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Vessel Dilator and C-Type Natriuretic Peptide Enhance the Proliferation of Human Osteoblasts

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ABSTRACT: C-natriuretic peptide (CNP) has been shown to regulate proliferation of mouse and rat osteoblasts. Genetic deletion of CNP results in dwarfism. Overexposure of CNP has been associated with arachnodactyly of hands and feet with a very long hallux bilaterally in a 14-y-old girl. CNP effects on bone growth involve inhibition of MEK 1 and ERK 1/2 kinases mediated *via* the intracellular messenger cGMP. Vessel dilator is another natriuretic peptide synthesized by the atrial natriuretic peptide gene whose biologic half-life is 12 times longer than CNP. Vessel dilator's biologic effects on proliferating cells are mediated *via* inhibiting MEK 1/2 and ERK 1/2 kinases *via* cGMP. Vessel dilator has never been studied on osteoblasts. CNP at 10 (nanomolar) nM ($p = 0.02$) and vessel dilator at 10 nM, 1 nM, 100 (picomolar) pM, and 10 pM ($p \leq 0.01$) in dose-response studies enhanced human osteoblasts' proliferation. This first study of human osteoblasts would suggest that vessel dilator with a much longer biologic half-life and with osteoblast-stimulatory effects at lower concentrations than CNP may have therapeutic potential in human achondroplasia, short stature, and osteoporosis. Vessel dilator stimulates osteoblast proliferation whereas most current therapies of osteoporosis target osteoclasts. (***Pediatr Res* 68: 405–408, 2010**)

Bone formation and longitudinal bone growth in long bones, ribs, and vertebrae occurs *via* endochondral ossification in the cartilaginous growth plate, which is located at both ends of the growth plate (1,2). One autocrine regulator of bone growth is C-type natriuretic peptide (CNP) (3–7), a member of the natriuretic peptide hormone family that circulates at a very low level, suggesting that it has very little systemic activity on bone (8,9). Studies using primary cultures of osteoblast-like cells and chondrocytes have revealed that natriuretic peptides with short half-lives such as CNP and atrial natriuretic peptide (ANP) can regulate proliferation and differentiation of osteoblasts and chondrocytes (3–7). CNP stimulates the intracellular messenger cGMP 10-fold more in chondrocytes than ANP (3). cGMP itself is important for bone development and plays a role in regulating growth and differentiation of osteoblasts (4–7,10,11). Genetic deletion of CNP or its signaling results in severe skeletal dysplasias caused by reduced chondrocyte proliferation and differentiation (12,13). In mice lacking CNP, dwarfism and early death occur (12). At birth, these mice have a 10% reduction in bone length, but the growth retardation becomes more severe postnatally and 70% of the mice die in the first 100 d after birth (12). Cartilage-specific overexpression of CNP partially rescues the achondroplasia dwarfism of the CNP-deficient mice, suggesting that CNP stimulates bone growth through direct effects on chondrocytes (11). Contrarily, mice with overexpression of CNP in cartilage have prominent skeletal overgrowth (11). Overexpression of CNP has also been associated with overgrowth and bone abnormalities in a 14-y-old girl (14). Functional inactivation of the natriuretic peptide (NPR)-B receptor that binds CNP (15,16) or gene encoding for cGMP protein kinase II through which cGMP effects are mediated also produces dwarfism (10,17,18).

CNP and ANP are ring-structured natriuretic peptides with very short half-lives of ≤3 min in the circulation (8,18–20). Their biologic effects last for ≤30 min (8,18–20). Vessel dilator is a linear natriuretic peptide synthesized by the *ANP* gene (21–23) that has a circulatory half-life of 107 min (24) and its biologic effects last >6 h (25). Vessel dilator, similar to CNP, has many of its effects mediated by cGMP (22,23). Because vessel dilator is a natriuretic peptide hormones with similar cGMP mechanism of action but much longer biologic effects than CNP or ANP (8,18–20,25), the present investigation was designed to determine whether a natriuretic peptide with at least 12-fold longer biologic effects (25) might increase osteoblasts' proliferation like CNP. Vessel dilator and CNP were compared directly against each other in dose-response curves to determine their comparative ability to enhance osteoblast proliferation.

