Forced Homodimerization by Site-Directed Mutagenesis Alters Guanylyl Cyclase Activity of Natriuretic Peptide Receptor B

Thomas Langenickel, Jens Buttgereit, Ines Pagel, Rainer Dietz, Roland Willenbrock, Michael Bader

Abstract—Natriuretic peptides mediate their physiologic effects through activation of membrane-bound, guanylyl cyclase–coupled receptors (NPRs). Receptor dimerization is an important feature of signal transduction. This study was aimed at characterizing structurally important residues of the extracellular ligand-binding domain of NPR-B for receptor dimerization and cGMP generation. Deletion mutagenesis was used to replace cysteine residues at positions 53 (C53S), 417 (C417S), and 426 (C426S) by serine. Receptor expression, dimerization, whole-cell cGMP response, and guanylyl cyclase activity of membrane fractions were determined in stably transfected COS-7 cells. C53S, C417S, and C426S mutants were expressed and found to form disulfide-bridged covalent dimers. In contrast to NPR-B and C53S, C417S and C426S mutants displayed constitutive activity in whole cells (C417S, 146±12%, P<0.01; C426S, 153±7% of ligand-independent NPR-B cGMP generation, P<0.01). The cGMP response of C417S and C426S mutants in whole cells was dose dependent and ∼4 times lower than that in NPR-B, whereas it was blunted in C53S-transfected cells (1 μmol/L CNP, NPR-B 2868±436%; C53S, 206±16% of control, P<0.001 vs NPR-B, C417S, and C426S). Guanylyl cyclase assay in transfected cells confirmed the constitutive activity of C417S and C426S mutants. These data suggest that receptor dimerization by covalent disulfide bridges alters ligand-independent as well as ligand-dependent receptor activity. Localization of the crosslink in relation to the cell membrane is important for configuration of the extracellular domain and the consequent signal transduction. (Hypertension. 2004;43[part 2]:460-465.)

Key Words: natriuretic peptides ■ receptors, natriuretic peptides ■ cyclic GMP ■ signal transduction
was aimed at characterizing the role of structurally important residues within the extracellular domain of NPR-B for receptor dimerization and cGMP generation.

Methods

Generation of Rat NPR-B Mutants
Full-length cDNA of rat NPR-B (a generous gift from Dr Lincoln Potter, University of Minnesota, Minneapolis) was subcloned into the pcDNA3.1(−)-myc/His vector (Invitrogen) and expressed as the c-Myc/6×His fusion protein under control of a cytomegalovirus promoter. The point mutations C53S, C417S, and C426S were inserted by using a site-directed mutagenesis kit (QuickChange, Stratagene) and were confirmed by sequencing.

Cell Culture and Transfection
All culture media and supplements were obtained from Sigma. COS-7 cells were grown in Dulbecco modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. For transfection, cells were grown in a 35-mm tissue-culture plates. Transfection was performed with Lipofectin reagent (GibcoBRL) according to the manufacturer’s protocol. Twenty-four hours after transfection, cells were selected with DMEM containing 600 μg/mL G-418 (Promega).

Qualitative RNA Expression Analysis
Cells were harvested and washed with phosphate-buffered saline. RNA was extracted from the cell pellet with Trizol reagent (GibcoBRL) according to the manufacturer’s protocol. Two micrograms of total RNA was transcribed into cDNA with an oligo-dT primer (Stratagene RT-PCR kit). Polymerase chain reaction (PCR) was performed to amplify NPR-B and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control.

Membrane Preparations
Cells were washed twice with phosphate-buffered saline and homogenized in ice-cold homogenization buffer (20 mmol/L Tris, 2 mmol/L EDTA, 2 mmol/L EGTA, 20 mmol/L iodoacetamide, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μmol/L pepstatin A, 10 μmol/L benzamidine, and 1 μmol/L leupeptin). The homogenate was centrifuged (45 minutes, 40 000g, 4°C), and the resulting pellet was resuspended in homogenization buffer. The protein concentration was measured with the Bradford reagent.

