (data not shown) and aggregation of cells into nodular areas. In postconfluent, differentiating osteoblasts, NCC protein expression levels in both FRC and MG63 cells gradually rose and peaked at approximately 2 wk after confluence, indicating that NCC acts as a novel potential osteoblast differentiation marker. Having ascertained the presence of NCC in postconfluent, differentiating osteoblasts, we then tested the effects of thiazides on the expression levels of known osteoblast markers.

Type I collagen is the major structural component of the organic matrix of bone and one of the earliest marker of osteoblast differentiation. Our data show that, at a stage when NCC protein expression is absent (i.e., 2 d after confluence), neither metolazone nor chlorothiazide had any effect on the production of type I collagen. In contrast, metolazone produced a concentration-dependent increase in the expression of Runx2, a master osteoblast-specific transcription regulator, in both FRC and MG63 cells. The ability of thiazides to regulate the osteoblast differentiation process was tested by measurement of the levels of osteopontin, a soluble, secreted phospho-
protein that is a component of the bone mineralized extracellular matrix (also called bone sialoprotein 1, secreted phosphoprotein, 2ar, and bp69). In both FRC and MG63 cells, metolazone significantly increased osteopontin production. These data, taken together with our observations that metolazone was ineffective during stages when NCC expression was undetectable and upregulated NCC expression in MG63 cells, suggest that thiazides act directly on differentiating osteoblasts through NCC.

The ultimate osteoblast differentiation marker is the formation of new bone, and FRC cells in culture form calcified nodules that can be visualized by von Kossa staining. Metolazone treatment of postconfluent FRC cells induced a dramatic, concentration-dependent increase in mineralized nodule formation. This effect was specific to thiazide diuretics because it could be mimicked by chlorothiazide but could not be emulated by the loop diuretic bumetanide, even though our results show that osteoblast models express NKCC1, the molecular target for loop diuretics.

As proof of concept, we used plasmid delivery of NCC antisense and sense cDNA in FRC cells and assessed the effects of metolazone on mineralized nodule formation. Overexpression of NCC resulted in a significant increase in metolazone-induced mineralized nodule formation. This increase was completely prevented by NCC knockdown with the antisense construct. This effect is even more striking when one considers that the efficiency of transfection of the plasmid in primary cells is only approximately 10% (estimated with co-transfection with a fluorescence reporter; data not shown). The evidence that the increase in mineralization is observed only in the presence of thiazides suggests the possibility that NCC might act as receptor for thiazides, rather than a co-transporter, although such an interpretation would not explain why patients with Gitelman syndrome exhibit an increased bone mineral density.

Finally, it has been suggested that thiazides prevent bone loss because they reduce acid production by inhibiting carbonic anhydrase activity in osteoclasts. It is interesting that we demonstrate NCC immunostaining in some osteoclasts of cryoprepared rat femora but not human bone. Given that osteoclast staining was observed in five different preparations, with the appropriate positive and negative controls, we believe that NCC immunofluorescence in a subpopulation of osteoclasts is real. This observation opens the possibility that thiazides might affect osteoclastic function through NCC in addition to creating alkalinization of the resorption milieu. The dual action of thiazide drugs on both osteoblast and osteoclast function could account for the observed reduced remodeling in patients taking such drugs in the absence of changes in plasma circulating parathyroid hormone levels.

The main finding of this study is the demonstration that thiazides directly stimulate osteoblast differentiation and mineral production independent of their renal action. This effect of thiazides is concentration dependent, is not mimicked by loop diuretics, is not due to increased osteoblast proliferation, and is enhanced by NCC overexpression. Together with the observations that thiazide treatment and inactivating mutations of NCC are associated with an increased bone mineral density in humans and in knockout murine models, our findings support a pivotal role for the osteoblast NCC in mediating thiazide-induced bone formation. Thiazide diuretics are inexpensive and exhibit a good safety profile. Our findings suggest that it might be possible to develop osteoblast-specific thiazides as part of osteoporosis prevention and therapeutic programs.

**CONCISE METHODS**

**Animals**

Sprague-Dawley rats (Charles River Laboratories, Wilmington, Kent, UK) were killed by cervical dislocation and used in accordance to the UK Animals Scientific Procedures Act of 1986.

**Cell Culture**

The human osteoblast cell line MG-63 was cultured as described previously. FRC cells were isolated as described previously; FRC, rat UMR-106, and mouse osteoblast-derived 2T3 cells were cultured as described previously. Metolazone (Sigma-Aldrich, Poole, Dorset, UK) was dissolved at 37°C for 2 h in the culture medium before being added to the cells. This procedure was repeated every 3 d. From confluence onward, the media were supplemented with ascorbic acid (284 μM for MG-63, UMR-106, and 2T3 and 568 μM for FRC cells; Sigma-Aldrich) and β-glycerophosphate (3 mM; Sigma-Aldrich).

