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The Receptor Attributable to C-Type Natriuretic Peptide-Induced Differentiation of Osteoblasts Is Switched From Type B- to Type C-Natriuretic Peptide Receptor With Aging

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Abstract C-type natriuretic peptide (CNP) stimulates the differentiation and inhibits the proliferation of osteoblastic lineage cells. In this study, we examined whether the effects of CNP on osteoblastic functions change with aging using calvarial osteoblast-like cells from 25-week-old (young) and 120-week-old (aged) rats. CNP inhibited DNA synthesis and stimulated collagen synthesis and mineralized bone nodule formation. These effects were less pronounced in aged rat cells, suggesting the age-related attenuation of CNP-induced signaling. They were also blocked by the treatment of young rat cells with KT5823, a protein kinase G (PKG) inhibitor, but not by the treatment of aged rat cells with KT5823. CNP stimulated cGMP production in young rat cells, but not in aged rat cells. Natriuretic peptide receptor (NPR)-B, which has a guanylyl cyclase activity domain, and NPR-C, which has no enzyme activity domain, were predominantly expressed in young and aged rat cells, respectively. C-ANF, an NPR-C agonist, mimicked the effects of CNP on the proliferation and differentiation of aged rat cells; these effects were inhibited by the treatment with pertussis toxin (PTX), a Gi protein inhibitor. CNP and C-ANF evoked intracellular levels of inositol-1,4,5-triphosphate and Ca^{2+}, which are markers for phospholiase C (PLC) activation, in aged rat cells, and the effects of these two peptides were also blocked by the treatment with PTX. From these results, we concluded that CNP acts as a positive regulator of bone formation by osteoblasts and that the signaling pathway for CNP is switched from NPR-B/cGMP/PKG to NPR-C/G_i protein/PLC with aging. J. Cell. Biochem. 103: 753–764, 2008. © 2007 Wiley-Liss, Inc.

Key words: osteoblast; aging; natriuretic peptide; natriuretic peptide receptor; cyclic GMP

C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family that includes atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) [Rosenzweig and Seidman, 1991; Potter and Hunter, 2001]. Three distinct types of receptor have been identified for these peptides and termed natriuretic peptide receptor (NPR)-A, NPR-B, and NPR-C have been identified [Schulz and Waldman, 1999; Silberbach and Roberts, 2001]. NPR-A and NPR-B, which are coupled with guanylyl cyclase, mediate the biological functions through cGMP production. Although NPR-A and NPR-B are structurally and functionally very similar, they have distinct ligand specificities. CNP binds to NPR-B, whereas ANP and BNP bind to NPR-A [Koller and Goeddel, 1992; Suga et al., 1992]. The role of NPR-C uncoupled with guanylyl cyclase has initially been considered to have a role in the clearance of bound ligands by internalization and degradation [Anand-Srivastava and Trachte, 1993]. However, recent data suggest

Abbreviations used: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; NPR, natriuretic peptide receptor; PKG, cGMP-dependent protein kinase; PLC, phosphatidylinositol-specific phospholiase C; Br-cGMP, 8-bromo-cGMP; IBMX, 3-isobutyl-1-methyl-xanthine; PTX, pertussis toxin; PI, phosphoinositide; IP_3, inositol-1,4,5-triphosphate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; BN, mineralized bone nodules; FBS, fetal bovine serum; BSA, bovine serum albumin.

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that NPR-C mediates several cell signaling and biological effects through a G_i protein [Anand-Srivastava et al., 1996; Anand-Srivastava, 1997].

Osteoporosis in the elderly is characterized by the loss of absolute bone mass most likely resulting from the suppression of bone formation by osteoblasts with aging [Carrington, 2005]. It is recognized that osteoblastic bone formation is an event regulated by local factors [Manolagas, 1998]. CNP and NPR-B expressed in osteogenic lineage cells regulate the proliferation and differentiation of these cells in an autocrine/paracrine fashion [Hagiwara et al., 1996; Inoue et al., 1996, 1999, 2002; Suda et al., 1996, 1999]. In addition, genetic studies have demonstrated that the most evident physiological effect of CNP is the growth of long bone through NPR-B. The inactivation of mutations in the genes coding for CNP [Chusho et al., 2001], NPR-B [Bartels et al., 2004] and cGMP-dependent protein kinase (PKG) II [Pfeifer et al., 1996; Miyazawa et al., 2002] causes dwarfism, whereas the transgenic overexpression of CNP causes skeletal overgrowth [Yasoda et al., 2004], suggesting that the CNP/NPR-B/cGMP/PKG signaling pathway is important in bone metabolism.

In this study, we examined the effects of CNP on osteoblastic functions using calvarial cells from 25-week-old (young) and 120-week-old (aged) rats and found that the receptor attributable to CNP-mediated functions is switched from NPR-B to NPR-C with aging.

MATERIALS AND METHODS

Materials

CNP and C-ANF were purchased from Peptide Institute (Osaka, Japan). Pertussis toxin (PTX), 8-bromo-cGMP (Br-cGMP), 3-isobutyl-1-methyl-xanthine (IBMX), KT5823, and the antibody for β-actin were purchased from Sigma (St. Louis, MO). The antibodies for NPR-B and NPR-C were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

The cells enriched for osteoblast phenotype were enzymatically isolated from calvariae of 25- and 120-week-old female Wistar rats (CLEA, Tokyo, Japan) as described previously [Kaneki et al., 1999, 2000]. In brief, frontal and parietal bones from rats were stripped of soft tissue and periosteum, minced with scissors, and digested with a mixture of 0.2% collagenase (Wako, Tokyo, Japan) and 0.25% trypsin at 37°C for 20 min. The released cells were collected and immediately suspended in F-12 medium (Invitrogen, Rockville, MD) supplemented with 10% fetal bovine serum (FBS, Invitrogen). This procedure was repeated five times every 20 min. The released cells from the last four fractions were grown in F-12 medium supplemented with 10% FBS at 37°C. After reaching subconfluence, the cells were collected by a trypsin treatment, plated in the 4-, 12-, or 24-well plates at a density of 2×10^3 cells/cm^2, and grown in the same medium for 5 days. On day 6, the medium was changed to α-MEM medium (Invitrogen) supplemented with 10% FBS, 5 mM β-glycerophosphate and 0.1 mg/ml ascorbic acid and the cells were further grown for 20 days.