METHODS

Culture of human osteoblast cells. A cell line (ATCC number CRL-11372) of human osteoblast cells was purchased from the American Type Culture Association (ATCC), Manassas, VA. Propagation of the human osteoblast cells was in a 1:1 mixture of Ham's F-12 Medium and DMEM with 2.5 mM L-glutamine without phenol red. Base medium was supplemented with 0.3 mg/mL of geneticin (G418) antibiotic and 10% fetal bovine serum (26). Cells were incubated at a temperature of 34°C in 5% CO_2 at which they have rapid cell division, doubling every 36 h (26). Immunostaining of these postconfluent-differentiated human osteoblasts showed that high levels of osteopontin, osteonectin, bone sialoprotein, and type 1 collagen were expressed (26). Cells were dispensed into new flasks with subculturing every 6–8 d. The medium was changed every 3 d.

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Abbreviations: ANP, atrial natriuretic peptide; **CNP,** C-type natriuretic peptide; **ERK 1/2,** extracellular signal-regulated kinase 1/2; **MEK 1/2,** MAP kinase kinase 1/2; **MAPK,** mitogen-activated protein kinase

Research protocol. After the osteoblast cells were subcultured for 24 h, ~5000 cells in 200 μL of the above media were then seeded (d 1) into 96-well plates (Nuclon, Roskilde, Denmark). After overnight incubation at 34°C in 5% CO_2, the media was removed (d 2), and 50 μL of fresh media was added to control wells, blank wells (with no cells inside), and 50 μL of media with 10 picomolar (pM), 100 pM, 1 nanomolar (nM), or 10 nM of CNP or vessel dilator. At d 5, in these experiments, 50 μL of fresh media was added to the controls, blank wells, and 50 μL of media with 1 nM, 10 nM, 10 pM, and 100 pM of the respective natriuretic hormones for a total volume of 100 μL of media in each well. At d 7, 20 μL of Cell Titer 96 Aqueous One Solution (Promega Corporation, Madison, WI) was added to each well containing 100 μL of medium and allowed to incubate for 4 h in 5% CO_2 atmosphere before recording absorbance at 490 nm with a 96-well plate reader (27). There were 15 observations of vessel dilator at each concentration and 16 observations of CNP at each concentration. The peptide hormones used in this investigation were from Phoenix Pharmaceuticals, Inc., Burlingame, CA.

Cell proliferation. Cell proliferation of human osteoblasts was examined with the Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega Corp.). This colorimetric method determines the viable cells' proliferation by recording the absorption at 490 nm with a 96-well plate reader (27) after incubating the respective cells at 37°C for 4 h in a 5% CO_2 atmosphere. Approximately 5000 human osteoblast cells were in each well. This proliferation assay detects the number of viable cells in proliferation using a tetrazolium compound (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent [phenazine ethosulfate (PES)]. PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution (27). The MTS tetrazolium compound (Owen's reagent) is bioreduced by living cells into a colored formazan product that is measurable at 490 nM in a spectrophotometer, thereby eliminating any nonviable (*i.e.* dead) cells that would not be proliferating (27). With this method, only viable cells' proliferation is measured because dead cells are unable to reduce the MTS tetrazolium compound to a colored formazan product.

Statistics. All data are expressed as mean ± SEM. Statistical significance was determined by the Mann-Whitney test (also called Wilcoxon rank-sum test) for different sample sizes. For the CNP group, there were 16 data points for each concentration and eight controls. For the vessel dilator group, there were 15 data points for each concentration and 24 controls.

RESULTS

CNP stimulates human osteoblast proliferation. CNP at its 10 nM concentration enhanced human osteoblast proliferation 27% ($n = 16$) compared with controls ($n = 8$; $p = 0.02$; Fig. 1). There was no significant enhancement of osteoblast proliferation at CNP concentrations of 1 nM, 100 pM, and 10 pM (Fig. 1). Thus, at 1 nM, there was a minus 1% enhancement, and at 100 pM, there was a minus 16% enhancement of osteoblast proliferation with CNP (Fig. 1).

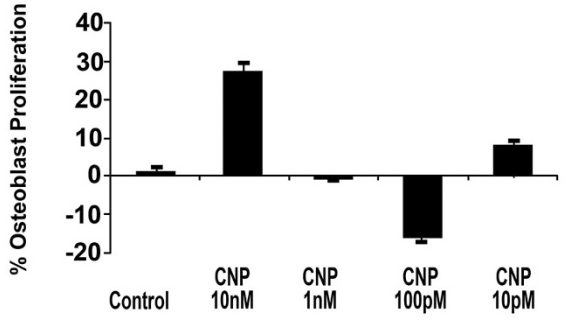

Figure 1. CNP enhances human osteoblast proliferation at its 10 nM concentration by 27% ($p = 0.02$) when evaluated by the Mann-Whitney (Wilcoxon rank-sum test). CNP did not significantly enhance human osteoblast proliferation at its 1 nM, 100 pM, and 10 pM concentrations when evaluated by Mann-Whitney test.