**Western Blotting**

SDS-PAGE immunoblotting of MG-63, UMR-106, 2T3, and FRC cells (whole-cell lysates) and human and rat kidney was performed as described previously, using the following primary antibodies: Affinity-purified rabbit anti-human NCC polyclonal antibodies (1:1000); affinity-purified rabbit anti-rat NCC polyclonal antibodies (1:5000); affinity-purified rabbit anti-rat NKCC1 (a gift of Dr. R. James Turner, National Institute of Dental and Craniofacial Research, Bethesda, MD; 1:1000); affinity-purified rabbit anti-rat NKCC2 polyclonal antibodies (1:1000); mouse anti-human Runx2 mAb (a gift of Dr. Andre von Wijnen, University of Massachusetts, Worcester, MA; 1:4000); mouse anti-rat osteopontin mAb (Iowa Hybriodma Bank, Iowa City, IA; 1:4000); and mouse anti-β-actin mAb (Abcam, Cambridge, Cambridgeshire, UK; 1:10,000). For SDS-PAGE, samples were heated to 60°C for 10 to 15 min in a 5X Laemmli sample buffer, in the presence of dithiothreitol (30 mg/ml) or β-mercaptoethanol (143 mM). Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes before blocking (30 min; PBS containing 5% semiskim milk powder or Odyssey blocking buffer; Li-Cor, Lincoln, NE) and antibody incubations (1 to 12 h). Membranes were washed in Tween-Tris–buffered saline (15 mM Tris [pH 8], 150 mM NaCl, and 0.1% [vol/vol] Tween 20). Antibody binding was visualized by an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Human kidney samples for immunoblotting were obtained from the unaffected portion of a kidney that had been resected because of a renal tumor.
were treated with metolazone (1 to 100 μM) for up to 2 wk. At each time point, triplicate wells were trypsinized and the cells were counted with a Coulter counter (Beckman Coulter, High Wycombe, Buckinghamshire, UK).

Immunohistochemistry
Undecalcified, snap-frozen rat femora (2 mo) and human mandible resections and EDTA-decalcified rat femora were prepared and used for immunofluorescence and immunoperoxidase experiments as described previously. Anti-human and anti-rat NCC polyclonal antibodies (see previous section) were used at 1:10 and 1:100 dilutions, respectively. The human mandible tissue was from neck resections from patients with squamous cell carcinoma invading bone (ethical approval and signed informed patient consent were obtained).

Effect of Metolazone on Osteoblast Proliferation
Cells were plated in 12-well plates at a density of 5000 cells/cm² and were treated with metolazone (1 to 100 μM) for up to 2 wk. At each time point, triplicate wells were trypsinized and the cells were counted with a Coulter counter (Beckman Coulter, High Wycombe, Buckinghamshire, UK).

Effect of Metolazone on Collagen 1A Content
The effects of metolazone on collagen 1A content were quantified as measurements of the hydroxyproline content in FRC cells after 48 h of treatment. After HCl digestion for 24 h at 110°C, the samples were freeze-dried to remove the acid, diluted in distilled water, and oxidized with chloramine followed by coupling with dimethylamino benzaldehyde at 70°C for 10 to 20 min. The colored product was measured at 550 nm. Standards of 1 to 10 μg/ml hydroxyproline were used to calculate the standard curve.

Effect of Metolazone on Osteoblast Differentiation
FRC and MG63 cells were cultured in 35-mm dishes (20,000 cells/cm²) and treated with metolazone or chlorothiazide, for 2 d after confluence for early mineralization experiments. For immunoblotting, the cells were washed in PBS and lysed in RIPA buffer as described previously. Semiquantitative changes in Runx2 and osteopontin immunoreactivities were normalized for the levels of β-actin (mouse monoclonal; Abcam, Cambridge, Cambridgeshire, UK).

Effect of Thiazides on Mineralized Nodule Formation
FRC cells were treated from confluence up to 3 wk. Mineralized nodules were visualized by von Kossa staining, as described previously. The images of mineralized nodules were captured on a flatbed scanner, and image analysis software (Scion Image, Frederick, MD) was used to count the number of mineralized nodules.

Statistical Analyses
The statistical significance was assessed by one-way ANOVA with the Tukey post hoc test or with the unpaired t test, as appropriate. Observations were considered to be statistically significant different at P ≤ 0.05.

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DISCLOSURES
None.

REFERENCES


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