To determine the effects of CNP, C-ANF, or Br-cGMP on the proliferation and differentiation of osteoblasts, the cells were treated with various concentrations of test agent in serum-free medium for 24 h on day 5. To determine the effects of subculture on the expression levels of natriuretic peptides.

Repeated Passage of Young Rat Cells

Young rat cells in 6-well plates at a density of 2×10^3 cells/cm^2 were grown in F-12 medium supplemented with 10% FBS at 37°C. At confluence, cells were collected by a trypsin treatment and subjected to the repeated passage under the same condition.

Determination of Collagen Synthesis

The cells in the 12-well plates were washed twice with serum-free α-MEM medium on day 7, and incubated for 5 h in the same medium containing [2,3-^{3}H]proline (1.5 μCi/ml; DuPont, Wilmington, DE). The cells were washed three times with cold PBS to remove unincorporated radioactivity, lysed by freezing-thawing, extracted in 0.5% Triton X-100, and precipitated with cold 10% trichloroacetic acid. After the collection of acid-precipitable materials by centrifugation, precipitates were extracted with acetone, dried, resolubilized in 0.5 M acetic acid, and neutralized with 1 M NaOH. The amount of [^{3}H]proline incorporated into the collagenase-digestible proteins was measured using bacterial collagenase (Sigma) by the method of Peterkofsky and Diegelmann [1971].

Determination of Calcium Content in Mineralized Bone Nodules

On day 26, the cells in the 4-well plates were washed twice with PBS, incubated with 1 ml of 0.5 M HCl overnight with gentle shaking to solubilize mineralized bone nodules (BN), and sonicated twice for 10 s at 4°C. The calcium content in the lysate was determined with a commercial kit (Wako) by a method using 3, 3′-bis[*N*,*N*-bis(carboxymethyl)amino-methyl]-*o*-cresolphtalein.

Proliferation Assay

On day 6, the cells in the 24-well plates were washed twice with serum-free α-MEM medium and further incubated for 3 h in the same medium containing [methyl-^{3}H]thymidine (1.25 μCi/ml; DuPont). The cells were washed three times with cold PBS to remove unincorporated radioactivity, followed by two washes with 10% trichloroacetic acid. The cell layers were solubilized in 1 M NaOH, and aliquots of the solubilized cells were diluted with liquid scintillation fluid after neutralization with HCl. The amount of [^{3}H]thymidine incorporated into the cells then counted using a beta counter.

Determination of Intracellular cGMP Concentration

On day 5, the cells cultured in the 12-well plates were washed three times with serum-free medium and further cultured for 24 h. The cells were incubated in serum-free F-12 medium containing 5×10^{-4} M IBMX for 20 min to inhibit phosphodiesterase activity, and then treated with a vehicle or a test agent in the same medium for 15 min. The reaction was terminated by aspiration. Then 1 ml of 90% 1-propanol was added to the reaction mixture to extract cGMP. The extract was dried and resolubilized in 0.05 M sodium acetate. The amount of cGMP was determined by radioimmunoassay using a commercial kit (Yamasa, Chiba, Japan) according to the manufacturer's instruction.

Determination of Intracellular Ca^{2+} Concentration

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by the method of Grynkiewicz et al. [1985]. In brief, the cells were incubated in the serum-free F-12 medium containing 0.01% bovine serum albumin (BSA) for 18 h on day 5. During the last 2 h of the incubation, 10 μM fra 2-AM (Sigma), a cell permeable fluorescent probe for Ca^{2+}, was included in the medium. The cells were washed three times with PBS, and treated with a vehicle or a test agent. The ratio of fluorescence intensity excited at 340 nm and 380 nm (500-nm emission) was measured with a Cyto Fluor II automatic plate-reading fluorometer (PerSeptive Biosystems, Tokyo, Japan).

Determination of Intracellular Inositol Triphosphate Concentration

To determine the intracellular inositol-1,4, 5-triphosphate (IP_3) concentration, the cells were incubated in the inositol- and serum-free F-12 medium containing 0.01% BSA for 18 h on day 5. During the last 1 h of the incubation, 1×10^{-4} M LiCl was included in the medium. Then the cells were treated with a vehicle or a test agent in the same medium for 30 s. The reaction was terminated by the removal of the medium, and the cells were scraped into 1 ml of 10% trichloroacetic acid (TCA). The dishes were washed twice with 1 ml of 10% TCA, and the combined solution was mixed with 1 ml of water-saturated diethyl ether. The water layer was applied to an anionic-exchange resin column (AG 1-X8). IP_3 was eluted from the column with 0.1 M formic acid containing 1 M ammonium formate. The amount of IP_3 was determined by radioimmunoassay using a commercial kit (GE Healthcare Bio-Science, Piscataway, NJ) according to the manufacturer's instruction.

Tissue Preparation

The 25- and 120-week-old rats were killed by decapitation. The bone (femur), brain (cerebrum and cerebellum), heart, kidney, liver, lung, mesentery, ovary, thymus, skin, spleen, and stomach were excised from eight female rats. They were frozen immediately in liquid nitrogen and stored at −80°C until use.

RT-PCR Analysis

On day 6, the cells were homogenized using 1 ml of TRIzol reagent (Invitrogen), and total RNA was extracted according to the manufacturer's protocol. cDNA was synthesized using 20 μl of reverse transcription reaction solution containing 1 μg of total RNA, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl,

5 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, 2.5 μM random hexamers, 20 U of RNase inhibitor, and 50 U of Moloney murine leukemia virus reverse transcriptase (all from Roche Applied Science, Indianapolis, IN). Quantitative real-time PCR amplification was performed in an iCycler real time PCR machine using an iQ SYBR Green supermix (both from Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instruction. The sequences of the primer sets for NPR-B, NPR-C, and β-actin mRNAs, the target sites on mRNAs and the product sizes determined by PCR are shown in Table I. To minimize the background of the products amplified from genomic DNAs, the primers were designed to exist on two different exons. The quantities of NPR-B and NPR-C mRNA in each sample were normalized using the C_T (threshold cycle) value obtained for β-actin mRNA amplification.

