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Direct fusion of subunits of heterodimeric nitric oxide sensitive guanylyl cyclase leads to functional enzymes with preserved biochemical properties: Evidence for isoform specific activation by ciguates

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ABSTRACT

Nitric oxide sensitive guanylyl cyclase (NOsGC) is a heterodimeric enzyme consisting of an α and a β subunit. Two heterodimeric enzymes are known to be important for NO-signalling in humans: α_1/β_1 and α_2/β_1. No difference had so far been detected with respect to their pharmacological properties, but as we show in the present paper the new drugs cinaciguat and ataciguat activate the α_1/β_1 form more effectively. Recent evidence suggests that homodimeric complexes of α and β subunits exist in vivo and that these non-heterodimerizing subunits have a separate function from cGMP signaling. To isolate the effect of the α_1/β_1 or α_2/β_1 heterodimeric enzyme in overexpression experiments from potential effects of non-heterodimerizing α_1, β_1 or α_2 subunits, we cloned constructs that guarantee a 1:1 stochiometry between α and β subunits and rule out the presence of homodimers. The carboxy-terminus of the β_1 subunit was directly fused to the amino-terminus of either the α_1 or α_2 subunit. The two different "conjoined" NOsGCs faithfully reproduced the biochemical and pharmacological properties of the α_1/β_1 and α_2/β_1 heterodimeric enzymes including the differential activation by ciguat-activators. Conjoined NOsGCs can be used for isoform specific overexpression in transgenic animals and therapeutic overexpression may be an application in the future. In both cases possible side effects of homodimeric α or β subunits are avoided. Crystallization with the goal of structure determination may also be easier for conjoined NOsGCs because enzyme preparations are more homogenous and are free of "contaminating" homodimers.

1. Introduction

Nitric oxide sensitive guanylyl cyclase (NOsGC) is the major physiological receptor for nitric oxide (NO) throughout the cardiovascular and central nervous system (for reviews see [1] and [2]). Two heterodimeric enzymes are known to be important for NO-signalling in humans: α_1/β_1 and α_2/β_1. Both catalyze the production of the second messenger molecule cGMP from GTP. Only the α_2/β_1 isoform can interact with the PDZ containing protein PSD-95 via its carboxy-terminal sequence and seems to adhere to specialized membrane compartments in some tissues [3]. No other difference has so far been detected in the biochemical and pharmacological properties of both purified enzyme isoforms [4].

It has been shown that co-expression of the α_1 and β_1 subunit in overexpression systems leads to the formation of α_1 homodimers and β_1 homodimers in addition to heterodimers [5]. Since the homodimers are inactive with respect to cGMP formation Zabel et al. suggested the possibility of a physiological equilibrium between homo- and heterodimeric NOsGC complexes and a possible regulation of NOsGC activity by the extent of homodimerization vs heterodimerization. In support of such a concept we have recently shown that NOsGC activity decreases during cerebral postnatal development because of a reduction in heterodimerization and have demonstrated the occurrence of non-heterodimerizing α_1 or β_1 subunits in vivo [6]. Intriguingly there is recent evidence to suggest that both the isolated α_1 subunit and the isolated β_1 subunit have a separate function from cGMP signalling: the α_1 subunit seems to act as an important mediator of the procarcinogenic effect of androgens [7,8]. The β_1 subunit has been suggested to regulate chromatin condensation and cell cycle progression [9].

Abbreviations: BSA, bovine serum albumin; CFP, cyan fluorescent protein; CV, column volume; DTT, dithiothreitol; GFP, green fluorescent protein; NO, nitric oxide; NOsGC, NO sensitive guanylyl cyclase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; S, STREP tag II; TEA, triethanolamine; YFP, yellow fluorescent protein.