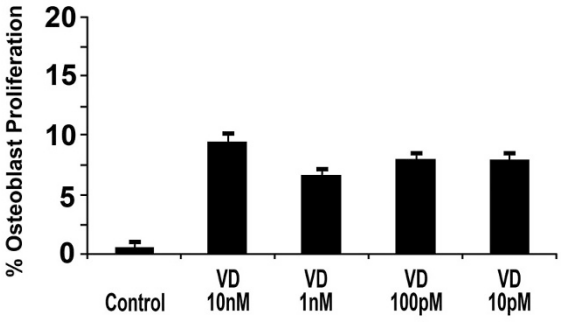

Figure 2. Vessel dilator enhanced the proliferation of human osteoblasts over a concentration range of 10 nM to 10 pM ($p \leq 0.01$) when evaluated by Mann-Whitney test. The 100 pM and 10 pM concentrations in this graph are in the circulating physiologic range of vessel dilator.

Vessel dilator enhances the proliferation of human osteoblasts. Vessel dilator at its 10 nM concentration ($n = 15$) enhanced the proliferation of human osteoblasts 8% compared with controls ($n = 24$; $p = 0.0018$, Fig. 2). Decreasing the concentration of vessel dilator 10-fold to 1 nM resulted in a 6% enhancement of the proliferation of human osteoblasts ($p < 0.01$). With a 100-fold decrease in the concentration of vessel dilator to 100 pM, there was still a 7% enhancement of the proliferation of human osteoblasts ($p = 0.0073$, Fig. 2). Vessel dilator at 10 pM stimulated human osteoblast proliferation 8% ($p = 0.01$).

Comparison of varying concentrations of CNP and vessel dilator on their ability to enhance human osteoblasts. Comparing the effects of CNP and vessel dilator on human osteoblast proliferation (Fig. 1 *versus* Fig. 2) revealed that CNP-stimulated osteoblast proliferation to a greater extent at its 10 nM concentration *versus* 10 nM concentration of vessel dilator ($p = 0.048$). However, at their respective 1 nM and 100 pM concentrations vessel dilator caused a more significant ($p < 0.05$) enhancement of human osteoblast proliferation.

DISCUSSION

CNP is expressed in fetal bones and accelerates longitudinal growth of fetal rat metatarsal bones in organ culture (7). CNP in this investigation was found to stimulate human osteoblast proliferation for the first time, extending previous findings that CNP can enhance osteoblast proliferation in rat (4) and mouse (5) osteoblasts. In this investigation of CNP on human osteoblasts, dose-response studies revealed that at 10 pM, which is CNP's physiological circulating concentration (9), CNP could not enhance human osteoblast proliferation suggesting that CNP may not be a systemic physiologic regulator of osteoblast function. This would confirm previous studies of CNP on osteoblast function on mice osteoblasts (5), rat osteoblasts (4), and rat chondrocytes (3,7) where CNP did not have any effects on osteoblasts in the pM range. However, the importance of CNP in bone growth is illustrated by genetic deletion of CNP resulting in skeletal dysplasia (12,13) with mice lacking CNP having dwarfism (12). Further evidence of CNP importance for bone growth is that mice overexpressing CNP in cartilage

have skeletal overgrowth (11), and a 14-y-old girl with overexpression of CNP, with a doubling of CNP in plasma, had bone overgrowth and who was >97° percentile in length at birth and had arachnodactyly of hands and feet with a very long hallux bilaterally at the age of 14 y (14). These studies would suggest that because CNP does not stimulate human, rat, or mouse osteoblasts at its circulating physiologic concentrations, its effects on bone are *via* an autocrine/paracrine process. The gene for CNP is expressed in bone (7) to allow it to be an autocrine/paracrine regulator of bone.

This is the first investigation demonstrating that vessel dilator, a linear structured peptide hormone as opposed to a ring-structured CNP (21–23), can stimulate osteoblast proliferation. That vessel dilator can enhance human osteoblast proliferation is important because its circulating half-life is 36-fold longer than CNP [*i.e.* 107 min for vessel dilator *versus* <3 min for CNP; (8,18–20,24)] and its biologic effects last for >6 h compared with <30 min for ring-structured natriuretic peptides such as CNP and ANP (25), which also has enhancing effects in bone growth (4). Vessel dilator, but not CNP, was found to enhance human osteoblast proliferation at its physiologic concentrations in the circulation (25), further suggesting that vessel dilator may be important for physiologic regulation of bone growth by stimulating osteoblasts. Increasing the concentration of vessel dilator above the physiologic range to pharmacological concentrations did not cause a further increase in its ability to enhance osteoblast proliferation. This information would suggest that bone proteases may be proteolytically degrading this peptide hormone at its higher concentrations. With more vessel dilator present in bone, the bone proteases may become more active in a negative feedback manner, cleaving this peptide hormone resulting in loss of any enhanced biologic activity beyond that observed with physiologic concentrations of vessel dilator.