Western Blot Analysis

On day 5, the cells in a 100-mm dish were washed with cold PBS and whole cell lysate was prepared by adding an M-PER mammalian protein extraction reagent (PIERCE, Rockford, IL) containing a protease inhibitor cocktail (Roche Applied Science) to the cells. Twenty micrograms of protein was loaded per lane and fractionated on a 10% polyacrylamide gel; the fractionated proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) by electroblotting. The membrane was blocked for nonspecific binding in 3% nonfat dry milk, followed by incubation with the antibody for NPR-B, NPR-C, or β-actin at 4°C. After the membrane was washed, the blots were probed with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories) and visualized using an enhanced chemiluminesence system (Amersham Biosciences, Piscataway, NJ) according to manufacturer's instructions.

Statistical Methods

Data were analyzed by Student's *t*-test or one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. $P < 0.01$ was considered significant. All data are represented as the mean ± SD of four cultures.

RESULTS

Effects of CNP on Proliferation and Differentiation

We first examined the effects of CNP on the proliferation and differentiation of young and aged rat cells. CNP dose-dependently enhanced the amount of [^{3}H]proline incorporated into the collagenase-digestible proteins (Fig. 1A; $EC_{50} = 9.1 \times 10^{-10}$ M) and increased calcium content in BN (Fig. 1B; $EC_{50} = 2.0 \times 10^{-9}$ M) in young rat cells, whereas it suppressed the amount of [^{3}H]thymidine incorporated into the cells (Fig. 1C; $IC_{50} = 1.0 \times 10^{-9}$ M). In aged rat cells, CNP also enhanced collagen synthesis (Fig. 1A) and calcification (Fig. 1B) and suppressed DNA synthesis (Fig. 1C) similarly to, but to a lesser extent than those in young rat cells. These results indicate an age-dependent suppression of the effects of CNP on osteoblastic functions.

Effects of CNP on cGMP Production

CNP caused a dose-dependent increase in cGMP production in young rat cells (Fig. 2). The EC_{50} of cGMP production was 8.8×10^{-10} M, and an approximately 30-fold increase in cGMP production over the control level was observed at 1×10^{-8} M or higher. No significant increase in cGMP production by CNP was observed in aged rat cells. These results indicate that

TABLE I. Sequences of Primers Used in Real-Time PCR Analysis

Gene	GenBank™ accession number	Sequence of primers	Target site of genes	Product size (bp)	References
NPR-B	NM_053838	F: 5′-TGAGCAAGCCACCCACTTC-3′ R: 5′-CAGCGGGCCGCAGATA-3′	652–762	111	Sellitti et al. [2004]
NPR-C	NM_012868	F: 5′-GGGCAGTGAGCGAGTGTT-3′ R: 5′-CCCCATCCTTCTTGCTGT-3′	757–1,157	401	Piao et al. [2004]
β-actin	NM_031144	F: 5′-CATGAAGATCAAGATCATTGCTCCT-3′ R: 5′-CTGCTTGCTGATCCACATCTG-3′	1,053–1,161	109	Rollin et al. [2005]

F, forward primer; R, reverse primer.

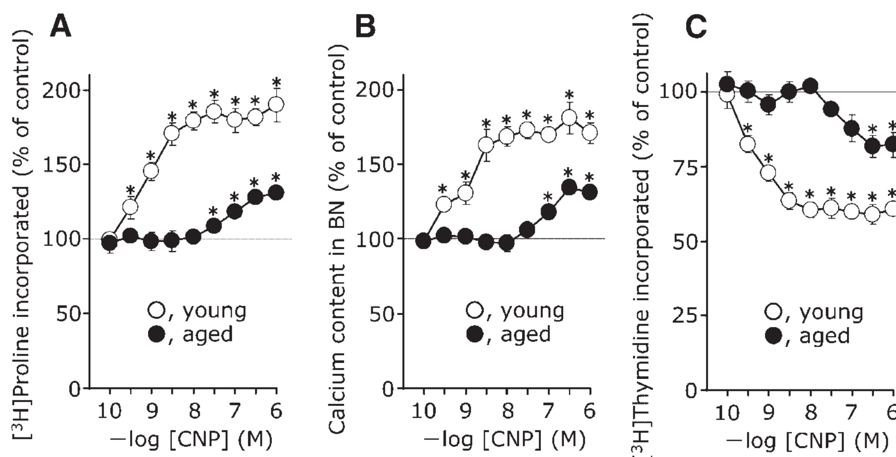

Fig. 1. Effects of CNP on proliferation and differentiation of young and aged rat cells. The cells were stimulated with the indicated CNP concentrations on day 5, and then proliferation and differentiation markers were determined as described in Materials and Methods. **A**: The amount of [^{3}H]proline incorporated into the collagenase-digestible proteins (basal levels in young and aged rat cells were 10,600 ± 840 and 8,320 ± 420 dpm/well, respectively). **B**: Calcium content in BN (basal levels in young and aged rat cells were 167 ± 28 and 118 ± 16 pg/well, respectively). **C**: The amount of [^{3}H]thymidine incorporated into the cells (basal levels in young and aged rat cells were 10,200 ± 627 and 6,420 ± 315 dpm/well, respectively). Each point and vertical bar represents the mean ± SD of four cultures, respectively. *$P < 0.01$; compared with basal levels. The experiment was repeated twice and the results were essentially the same as those depicted.

NPR-B expressed in young rat cells leads to cGMP production in response to CNP stimulation.

KT5823 (1×10^{-6} M), a PKG inhibitor, almost completely inhibited the CNP-induced enhancement of collagen synthesis (Fig. 3A) and calcification (Fig. 3B) and the suppression of DNA synthesis (Fig. 3C) in young rat cells. The PKG inhibitor had no effect on the CNP-induced increase in the level of differentiation markers (Fig. 3A,B) and a decrease in the level of proliferation markers (Fig. 3C) in aged rat cells. These results indicate that CNP regulates osteoblastic functions through PKG in young rat cells, but not in aged rat cells.