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To isolate the effect of the α_1/β_1 and α_2/β_1 heterodimeric enzyme in overexpression experiments from potential effects of non-heterodimerizing α_1, β_1 or α_2 subunits, we cloned constructs that guarantee a 1:1 stochiometry between α and β subunits and rule out the presence of homodimers. Here we provide the pharmacological and biochemical characterization of these "conjoined" NOsGCs. Conjoined NOsGCs will allow facilitated overexpression in transgenic animals and will exclude overexpression of non-heterodimerizing subunits that have effects independent of cGMP signalling. Crystallization with the goal of structure determination should be easier for conjoined NOsGC because enzyme preparations are more homogenous and are free of "contaminating" β_1 homodimers or α homodimers. In addition to providing a novel tool for research, we provide the first evidence of a pharmacological difference between α_1/β_1 and α_2/β_1 heterodimeric enzymes: the ciguat-activators which are currently being developed clinically for the treatment of heart failure and other cardiovascular disease states are more effective activators of the α_1/β_1 isoform.

2. Materials and methods

2.1. Materials

3-(5′-Hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO) and all other chemicals, in the highest grade of purity, were obtained from Sigma–Aldrich (Munich, Germany). Cinaciguat (BAY 58-2667) was a generous gift from Johannes-Peter Stasch (Bayer Schering Pharma, Wuppertal, Germany). Ataciguat (HMR 1766) was a generous gift from Sanofi-Aventis (Frankfurt, Germany). Products for Sf9 cell culture were from Invitrogen (Karlsruhe, Germany). Restriction enzymes and polymerases were obtained from NEB (Frankfurt, Germany).

2.2. Cloning of fluorescent tagged NOsGC subunits

The β_1 subunit cDNA was amplified by RT-PCR from rat heart using cDNA Polymerase Mix and the primer pair P141 (5′-CCG ACA CCA TGT ACG GTT TTG TGA-3′) and P180 (5′-GGG CCC AGT TTT CAT CCT GGT TTG TTT CCT-3′). The resulting PCR product was subcloned into pcDNA3.1/V5/His-TOPO. From this construct the β_1 subunit cDNA was cloned HindIII/ApaI into pEYFP-N1. β_1YFP construct was cloned SpeI/XbaI from pEYFP-N1 into pFASTBAC (Invitrogen; Karlsruhe, Germany).

The NOsGC α_1 subunit was amplified from rat cDNA using the primer pair: P178 (5′-ACA CCG GCT AAT AAG GAG GAA ACC AC-3′) and P179 (5′-ATC TAC CCC TGA GGC CTT GCC TAA GAA-3′). The resulting PCR product was subcloned into pcDNA3.1/V5/His-TOPO (Invitrogen; Karlsruhe, Germany). From this construct the α_1 cDNA was cloned HindIII/XhoI into HindIII/SalI pEYFP-N1 (Clontech; Saint-Germain-en-Laye, France). The carboxy-terminally YFP tagged α_1 subunit was cloned NheI/XbaI into SpeI/XbaI pFASTBAC.

The α_2 NOsGC subunit was cloned BamHI/SspI from pFASTBAC [10] into BglII/BamHI (Klenow fill in) in pEGFP-N1 (Clontech; Saint-Germain-en-Laye, France). From this construct the α_2 subunit was cloned Eco47III/NotI into pECFP-N1(Clontech; Saint-Germain-en-Laye, France) to get the α_2CFP construct. The carboxy-terminally CFP tagged α_2 subunits was cloned Eco47III/NotI into StuI/NotI pFASTBAC vector. ECFP was exchanged with EYFP from pEYFP-C1 (Clontech; Saint-Germain-en-Laye, France) using AgeI/KpnI restriction sites.

2.3. Cloning of conjoined NOsGC

For $\beta_1\alpha_1$ conjoined NOsGC: the α_1 subunit in pcDNA3.1/V5/His-TOPO was amplified by PCR using the primer pair: P270 (5′-GAT CGT GTA CAA GAT GTT CTG CAG GAA GTT CAA AG-3′) and P269 (5′-GAT CGT CTA GAT TAA TCT ACC CCT GAG GCC TTG CC-3′) to create new restriction sites 5′ (BsrGI) and 3′(XbaI) of the α_1 gene. The resulting PCR product was subcloned into pCR2.1TOPO (Invitrogen; Karlsruhe, Germany). From this construct the α_1 NOsGC subunit was cloned BsrGI/XbaI from pCR2.1TOPO 3′ of β_1YFP in pEYFP-N1 to get a β_1YFPα_1 construct. YFP was deleted by the restriction with BsrGI/ApaI and blunt ends were created by mung bean nuclease (NEB; Frankfurt, Germany) treatment. The $\beta_1\alpha_1$ conjoined NOsGC in pEYFP-N1 was cloned SpeI/XbaI in pFASTBAC.