With respect to the mechanisms of vessel dilator and CNP's enhancement of osteoblast proliferation, cGMP would seem to be an important mediator of their effects because CNP can increase this intracellular mediator in chondrocytes (3) and the majority of vessel dilator's effects are mediated *via* cGMP (21–23,28). cGMP itself is important for bone development, which have been shown to regulate proliferation and differentiation of osteoblasts and chondrocytes (4–7,10,11). Inactivation of the gene encoding for cGMP protein kinase II through which cGMP effects are mediated in bone also produces achondroplastic dwarfism (10,17,18). Overexpression of CNP in chondrocytes rescues achondroplasia through inhibition of MEK 1 kinase in the mitogen-activated protein kinase (MAPK) pathway (11). Constitutive activation of MEK 1 kinase in chondrocytes causes achondroplasia-like dwarfism in mice (29). Vessel dilator inhibits the activation, *i.e.* phosphorylation of MEK 1/2 kinases by 98% in proliferating prostate cancer cells (28). Vessel dilator's ability to inhibit MEK 1/2 kinases in proliferating cells is mediated by cGMP as evidenced by 1) using a cGMP antibody that blocks vessel dilator effects on MEK 1/2 kinases and 2) cGMP itself could inhibit MEK 1/2 kinases in proliferating cells (28). CNP and 8-bromo cGMP also inhibit mitogen- (fibroblast growth factor) stimulated ERK 1/2 kinases' phosphorylation in ATDC5 cells, a mouse chondrogenic cell line (30). Vessel dilator inhibits 96% of the phosphorylation of basal activity of ERK 1/2 kinases in proliferating cells (31) and completely blocks mitogen [epidermal growth factor, (EGF)] stimulation of ERK 1/2 kinases (32). Thus, both vessel dilator and CNP seem to have identical molecular mechanisms of action of stimulating osteoblasts and bone growth *via* the inhibiting MAP kinases MEK 1/2 and ERK 1/2, mediated at least in part by cGMP (11,29–32).

With respect to potential treatment of bone diseases, CNP has been suggested to be a new treatment strategy for achondroplasia (30). Vessel dilator, with its 36-fold longer half-life and significantly longer biologic effects than CNP (*i.e.* >12 times longer; 25), would seem to be a better choice for treatment of bone disease such as dwarfism because it could be given less frequently with similar therapeutic results. Furthermore, as evidenced in this investigation, vessel dilator stimulates osteoblastic proliferation over a concentration range of 10 nM through 10 pM whereas CNP at concentration <10 nM did not significantly enhance human osteoblast proliferation. CNP's half-life is very short (3 min), *in vivo* whereas vessel dilator's half-life of >6 h (25) would suggest it could be given four times per day to affect bone growth. As vessel dilator can be given on a reasonable schedule of four times per day, it may have a role in the treatment of short stature in children by enhancing their osteoblast proliferation.

In addition to growth disorders in children, CNP and vessel dilator may have a therapeutic role in treating a common bone disease in adults, *i.e.* osteoporosis. Current therapeutic agents for osteoporosis concentrate on inhibiting osteoclasts (33). Bisphosphonates such as alendronate, PTH, calcitonin, and 1, 25-dihydroxy vitamin D, all work *via* inhibiting osteoclasts (33). Sex steroids such as estrogens and testosterone do stimulate osteoblasts (33) but are usually given only in cases of documented low testosterone and/or estrogens because of their side effects. Estrogens, for example, are not currently given for osteoporosis even when the person is postmenopausal with low estrogen levels by some physicians because of their potential cardiovascular risk (33). Sodium fluoride stimulates osteoblasts and has been used for vertebral fractures but even though bone mass increased secondary to sodium fluoride, it does not decrease the incidence of fractures (33). An agent that stimulates osteoblasts without the side effects of sodium fluoride or sex steroids and that will cause bone formation *via* osteoblasts rather than inhibiting old bone in place (*via* osteoclasts) has been sought for decades (33). This investigation where vessel dilator was demonstrated to stimulate human osteoblasts suggests that it may provide new therapeutic option for bone disease. Vessel dilator would be a preferred option over CNP because of its much longer biologic activity for >6 h compared with <30 min for CNP (25). Treatment every 30 min with CNP would be very impractical.

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