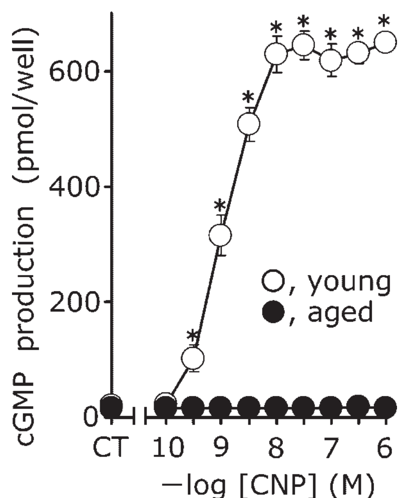

Fig. 2. Effects of CNP on cGMP production in young and aged rat cells. After pretreatment with 5×10^{-4} M IBMX for 20 min, the cells were stimulated with the indicated CNP concentrations for 15 min on day 5. The amount of cGMP was determined as described in Materials and Methods. Each point and vertical bar represents the mean ± SD of four cultures, respectively. The basal levels of intracellular cGMP (CT) in young and aged rat cells were 22.3 ± 0.99 and 15.7 ± 0.24 pmol/well, respectively. *$P < 0.01$; compared with control (CT). The experiment was repeated twice and the results were essentially the same as those depicted.

Expression Levels of Natriuretic Peptide Receptors

We determined the mRNA and protein levels of NPRs in untreated cells by real-time RT-PCR and Western blot analyses, respectively. The NPR-B mRNA level in young rat cells was approximately 60-fold higher than that in aged rat cells (Fig. 4A), whereas the NPR-C mRNA level in aged rat cells was approximately 20-fold higher than that in young rat cells (Fig. 4B). Consistent with these results, the NPR-B protein level in young rat cells was higher than that in aged rat cells, and the NPR-C protein level in aged rat cells was higher than that in young rat cells (Fig. 4C). These results indicate that the signaling pathway for CNP changes with aging.

Next, we determined the mRNA levels of NPRs in several tissues from young and aged rats by real-time PCR analysis (Table II). The mRNA expression of NPR-B and NPR-C was

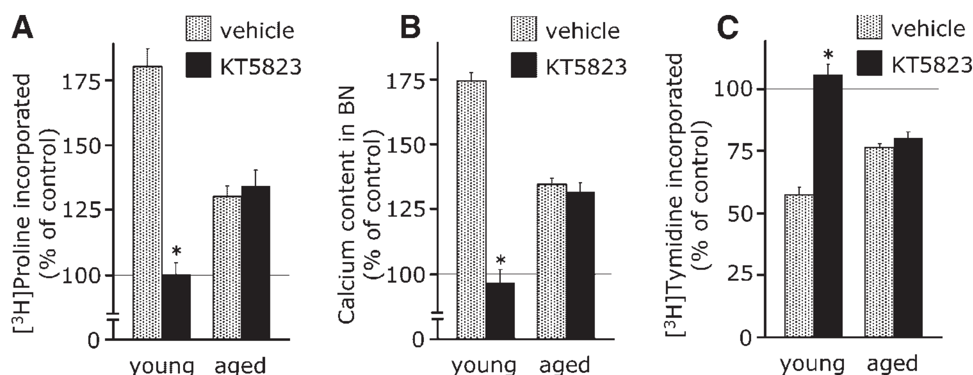

Fig. 3. Effects of PKG inhibitor on CNP-induced osteoblastic functions in young and aged rat cells. The cells were pretreated with vehicle (PBS) or KT5823 (1×10^{-6} M) during the last 3 h of day 4, and then incubated with 1×10^{-6} M CNP for 24 h. The amount of [^{3}H]proline incorporated into the collagenase-digestible proteins (**A**), calcium content in BN (**B**) and the amount of [^{3}H]thymidine incorporated into the cells (**C**) were determined under the same conditions described in Figure 1.

detected in bone, brain, heart, kidney, liver, lung, mesentery, ovary, and thymus from young and aged rats, but not in skin, stomach, and spleen. The NPR-B mRNA levels in bone, mesentery, ovary, and kidney decreased to $47.6 \pm 10.2\%$, $51.2 \pm 10.3\%$, $62.4 \pm 11.1\%$, and $66.1 \pm 10.2\%$, respectively, in aged rats compared to the levels found in young rats. In contrast, the NPR-C mRNA levels in bone, mesentery, kidney and heart increased to $235.9 \pm 21.4\%$, $209.1 \pm 18.3\%$, $175.2 \pm 16.1\%$, and $172.8 \pm 12.5\%$, respectively, in aged rats compared to the levels found in young rats. These results indicate that the signaling pathway for CNP changes with aging not only in bone but also other tissues.

Effects of Repeated Passage on Expression Levels of Natriuretic Peptide Receptors

We employed young rat cells to investigate the effects of repeated passage on the mRNA levels of NPRs (Table III). Insulin-like growth factor-I (IGF-I) was used as an aging-related maker in osteoblasts. A significant decrease in mRNA levels of NPR-B and IGF-I was observed after passage 8, and the levels decreased to $21.4 \pm 2.0\%$ and $30.9 \pm 3.2\%$, respectively, at passage 16 compared to the levels at the first

