Bone 56 (2013) 375–382

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

A human skeletal overgrowth mutation increases maximal velocity and blocks desensitization of guanylyl cyclase-B[☆]

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ARTICLE INFO

Article history:
Received 18 February 2013
Revised 12 June 2013
Accepted 24 June 2013
Available online 1 July 2013

Edited by: R. Baron

Keywords:
Natriuretic peptides
Guanylate cyclase
Bone growth
cGMP
Dwarfism
Achondroplasia

ABSTRACT

C-type natriuretic peptide (CNP) increases long bone growth by stimulating guanylyl cyclase (GC)-B/NPR-B/NPR2. Recently, a Val to Met missense mutation at position 883 in the catalytic domain of GC-B was identified in humans with increased blood cGMP levels that cause abnormally long bones. Here, we determined how this mutation activates GC-B. In the absence of CNP, cGMP levels in cells expressing V883M-GC-B were increased more than 20 fold compared to cells expressing wild-type (WT)-GC-B, and the addition of CNP only further increased cGMP levels 2-fold. In the absence of CNP, maximal enzymatic activity (Vmax) of V883M-GC-B was increased 15-fold compared to WT-GC-B but the affinity of the enzymes for substrate as revealed by the Michaelis constant (Km) was unaffected. Surprisingly, CNP decreased the Km of V883M-GC-B 10-fold in a concentration-dependent manner without increasing Vmax. Unlike the WT enzyme the Km reduction of V883M-GC-B did not require ATP. Unexpectedly, V883M-GC-B, but not WT-GC-B, failed to inactivate with time. Phosphorylation elevated but was not required for the activity increase associated with the mutation because the Val to Met substitution also activated a GC-B mutant lacking all known phosphorylation sites. We conclude that the V883M mutation increases maximal velocity in the absence of CNP, eliminates the requirement for ATP in the CNP-dependent Km reduction, and disrupts the normal inactivation process.

Introduction

C-type natriuretic peptide (CNP) stimulates long bone growth and inhibits meiotic resumption in oocytes by activating the enzyme variously known as guanylyl cyclase (GC)-B, natriuretic peptide receptor (NPR)-2 or NPR-B, which catalyzes the synthesis of the intracellular signaling molecule, cGMP [1–3]. GC-B is a homodimer containing an extracellular ligand-binding domain, a single membrane-spanning region, and an intracellular highly phosphorylated kinase homology domain, dimerization domain and C-terminal GC catalytic domain [4].

CNP binding increases GC-B activity by two mechanisms. It increases the maximal rate of cGMP production called maximal velocity (Vmax) and it also increases the affinity of the enzyme for GTP that is observed as a reduction in the Michaelis constant — the GTP concentration required to reach half the Vmax. Under non-physiologic conditions such as an enzyme assay where ATP is not present, the activity of GC-B is positive cooperative as demonstrated by a Hill coefficient of greater than 1. This means that GTP binds an allosteric site that increases the affinity of the catalytic site for GTP. However, under biological conditions where ATP concentrations are at or above 1 mM, the Hill coefficient of GC-B is 1 because the allosteric site is occupied by ATP not GTP. Recently, we demonstrated that ATP is required for the CNP-dependent reduction in the Km of GC-B [5,6]. Finally, in broken cell assays, ATP also increases GC-B activity by providing the phosphate that is added to the serine and threonine residues on the enzyme that is necessary for activation by CNP [7,8].

GC-B was identified in rat chondrocytes in 1994 [9], but the ability of natriuretic peptides to stimulate skeletal growth was first observed in transgenic mice overexpressing BNP in 1998 [10]. Subsequent bone culture studies indicated that CNP, not BNP, increased the proliferative and hypertrophic zones of the murine growth plate, which increases the length of long bones [10]. CNP also increases the earliest stage of endochondral bone development – the condensation of mesenchymal precursor cells – as well as stimulates glycosaminoglycan synthesis and extracellular matrix production [11,12]. Consistent with the requirement of CNP and GC-B in normal long bone growth in mammals, mice lacking either CNP or GC-B were dwarfs [13,14], and mice lacking the

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Abbreviations: CNP, C-type natriuretic peptide; GC, guanylyl cyclase; NP, natriuretic peptide; WT, wild type.

[☆] Disclosure statement: The authors have nothing to declare.

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http://dx.doi.org/10.1016/j.bone.2013.06.024

natriuretic peptide clearance receptor (NPR-C) that degrades CNP exhibited skeletal hyperplasia [15,16]. In contrast, mice lacking BNP display no skeletal abnormalities [17]. Importantly, CNP and CNP analogs were recently shown to increase long bone growth in murine models of achondroplasia [18–20].

Homozygous inactivating mutations in both alleles of GC-B were identified in humans with acromesomelic dysplasia, type Maroteaux (AMDM) dwarfism [21–23], and heterozygous mutations in GC-B were associated with non-pathological reductions in human stature [24]. Conversely, mutations associated with CNP overexpression were identified in patients with skeletal overgrowth [25,26], and a genome-wide association study identified correlations between genetic mutations that regulate CNP or NPR-C expression and height in Northwestern European populations [27].

In 2012, Miura et al. identified a conserved valine to methionine missense mutation at position 883 in the catalytic domain of human GC-B (V883M-GC-B) in three generations of a Japanese family with skeletal overgrowth, fragile bones and elevated blood cGMP concentrations [28]. Importantly, how this mutation increases GC-B activity was not determined. Here, we show that this single residue substitution increases the maximal velocity of GC-B in the absence of CNP and that CNP reduces the Km of V883M-GC-B an order of magnitude without ATP or without increasing maximal velocity. Unexpectedly, the V883M substitution blocked the normal inactivation process.