Western Blotting and Immunodetection
Twenty to 50 μg of membrane proteins was separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The epitope-tagged NPR-B and NPR-B mutants were detected with a primary mouse monoclonal antibody against the C-terminal c-myc (Invitrogen), and then probed with a secondary horseradish peroxidase–coupled anti-mouse antibody with use of an enhanced chemiluminescent system (ECL western blotting analysis system, Amersham).

Whole-Cell Guanylyl Cyclase Stimulation
Stable clones expressing either NPR-B or NPR-B mutants were cultivated on 24-well cluster plates. For guanylyl cyclase stimulation, cells were first incubated twice with serum-free DMEM for 15 minutes and thereafter incubated in 400 μL serum-free DMEM containing 1 mmol/L 3-isobutyl-1-methylxanthine (IBMX), 10 mmol/L theophylline, 10 mmol/L creatine phosphate, 10 U creatine kinase, 1 mmol/L GTP, and 4 mmol/L MnCl₂. Stimulation was carried out with 1% Triton X-100, 4 mmol/L MnCl₂, 1 mmol/L GTP, 1 mmol/L ATP, 1 μmol/L CNP or 1 mmol/L ATP, and 1 μmol/L CNP. cGMP was separated from GTP by chromatography and measured by radioimmunoassay.

Statistics
All values are expressed as mean±SEM. Data were compared by ANOVA, and a probability value <0.05 was considered statistically significant.

Results

mRNA Expression of NPR-B and NPR-B Mutants in COS-7 Cells
Clones of stably transfected COS-7 cells were studied regarding mRNA content of either native or mutated NPR-B. No endogenous NPR-B mRNA could be detected in nontransfected COS-7 cells, and no cGMP response could be achieved by stimulation of nontransfected cells with either ANP or CNP (data not shown). Reverse transcription (RT)–PCR revealed mRNA expression of all transfected constructs, NPR-B, and NPR-B mutants (Figure 1A). GAPDH mRNA expression served as an internal control.

Protein Expression and Structure of NPR-B Mutants
Protein expression and dimer formation of NPR-B and NPR-B mutants were shown in membrane fractions by SDS-PAGE under reducing and nonreducing conditions, followed by Western blotting and immunodetection. Iodoacetamide was used for protein preparation to prevent formation of disulfide bridges during protein isolation. NPR-B was detected under reducing as well as nonreducing conditions as an ∼130-kDa protein, clearly corresponding to the receptor monomer (Figure 1B). To prove our hypothesis that replacement of a cysteine by serine would lead to free reactive cysteine with consecutive formation of disulfide-bridged NPR-B dimers, membrane fractions of cells transfected with C53S, C417S, or C426S mutants were run under reducing and nonreducing conditions. Under reducing conditions, signals were detected at ∼130 kDa, corresponding to receptor monomers, whereas under nonreducing conditions, signals were obtained at ∼260 kDa, corresponding to receptor dimers (Figure 1B). The protein expression levels of NPR-B and all mutants were found to be similar in all preparations.

Whole-Cell Guanylyl Cyclase Activity
To determine the influence of ligand-independent receptor dimerization induced by C53S, C417S, or C426S mutants on receptor activity, basal cGMP formation without ligand stimulation was measured and compared with that of NPR-B. As shown in Figure 2, basal guanylyl cyclase activity was increased in cells expressing C417S or C426S mutants compared with NPR-B (C417S, 146±12% of NPR-B, P<0.01 versus NPR-B; C426S, 153±7% of NPR-B, P<0.01 versus NPR-B). Expression of the C53S mutant had no influence on basal cGMP generation (11±7% of NPR-B).