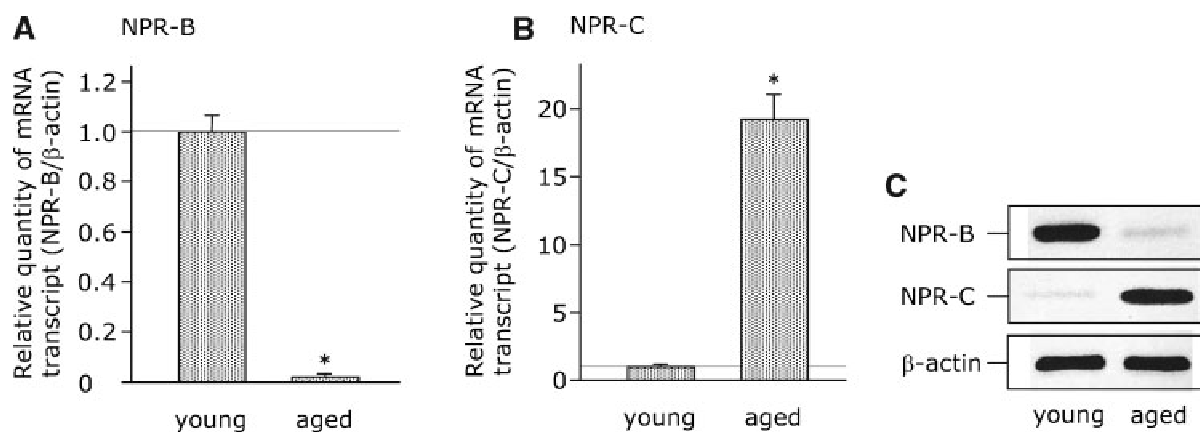

Fig. 4. Expression levels of NPR-B and NPR-C in young and aged rat cells. At the beginning of day 5, total RNA and protein were extracted from the cells and subjected to real-time RT-PCR (**A**,**B**) and Western blot (**C**) analyses as described in Materials and Methods. A,B: The relative expression levels of NPR-B and NPR-C were normalized to that of β-actin in the same sample. The relative quantitation of the mRNA transcripts of NPR-B and NPR-C was calculated using (relative level in aged rat cells)/(relative level in young rat cells). Each point and vertical bar represents the mean ± SD of four cultures, respectively. $^*P < 0.01$; compared with level in young rat cells. C: NPR-B, NPR-C, and β-actin protein levels. The experiment was repeated twice and the results were essentially the same as those depicted.

TABLE II. Age-Related Changes in mRNA Levels of Natriuretic Peptide Receptors

Tissues	Relative expression levels in aged rats (% of those in young rats)	
	NPR-B	NPR-C
Bone (Femur)	47.6 ± 10.2*	235.9 ± 21.4*
Brain	105.3 ± 14.4	119.6 ± 15.5
Heart	92.4 ± 13.8	172.8 ± 12.5*
Kidney	66.1 ± 10.2*	175.2 ± 16.1*
Liver	105.2 ± 12.7	124.1 ± 16.9
Lung	93.5 ± 13.8	102.4 ± 22.3
Mesentery	51.2 ± 10.3*	209.1 ± 18.3*
Ovary	62.4 ± 11.1*	132.5 ± 26.3
Thymus	93.5 ± 10.4	115.7 ± 13.8
Skin	ND	ND
Spleen	ND	ND
Stomach	ND	ND

Each data represents the mean ± SD of eight rats.
ND, not detected.
*$P < 0.05$; versus young rats.

passage. In contrast, a significant increase in NPR-C mRNA level was observed after passage 10, and the level increased to 225.8 ± 14.3% at passage 16 compared to the level at the first passage. These results indicate that the signaling pathway for CNP changes with in vitro cell aging.

Effects of NPR-C Agonist and cGMP Analogue on Proliferation and Differentiation

To determine if NPR-C-induced signaling regulates osteoblastic functions, the cells were treated with C-ANF, a selective agonist for NPR-C. C-ANF caused a dose-dependent enhancement of collagen synthesis (Fig. 5A) and calcification (Fig. 5B) and a dose-dependent suppression of DNA synthesis in aged rat cells (Fig. 5C), similarly to CNP, as shown in Figure 1, although no effects of C-ANF on proliferation and differentiation were observed in young rat cells. KT5823 (1×10^{-6} M) did not inhibit the C-ANF (1×10^{-6} M)-induced stimulation of collagen synthesis or calcification, or the inhibition of DNA synthesis in aged rat cells (data not shown). C-ANF failed to induce cGMP production in young and aged rat cells (Fig. 5D). These results suggest that NPR-C regulates the osteoblastic functions of aged rat cells in a cGMP-independent manner.

We next treated the cells with Br-cGMP, a membrane-permeable cGMP analogue. In contrast to C-ANF, in young and aged rat cells, Br-cGMP within a concentration range of 3×10^{-6} to 1×10^{-4} M showed similar dose-dependent enhancements of collagen synthesis (Fig. 6A) and calcification (Fig. 6B) and suppression of DNA synthesis (Fig. 6C), suggesting that the cGMP-induced signaling pathway is not affected by aging.

TABLE III. Effects of Subculture on mRNA Levels of Natriuretic Peptide Receptors and IGF-I

Passage number	Relative expression levels (% of those in the first passage)		
	NPR-B	NPR-C	IGF-I
1	100.0 ± 8.3	100.0 ± 4.5	100.0 ± 4.4
3	108.4 ± 5.2	95.3 ± 6.3	93.5 ± 6.2
5	91.6 ± 6.6	120.1 ± 9.9	104.6 ± 2.5
8	71.0 ± 8.2*	111.1 ± 10.3	80.2 ± 3.9*
10	45.4 ± 4.8*	146.0 ± 7.2*	57.3 ± 8.2*
13	56.2 ± 8.2*	155.5 ± 10.6*	36.6 ± 5.1*
16	21.4 ± 2.0*	225.8 ± 14.3*	30.9 ± 3.2*

Each data represents the mean ± SD of four cultures.
*$P < 0.05$; versus first passage cells.

Effects of PTX on CNP-Induced Stimulation of Differentiation and Inhibition of Proliferation

Since NPR-C has been reported to control phosphatidylinositol-specific phospholiase C (PLC) through the G_i protein without any cGMP response in several cell types other than osteoblasts, we examined the role of the G_i protein in the proliferation and differentiation of osteoblasts by exposing cells to CNP (1×10^{-6} M), C-ANF (1×10^{-6} M), and Br-cGMP (3×10^{-5} M) in the presence of PTX, a G_i protein inhibitor (Fig. 7). PTX almost completely inhibited the CNP- and C-ANF-induced stimulation of collagen synthesis (Fig. 7A) and calcification (Fig. 7B), and inhibition of DNA synthesis (Fig. 7C) in aged rat cells, whereas it had no significant effect in young rat cells (data not shown), suggesting that CNP regulates the proliferation and differentiation of aged rat cells through the NPR-C/G_i protein signaling pathway. PTX had no effects on the Br-cGMP-induced stimulation of collagen synthesis or calcification, or the inhibition of DNA synthesis in young and aged rat cells.

