Biochemical Pharmacology, Vol. 59, pp. 713–717, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00381-0

Expression of Nitric Oxide-sensitive Guanylyl Cyclase Subunits in Human Corpus Cavernosum

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ABSTRACT. The muscles of the corpus cavernosum of the penis relax in response to stimulation of non-adrenergic, non-cholinergic nerves or nitric oxide (NO)-donating drugs to elicit erection. It is generally assumed that NO mediates this effect via activation of soluble guanylyl cyclase and a subsequent increase in cyclic guanosine 3′,5′-monophosphate concentration. However, there are no data on the expression of this enzyme in human corpus cavernosum. The purpose of the present study was the molecular characterization of NO-sensitive guanylyl cyclase in human corpus cavernosum. RNA was extracted from tissue samples obtained from seven patients undergoing penile prosthetic surgery or correction of penile deviation. Reverse transcriptase–polymerase chain reaction (RT–PCR) with specific primers for the subunits of NO-sensitive guanylyl cyclase was performed, and PCR products were subcloned and sequenced. Specific amplification products encoding the α_1, β_1, α_2, and β_2 subunits were detected. In addition, we isolated a transcript encoding a novel variant β_2 subunit. To test whether this novel transcript arises by alternative splicing or whether it is encoded by a separate gene, a 4000-bp clone of the corresponding genomic DNA sequence was isolated. Sequence analysis suggests that the novel β_2 variant arises by alternative splicing from the same gene as the β_2 subunit on chromosome 13. In conclusion, our findings suggest the presence of different subunit mRNAs of NO-sensitive guanylyl cyclase in human corpus cavernosum. BIOCHEM PHARMACOL **59**;6:713–717, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. corpus cavernosum; guanylyl cyclase; nitric oxide; enzyme isoforms; erectile dysfunction

The muscles of the corpus cavernosum of the penis relax in response to stimulation of non-adrenergic, non-cholinergic nerves or NO§-donating drugs to elicit erection [1–3]. The relaxant effect of NO on corpus cavernosum tissue has been demonstrated *in vitro* [4–6], and NO has now been established as the physiological mediator of penile erection. Stimulation of the relaxant innervation in human corpus cavernosum produces increases in the tissue content of both NO and cyclic GMP [7]. The importance of cGMP is also underlined by the mechanism of action of sildenafil, an inhibitor of cGMP-specific phosphodiesterase type V [8].

NO-releasing drugs have been used in the treatment of erectile dysfunction. Clinical trials with topical nitroglycerin therapy [9] and injection therapy with the NO donor linsidomine chlorhydrate (SIN-1) [10–12] rely both on activation of soluble guanylyl cyclase and a subsequent increase in cGMP. The guanylyl cyclase modulator YC-1 alters soluble guanylyl cyclase sensitivity to NO and CO [13] and possibly represents a novel lead compound for research in erectile dysfunction. Soluble guanylyl cyclase, the pharmacological target of YC-1 as well as NO-releasing drugs, is a heterodimeric enzyme consisting of one α and one β subunit (for review see [14]). The prototypical α_1/β_1 isoform has been purified from native tissue and characterized extensively by several groups. This enzyme contains a prosthetic heme group that mediates activation by NO, leading to a more than 200-fold increase in formation of cGMP from GTP. The corresponding human enzyme has recently been thoroughly characterized after overexpression in Sf9 cells [15]. The natural occurrence of a second NO-sensitive guanylyl cyclase isoform has been demonstrated in human placenta [16]. This α_2/β_1 heterodimeric enzyme also contains a prosthetic heme group and displays similar pharmacological properties as the α_1/β_1 isoform [16]. Less is known about potential enzyme isoforms containing the β_2 subunit [17], although Gupta *et al.* have recently presented evidence for the formation of an NO-sensitive α_1/β_2 heterodimer *in vitro* [18]. However, homodimer formation has recently been described for the human α_1 and β_1 subunit, and by analogy the formation of β_2/β_2 subunit homodimers is also conceivable [19]. Despite the important role of soluble guanylyl cyclase in the physiological function of the human corpus cavernosum, no data are available on the expression of this enzyme and its subunit composition in this tissue.