For $\beta_1\alpha_2$ conjoined NOsGC: NOsGC α_2 rat was cloned BstEII (Klenow fill in)/BamHI from pFASTBAC [10] into pEYFP-C1 EcoRI (Klenow fill in)/BamHI. The YFPα_2 cDNA in pEYFP-C1 was digested with BsrGI/NheI to delete YFP from the vector. In this construct β_1YFP was cloned NheI/BsrGI from pEYFP-N1 resulting in the conjoined β_1YFPα_2 (in pEYFP-C1). YFP was deleted by the restriction with AgeI/BspEI and blunt ends were created by Pfu polymerase treatment. The $\beta_1\alpha_2$ conjoined NOsGC in pEYFP-C1 was cloned SpeI/NotI in pFASTBAC.

2.4. Cloning of STREP tagged (S) NOsGC subunits and conjoined NOsGC

The cDNA encoding the carboxy-terminal 228 amino acids of the rat NOsGC α_1 subunit was amplified from the construct above using an antisense primer designed to fuse a carboxy-terminal STREP tag to the α_1 subunit. The following primer pair was used: P289 (5′-AGC AGC TCT GGC AAG GAC AAA T-3′) and P290 (5′-TCT AGA TTA TTT TTC GAA CTG CGG GTG GCT CCA ATC TAC CCC TGA GGC CTT GCC TAA G-3′). The resulting PCR product was subcloned into pCR2.1TOPO. The DNA encoding the STREP tagged carboxy-terminal part of the α_1 subunit in pCR2.1TOPO was cloned BstEII/XbaI into the $\beta_1\alpha_1$ conjoined NOsGC in pFASTBAC replacing the respective carboxy-terminus. Rat α_1 NOsGC with a carboxy-terminal STREP tag was obtained by cloning the amino-terminal part of the rat α_1 NOsGC subunit in pCR2.1TOPO SpeI/BstEII in the preceding pFASTBAC construct thereby deleting the β_1 subunit from the conjoined NOsGC construct.

The cDNA encoding the carboxy-terminal 133 amino acids of the rat NOsGC α_2 subunit was amplified from pFASTBAC [10] using a primer designed to fuse a carboxy-terminal STREP tag to the α_2 subunit. The following primer pair was used: P280 (5′-AAG ACC CAT TCA GAT GCG GAT AGG-3′) and P281 (5′-TTA TTT TTC GAA CTG CGG GTG GCT CAA GAG GCT AGT TTC TCG GAG GAA CAT CG-3′). The resulting PCR product was subcloned into pCR2.1TOPO. The STREP tagged α_1 subunit fragment from pCR2.1TOPO was cloned NheI/NotI into the $\beta_1\alpha_2$ conjoined NOsGC in pFASTBAC (see above).

Rat α_2 NOsGC with a carboxy-terminal STREP tag was obtained by cloning the amino-terminal part of the rat α_2 NOsGC subunit in pFASTBAC [10] NheI/BssHII in the preceding pFASTBAC construct thereby deleting the β_1 subunit from the conjoined NOsGC construct.

The β_1 subunit was also fused with a carboxy-terminal STREP tag. The fluorescent YFP from β_1YFP in pFASTBAC was replaced SmaI/PvuI with the STREP tag cloned StuI/PvuI from the STREP tagged $\beta_1\alpha_1$ conjoined NOsGC in pFASTBAC.

2.5. Generation of recombinant baculovirus, Sf9 cell culture and expression of recombinant guanylyl cyclase subunits

Recombinant baculovirus was generated according to the BAC-TO-BAC™ System (Invitrogen; Karlsruhe, Germany). Sf9 cells were cultured in Sf-900 II serum-free medium supplemented with 1% penicillin/streptomycin and 10% fetal calf serum. Spinner cultures were grown at 27 °C at 140 rpm shaking and diluted to 2×10^6 cells/ml for infection.