To determine whether CNP and C-ANF stimulate PLC activity through NPR-C, we examined their effects on IP_3 production and $[Ca^{2+}]_i$ as phosphoinositide (PI) turnover markers using aged rat cells. CNP (1×10^{-6} M) caused a 3.9-fold increase in IP_3 production (Fig. 8A) and a 2.1-fold increase in $[Ca^{2+}]_i$

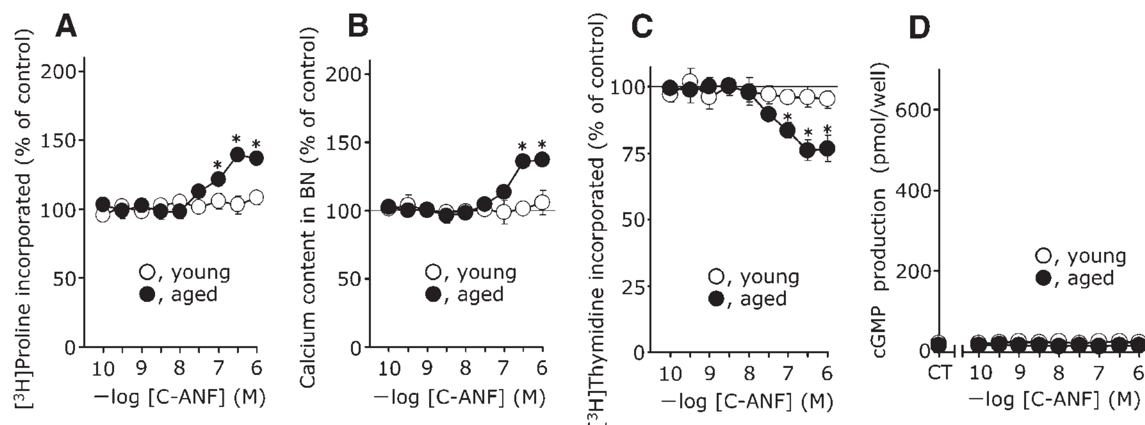

Fig. 5. Effects of C-ANF on proliferation and differentiation of and cGMP production in young and aged rat cells. The amount of [3H]proline incorporated into the collagenase-digestible proteins (**A**), calcium content in BN (**B**), and the amount of [3H]thymidine incorporated into the cells (**C**) were determined under the same conditions described in Figure 1. cGMP production (**D**) was determined under the same conditions described in Figure 2. Each point and vertical bar represents the mean ± SD of four cultures, respectively. *$P < 0.01$; compared with basal level. The experiment was repeated twice and the results were essentially the same as those depicted.

relative to those for the controls (Fig. 8B). C-ANF also caused a 3.7-fold increase in IP_3 production (Fig. 8A) and a 2.2-fold increase in $[Ca^{2+}]_i$ relative to those for the controls (Fig. 8B). The CNP- and C-ANF-induced stimulations of PI turnover were almost completely inhibited by treating the cells with PTX (100 ng/ml) but not with KT5823 (1×10^{-6} M), indicating that CNP regulates the osteoblastic functions of aged rat cells through the NPR-C/G_i protein/PLC signaling pathway.

DISCUSSION

CNP regulates the functions of osteoblastic lineage cells including MC-3T3-E1 cells and newborn rat calvarial cells, which have an ability to form BN in vitro, and C3H10T1/2 and ROB-C26 cells, which have multiple potentials to differentiate into not only osteoblasts but also adipocytes and myoblasts [Hagiwara et al., 1996; Inoue et al., 1996; Suda et al., 1996, 1999]. In this study, we found that CNP inhibits

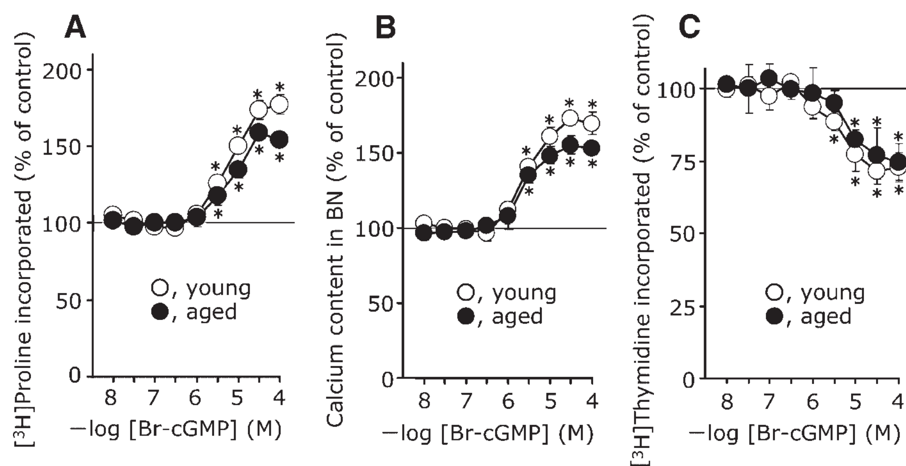

Fig. 6. Effects of Br-cGMP on proliferation and differentiation of young and aged rat cells. The amount of [3H]proline incorporated into the collagenase-digestible proteins (**A**), calcium content in BN (**B**), and the amount of [3H]thymidine incorporated into the cells (**C**) were determined under the same conditions described in Figure 1. Each point and vertical bar represents the mean ± SD of four cultures, respectively. *$P < 0.01$; compared with basal level. The experiment was repeated twice and the results were essentially the same as those depicted.