Ligand-dependent guanylyl cyclase activation was tested by stimulation of transfected cells with CNP at different

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concentrations (Figure 2). Cells transfected with NPR-B demonstrated a dose-dependent cGMP response on stimulation with 1 nmol/L to 1 μmol/L CNP (1 mmol/L, 174±11% of baseline, P<0.001 versus baseline; 1 μmol/L, 2868±435% of baseline, P<0.001 versus baseline). Cells expressing C417S displayed a cGMP response at 10 nmol/L CNP (638±16% of baseline, P<0.001 versus baseline), and the maximum was observed at 100 nmol/L CNP (827±15% of baseline, P<0.001 versus baseline). The response at all ligand concentrations was below that of NPR-B-transfected cells. C53S-transfected cells displayed a blunted cGMP response to 1 μmol/L CNP, **P<0.01 vs NPR-B; C426S, 201±22% of Triton/Mn2⁺, P<0.05 versus NPR-B). The addition of ATP, whose binding to the KHD is essential for receptor activation, resulted in an increase of cGMP formation in membranes from cells transfected with C417S and C426S mutants compared with NPR-B (NPR-B, 117±6%; C417S, 227±22%, P<0.01 versus NPR-B and C53S; C426S, 201±9% of Triton/Mn2⁺, P<0.01 versus NPR-B and C53S; Figure 3). Membranes from cells transfected with the C53S mutant did not show any cGMP response on ATP stimulation (C53S, 103±4% of Triton/Mn2⁺; Figure 3). These data indicate constitutive ligand-independent activity of the C417S and C426S mutants. In contrast, stimulation with the ligand CNP alone led to higher cGMP values in membranes with NPR-B compared with all mutants (NPR-B, 440±52%, P<0.01 vs all mutants; C53S, 158±9%; C417S, 279±26%, P<0.01 versus C53S and C426S; C426S, 243±21% of Triton/Mn2⁺, P<0.01 versus C53S; Figure 3). Stimulation with both CNP and ATP was expected to result in maximal cGMP activation in membranes from cells transfected with NPR-B. Indeed, consistent with CNP stimulation alone, we observed the most pronounced response in NPR-B membranes compared with C53S, C417S, and C426S (NPR-B, 842±39% of Triton/Mn2⁺, P<0.01 versus all mutants; C53S, 158±13%; C417S, 390±26%, P<0.01 versus C53S; C426S, 356±20%, P<0.01 versus C53S; Figure 3).
Discussion

The amino acid sequence within the extracellular domain of NPR-A and -B is highly conserved. Three intramolecular disulfide bridges within the extracellular domain are relevant for formation of the ligand-binding pocket and have been well characterized for NPR-A. This study was aimed at characterizing the cysteine residues of NPR-B at position 53, 417, and 426 regarding their influence on ligand-dependent and -independent receptor activity. We were able to demonstrate that substitution of these cysteines by serine led to formation of intermolecular disulfide linkages between 2 receptor monomers. Whereas the mutations C417S and C426S, which are located close to the cell membrane, resulted in constitutive active receptor dimers, the C53S mutation at a position distal from the cell membrane was inactive.

To analyze the ability of the NPR-B mutants to form receptor dimers, we performed SDS-PAGE on membrane fractions of transfected cells. Under reducing conditions, we detected all receptor mutants at a molecular weight of ~130 kDa, whereas under nonreducing conditions, all receptor mutants were found at a molecular weight of ~260 kDa. NPR-B was detected as an ~130-kDa protein under both reducing and nonreducing conditions, suggesting its presence as monomer. Thus, we conclude that introduction of either mutation led to formation of receptor dimers linked by disulfide bridges. These data are consistent with previously published results on NPR-A. Labrecque et al demonstrated that substitution of cysteine by serine led to formation of intermolecular disulfide linkages between 2 receptor monomers. Whereas the mutations C417S and C426S, which are located close to the cell membrane, resulted in constitutive active receptor dimers, the C53S mutation at a position distal from the cell membrane was inactive.