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§ *Abbreviations:* NO, nitric oxide; cGMP, cyclic guanosine 3′,5′-monophosphate; PCR, polymerase chain reaction; and RT–PCR, reverse transcriptase–polymerase chain reaction.

Received 1 June 1999; accepted 21 September 1999.

MATERIALS AND METHODS

Tissue Samples

Tissue samples were obtained from seven patients undergoing penile prosthetic surgery or correction of penile deviation under general anesthesia. Samples of approximately 100 mg were immediately frozen in liquid nitrogen. Informed consent was obtained from each patient prior to the surgery. The study was approved by the local ethics committee.

RNA Preparation

Total RNA was extracted with RNAzol (Biotecx Laboratories). The preparation was performed on ice. Corpus cavernosum tissue (100 mg) was minced in liquid nitrogen, transferred to a 2-ml Eppendorf cup with 1 mL of RNAzol solution, and homogenized additionally with a Polytron. Chloroform (100 μL) was added, and the samples were shaken vigorously to ensure mixing of the aqueous and organic phases. After centrifugation at 10,000 *g*, the upper aqueous phase (approximately 400 μL) was transferred to an Eppendorf cup together with 400 μL isopropanol and stored on ice for 30 min. The samples were centrifuged at 14,000 *g* for 60 min, the supernatant was discarded, and the pellet was washed with 300 μL of 75% ethanol and dried in an exsiccator for 5 min. The pellet was dissolved in sterile RNase free water and the concentration determined photometrically at 260 nm.

First Strand cDNA Synthesis

First strand synthesis was performed using a kit with Moloney murine leukemia virus reverse transcriptase (Stratagene). Five micrograms of total RNA was adjusted with sterile RNase free water to a final volume of 38 μL. Three microliters of random primers (100 ng/μL) was added and the samples were incubated at 65° for 5 min. After cooling of the samples for 10 min at room temperature, first strand buffer, RNase block inhibitor, and dNTPs were added according to the manufacturer's suggestions. One microliter of reverse transcriptase was added to the reactions and 1 μL sterile RNase free water to the negative control reactions. Samples were incubated at 37° for 1 hr and then at 42° for 30 min before being exposed to a final heat inactivation step at 90° for 5 min. The equivalent of 100 ng reverse-transcribed total RNA was used in the subsequent PCR reactions. Human kidney cDNA was obtained from Clontech Labs (Marathon-ready cDNA).

Genomic DNA

Genomic DNA was prepared from 10 g of human placental tissue. The tissue was minced in liquid nitrogen and was additionally homogenized with a Polytron in 20 mL of a buffer containing sarcosyl (1% w/v), EDTA 0.1 M (pH 8.0), and proteinase K (0.2 mg/mL). The lysate was rotated at 50° for 3 hr in a hybridization oven and subsequently extracted three times with buffer-saturated phenol and three times with diethyl ether. The aqueous phase was digested with RNase A (100 μg/mL) and incubated on ice for 1 hr. The lysate was precipitated with 0.5 volumes of a solution containing PEG (polyethylene glycol) 6000 30% (w/v) and NaCl 1.5 M. After centrifugation for 20 min at 10,000 *g*, the pellet was washed with 70% ethanol, dried in an exsiccator, dissolved in sterile water, and the concentration determined photometrically at 260 nm. Genomic DNA of the monochromosomal cell hybrids was a kind gift from Dr. S. Schweiger of the Max-Planck-Institute for Molecular Genetics (Berlin). An end concentration of 10 ng/μL genomic DNA was used in the polymerase chain reaction.