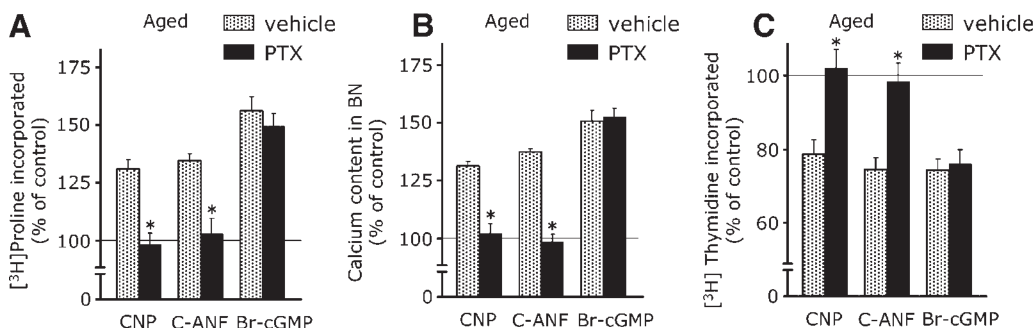

Fig. 7. Effects of PTX on proliferation and differentiation of aged rat cells. The cells were pretreated with a vehicle (PBS) or PTX (100 ng/ml) during the last 3 h of day 4, and further incubated in the same medium containing 1×10^{-6} M CNP, 1×10^{-6} M C-ANF, or 3×10^{-5} M Br-cGMP for 24 h. The amount of [^{3}H]proline incorporated into the collagenase-digestible proteins (**A**), calcium content in BN (**B**), the amount of [^{3}H]thymidine incorporated into the cells (**C**) were determined under the conditions described in Figure 1. Each point and vertical bar represents the mean ± SD of four cultures, respectively. *$P < 0.01$; between vehicle and PTX treatments of group. The experiment was repeated twice and the results were essentially the same as those depicted.

DNA synthesis and stimulates collagen synthesis and mineralized bone nodule formation, which are markers of osteoblastic differentiation. The degree of these effects is less pronounced in aged rat cells, suggesting the age-related attenuation of CNP-induced signaling.

CNP exerts its effect on osteoblastic functions through cGMP production [Hagiwara et al., 1996; Inoue et al., 1996; Suda et al., 1996, 1999]. The binding of CNP to NPR-B leads to guanylyl cyclase activation to produce cGMP, which activates bone formation through PKG activation. In this study, CNP stimulated cGMP production in young rat cells and the effects of CNP on the proliferation and differentiation of the cells were completely abolished by the treatment with KT5823, a PKG inhibitor, indicating the involvement of the NPR-B/cGMP/PKG-dependent mechanism in CNP-mediated effects. In aged rat cells, CNP failed to stimulate cGMP production and KT5823 showed no effects of CNP on the osteoblastic functions. These results indicate that CNP regulates the osteoblastic functions of aged rat cells through a mechanism independent of the NPR-B/cGMP/PKG signaling pathway.

The main function of NPR-C has originally been considered to be a clearance receptor for

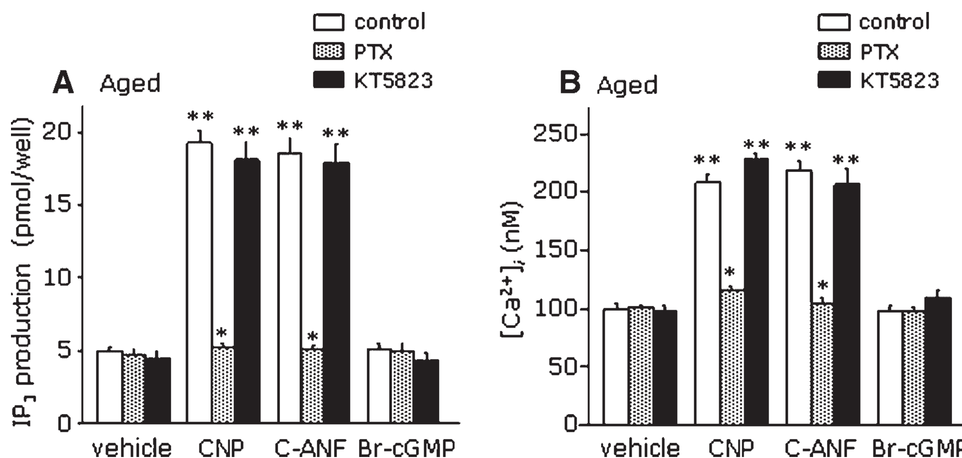

Fig. 8. Effects of CNP and CNP-related agents on PI turnover in aged rat cells. The cells were treated with a vehicle, PTX (100 ng/ml) or KT5823 (1×10^{-6} M) during the last 3 h of day 4, and further incubated in the same medium containing 1×10^{-6} M CNP, 1×10^{-6} M C-ANF, or 3×10^{-5} M Br-cGMP for 15 min. IP_3 production (**A**) and $[Ca^{2+}]_i$ (**B**) were determined as described in Materials and Methods. Each point and vertical bar represents the mean ± SD of four cultures, respectively. *$P < 0.01$; versus control. **$P < 0.01$; versus vehicle. The experiment was repeated twice and the results were essentially the same as those depicted.

internalizing and degrading natriuretic peptides because of the high affinity of all natriuretic peptides for NPR-C and the ability of NPR-C to be recycled rapidly in the presence or absence of natriuretic peptides [Nussenzveig et al., 1990; Suga et al., 1992]. NPR-C, unlike NPR-B, possesses only 37 amino acids in an intracellular domain without kinase and guanylyl cyclase activities [Koller and Goeddel, 1992; Anand-Srivastava and Trachte, 1993]. In this study, we found that NPR-B and NPR-C are dominantly expressed in young and aged rat cells, respectively, in terms of both mRNA and protein levels, suggesting a major role of NPR-C in the osteoblastic functions of aged rat cells.

NPR-C is expressed in many but not all tissues that express NPR-A and/or NPR-B [Anand-Srivastava, 2005]. However, there is no report on age-related changes in the expression of NPRs. We found in this study that NPR-B mRNA levels in bone, kidney, mesentery and ovary from young rats were significantly higher than those from aged rats, and NPR-C mRNA levels in bone, heart, kidney and mesentery from aged rats were significantly higher than those from young rats. These results indicate that the age-related change in the expression of NPRs is not limited in bone.