The influence of the inserted mutations on guanylyl cyclase activity compared with NPR-B was assessed in whole cells as well as in membrane preparations. In whole cells, C417S and C426S mutants had a significantly higher basal activity compared with NPR-B, showing constitutive activity of both mutants. Dimeric tightening of the juxtamembrane domain of NPR-B and guanylyl cyclase activity, as previously shown for NPR-A, are closely related. One might conclude that disulfide bridges close to the cell membrane are formed in C417S and C426S mutants, which result in tightening of 2 receptor monomers. This tightening initiates a conformational change of the receptor, with consecutive dimerization of the intracellular receptor domain and formation of the ATP-binding domain of NPR-B. van den Akker et al revealed the structure of the glycosylated extracellular NPR-A–ligand complex by x-ray crystallography. It was shown that the ligand-binding domain of an NPR-A dimer appears as a V-shape molecule, with a narrowing of the 2 monomers close to the juxtamembrane region. If one assumes high structural homology between NPR-A and NPR-B, these results could explain the constitutive activity of C417S and C426S, as well as the lack of activity of C53S (Figure 4). The intermolecular disulfide bridge of C53S is located at the N-terminus of the receptor, distant from the cell membrane. The resulting A shape of the receptor dimer might exclude a close tightening of both monomers within the juxtamembrane region. One might also speculate that this A-shape configuration impairs ligand binding. The result
The receptor dimer excludes close tightening of both monomers within the juxtamembrane region and impairs ligand binding. Basal cGMP production is therefore not different from that of NPR-B. In contrast, if contact of the 2 monomers is distal from the cell membrane, as it is for C53S, the resulting A shape of the receptor dimer excludes close tightening of both monomers within the juxtamembrane region and impairs ligand binding. Basal cGMP production is therefore not different from that of NPR-B, but the response of C53S to CNP is markedly reduced.

would be a lack of cGMP response on ligand stimulation in cells transfected with C53S, which we confirmed in this study. These data are consistent with recently published results of an NPR-A C74W mutant, which displayed a covalent dimer in an A-shape configuration and a reduced cGMP response to ANP.18 The C417S and C426S mutants responded dose-dependently, but the cGMP response was blunted compared with that of NPR-B. In contrast, if contact of the 2 monomers is distal from the cell membrane, as it is for C53S, the resulting A shape of the receptor dimer excludes close tightening of both monomers within the juxtamembrane region and impairs ligand binding. Basal cGMP production is therefore not different from that of NPR-B, but the response of C53S to CNP is markedly reduced.

Guanylyl cyclase activity was also investigated in vitro in membrane fractions from cells transfected with NPR-B and the NPR-B mutants C417S, C426S, and C53S. After prestimulation with CNP, the membranes were treated under different conditions, including GTP alone (basal), Triton/ATP, CNP, and ATP/CNP. Stimulation with CNP alone displayed the most pronounced cGMP response in membranes from NPR-B-transfected cells. The cGMP response in cells transfected with either mutant was lower than the cGMP response in NPR-B-transfected cells. Maximal catalytic activity of the receptor is achieved when stimulated with ATP/CNP. NPR-B-transfected cells showed the highest activity when stimulated with ATP/CNP compared with all mutants, which might be attributed to receptor desensitization or impaired ligand binding.20 However, the addition of ATP alone led to activation of the NPR-B mutants C417S and C426S. This observation confirmed the constitutive activity of both mutants.

**Acknowledgments**

This project has been supported by research fellowships of the Max-Delbrueck-Center for Molecular Medicine (T.L. and I.P.) and by a grant from Verbund Klinische Phamakologie Berlin-Brandenburg (T.L.). The authors would like to thank Rita Guenzel and Jeannette Mothes for technical assistance and Abdulhameed Aziz for editorial help.

**References**


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_Hypertension_. 2004;43:460-465; originally published online December 22, 2003;
doi: 10.1161/01.HYP.0000110907.33263.0b

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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