Materials and methods

Reagents

^{125}I-cGMP radioimmunoassay kits and ^{32}P-α-GTP were from Perkin Elmer (Waltham, MA). CNP-22 was purchased from Sigma (St. Louis, MO). The plasmids encoding the N-terminally HA-tagged form of WT human GC-B (HA-WT-GC-B) [22] and HA-V883M-GC-B plasmids [28] have been described. The plasmids expressing rat GC-B-7A and GC-B-7E were also previously described [29,30]. The ATDC5 chondrocytes were from ATCC (www.atcc.org).

Cells and transfections

293 neocells were maintained and transiently transfected by the HEPES–calcium-phosphate precipitation method as previously reported [30].

Whole cell cGMP elevation assays

Cyclic GMP concentrations were measured by radioimmunoassay in ethanol extracts of transiently transfected 293 cells that were pre-incubated with 1 mM isobutylmethyl xanthine, a general phosphodiesterase inhibitor, for 10 min before being incubated with increasing concentrations of CNP as previously described [31].

Guanylyl cyclase assays

Crude membranes were prepared at 4 °C in phosphatase inhibitor buffer consisting of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid — pH 7.4, 50 mM NaCl, 20% glycerol, 50 mM NaF, 1 mM EDTA, 0.5 μM microcystin and 1× Roche protease inhibitor cocktail. All assays were performed at 37 °C in a cocktail containing 25 mM HEPES pH 7.4, 50 mM NaCl, 0.1% BSA, 0.5 mM isobutylmethyl xanthine, 1 mM EDTA, 0.5 μM microcystin, 5 mM phosphocreatine, 0.1 μg/μl creatine kinase and 5 mM $MgCl_2$.

The single substrate concentration GC assays were performed using ^{32}P-GTP as substrate in the presence of 1 mM ATP and 1 mM GTP at 37 °C for 3 min as previously described [31]. For the desensitization assays, the reaction was performed using a pool of crude membranes. The reaction was initiated by the addition of pre-warmed cocktail. At the designated times, 0.1 ml aliquots were removed and added to ice-cold tubes containing 0.5 ml zinc acetate to stop the reaction. Alumina column chromatography purified the ^{32}P-cGMP, which was quantified by Cerenkov counting [32].

Substrate-velocity assays were performed for the indicated times with the indicated GTP concentrations. The resulting cGMP concentrations were determined by radioimmunoassay as described [33]. When included, free manganese concentrations in the assays were 2 mM. Because enzymatic activity was not completely linear with time, the kinetic parameters obtained under these conditions are considered “apparent”.

Western blotting

293T cells were transfected with the indicated constructs, immunoprecipitated, fractionated by reducing SDS-PAGE and blotted to an Immobilon membrane for immune-detection as previously described [34]. The blot was blocked and probed with at 1/2500 dilution of rabbit serum 6328 followed by incubation with a 1/20,000 dilution of goat anti-rabbit IRDye 680 conjugated antibody and visualized on a LI-COR instrument as previously described [35].

Statistical analysis

Statistics and graphs were generated with Prism 5 software. Student's paired *t*-test determined significance where $p \leq 0.05$ was considered significant. The vertical bars within the symbols represent the SEM. Where not visible the bars are contained within the symbol. EC_{50} values were calculated based on the nonlinear curve fitting equation $Y = Top * X / (EC_{50} + X)$. Substrate–velocity curves were analyzed using an allosteric sigmoidal model to generate Hill coefficients.

Results

Cyclic GMP is elevated more than twenty-fold in cells expressing GC-B-V883M

HEK293 cells were transiently transfected with human isoforms of HA-WT-GC-B or HA-V883M-GC-B. Two days later, the cells were incubated in the presence of increasing concentrations of CNP for 3 min and intracellular cGMP concentrations were determined (Fig. 1A). Basal (no CNP) cGMP concentrations were elevated 21-fold in cells expressing HA-V883M-GC-B compared to cells expressing HA-WT-GC-B. Maximal concentrations of CNP increased cGMP concentrations 29-fold in HA-WT-GC-B expressing cells but only 2-fold in cells expressing HA-V883M-GC-B. The EC_{50} for CNP activation was not significantly different between the WT and mutant enzymes, consistent with the mutation not affecting the affinity of CNP for GC-B.

Plasmids expressing WT and GC-B-V883M were also transiently transfected into ATDC5 mouse chondrocytic cells that endogenously express GC-B. Since these cells express phosphodiesterases 1 and 5, we pretreated them with a general phosphodiesterase to emphasize cGMP synthesis by GC-B [36]. Overexpression of WT-GC-B slightly elevated cyclic GMP concentrations in the ATDC5 cells, but overexpression of the GC-B-V883M mutant resulted in cGMP levels that were more than four-fold higher than those observed in cells transfected with the WT enzyme (Fig. 1B). These data indicate that the increased basal activity associated with the V883M mutation occurs in a natural cellular environment for GC-B and is consistent with the increased plasma cGMP concentrations measured in patients expressing V883M-GC-B [28].

Basal enzymatic activity of V883M-GC-B is elevated but expression is reduced

GC activity was measured in crude membranes from 293 cells expressing green fluorescent protein (GFP) as a control, WT-GC-B,