Recent studies have investigated the age-related alteration in responses to growth factors in a variety of cells senesced by the repeated passage in vitro [Bird et al., 2003]. The cellular senescence in vitro shows a good correlation with several characteristics of in vivo aging. Kveiborg et al. [2000] have employed human osteoblasts with typical characteristics of cellular senescence such as a limited proliferative capacity and an increased β-galactosidase activity. They demonstrate that osteoblast aging is accompanied by the decrease in the expression of IGF-I, an important stimulator of osteoblast functions in vivo and in vitro. We found in this study that the repeated passage of young rat cells resulted in the up-regulation of NPR-C and down-regulation of NPR-B and IGF-I. However, the detailed mechanisms leading to alteration in the expression of NPRs remain to be determined. Oxidative stress resulting in an increase of intracellular reactive oxygen species is primarily responsible for aged phenotype in a variety of cells and tissues [Lavrovsky et al., 2000; Lee et al., 2006]. It is likely that some of the general mechanisms, such as DNA damage accumulation, may be responsible for the alteration.

Recently, the intracellular domain of NPR-C has been reported to activate G protein-dependent pathways linked to several effector enzymes. Studies of smooth muscle cells have demonstrated the role of NPR-C as a signal transducer and identified the subtype of G proteins activated by NPR-C as the G_{i1} and G_{i2} proteins [Murthy et al., 1998; Teng et al., 1998]. PI turnover signaling has been recognized as a major signaling pathway for hormones mobilizing intracellular Ca^{2+}. The activation of G_i-protein-coupled receptors by hormones results in PLC-β activation leading to IP_3 production from phosphatidylinositol-4,5-diphosphate, which is involved in intracellular Ca^{2+} mobilization. We previously showed that the activation of PI turnover results in the stimulation of osteoblastic differentiation and the inhibition of proliferation in calvarial cells [Fujieda et al., 1999; Kaneki et al., 1999]. We therefore hypothesized that CNP regulates the proliferation and differentiation of aged rat cells through an NPR-C/Gi protein/PLC signaling pathway. We found in the present study that C-ANF, a NPR-C agonist, stimulated the differentiation and inhibited the proliferation of aged rat cells without cGMP production, indicating that NPR-C regulates the proliferation and differentiation of osteoblasts. Moreover, the effect of C-ANF described above was completely abolished by treating aged rat cells with PTX, a Gi protein inhibitor, and CNP mimicked these effects of C-ANF on the functions of aged rat cells, further supporting the notion that the NPR-C/Gi protein signaling pathway is involved in the action of CNP on aged rat cells. Finally, C-ANF and CNP stimulated IP_3 production and Ca^{2+} release in a PTX-sensitive manner, indicating the involvement of PLC activation in the CNP-mediated action on aged rat cells.

The knockout mice lacking NPR-C gene have a skeletal overgrowth recognized 1 week after birth [Matsukawa et al., 1999]. The NPR-C mRNA is readily detected in osteoblasts of developing wild-type mice by the in situ hybridization. We found in this study that the stimulatory effect of NPR-B-induced signaling on bone formation was more prominent compared to that of NPR-C-induced signaling in young rat cells. The observations in NPR-C knockout mice and our present study suggest

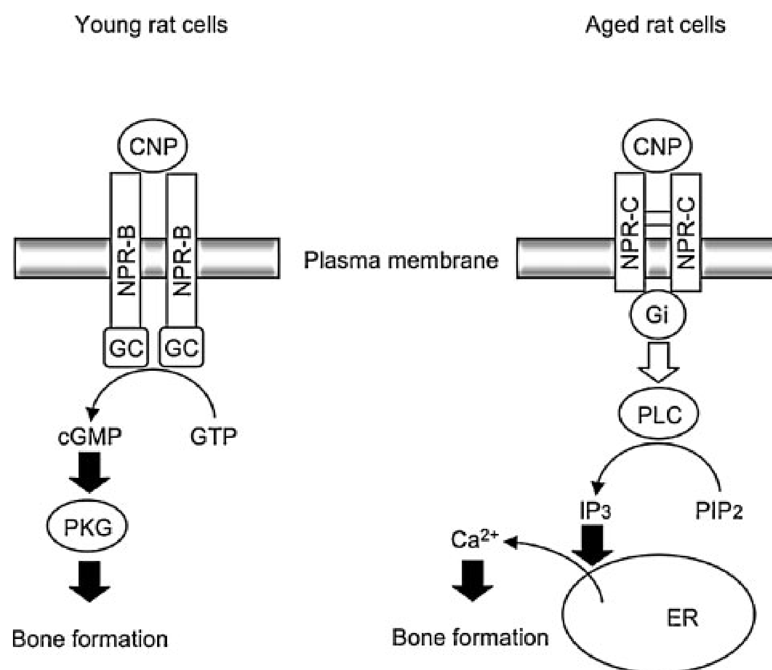

Fig. 9. Scheme of signaling pathways for CNP-induced cellular responses in rat calvarial cells. The functional natriuretic peptide receptors are NPR-B and NPR-C in young (**left panel**) and aged (**right panel**) rat cells, respectively. In young rat cells, the binding of CNP to NPR-B leads to guanylyl cyclase (GC) activation to produce cGMP, which activates of bone formation through PKG activation. In aged rat cells, the binding of CNP to NPR-C leads to PLC activation through G_i protein to produce IP_3, which binds to its receptor on the endoplasmic reticulum (ER), resulting in Ca^{2+} release. The increase in intracellular Ca^{2+} concentration activates bone formation.

that NPR-C in growing bone regulates the effects of locally produced CNP as a clearance receptor in young animals.

In summary, we demonstrated that CNP acts as a positive regulator of bone formation by osteoblasts and that the signaling pathway for CNP is switched from NPR-B/cGMP/PKG to NPR-C/G_i protein/PLC with aging, as illustrated in Figure 9. To the best of our knowledge, this is the first study that shows an age-related change in the responsiveness of osteoblast-like cells to natriuretic peptides.

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