Primers and Conditions for PCR Analysis

Amplification with AmpliTaq Gold (Perkin Elmer) was performed in a total volume of 50 μL with 40 μL mineral oil overlay and the following end concentration: primers 1 μM each, dNTPs 200 μM, $MgCl_2$ 1.5 mM, AmpliTaq Gold 0.025 U/μL. The following temperature profile was run on an MWG Biotech Thermocycler. Samples were denatured and the polymerase activated by an initial 95° for 10 min, and 35 to 40 cycles of denaturation (94°, 1 min), annealing (50–60°, 1 min), and extension (72°, 1 min) were followed by a final extension step at 72° for 10 min. The further conditions for the individual primer pairs and PCR product lengths were: *human* β_1 *subunit*: $\beta_1$hum1227 (8) TAA GAG CCC TGG AAG ATG AAA AGA and $\beta_1$hum2072 (7) TGG GGT AAT GGA CAA GGA CAA A; 846-bp PCR product, annealing temperature 60°; *human* α_2 *and* α_{2i} *subunit*: $\alpha_2$svsense (6) TGT ACA CCA GAT TTG ACC ACC AGT and $\alpha_2$svanti (5) ACG AGA CCG CGG AAT GAA TG; PCR products of 392 bp for α_2 and 485 bp for α_{2i} [20]; annealing temperature 60°; *human* α_{2i} *subunit*: ivssense (4) TTT TCT CCT TTC CTG TTT CCA TCC and $\alpha_2$svanti (5) ACG AGA CCG CGG AAT GAA TG; 274-bp PCR product; annealing temperature 55°; *human* α_1 *subunit*: α_3_1223 (48) TGC CTC CCT GCT TCC ATA AT and α_3_1710 (49) GTA GAG CCC TCG TCC TGT AAA ATC; 488-bp PCR product; annealing temperature 60°; *human* β_2 *subunit*: β_2-C (40): AGG GAA GAA GGA GCA TGT TGT GTT and β_2-D (41): TCT GCG GAT GCT GAA AAT GTT GA; 460-bp (GenBank Accession No. AF0384499) or 244-bp PCR product (novel variant isolated in this study); annealing temperature 60°. For detection of the corresponding genomic clone, the same primers as for amplification of the cDNA were used. Addition of a final concentration of 5% DMSO was crucial for successful amplification of the 4000-bp genomic β_2 product.

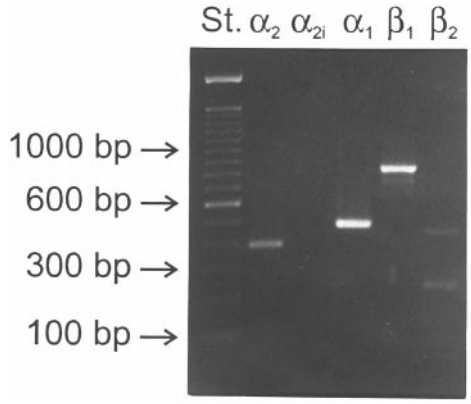

FIG. 1. RT–PCR analysis of guanylyl cyclase subunits in human corpus cavernosum. PCR was performed for 35 cycles using primer pairs specific to the indicated subunit with cDNA from human corpus cavernosum tissue as template. Products were separated on a 1% (w/v) agarose gel and stained with ethidium bromide. St., standard.

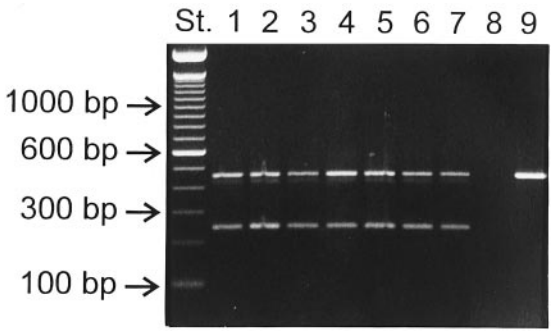

FIG. 2. RT–PCR analysis of guanylyl cyclase subunits in human corpus cavernosum. PCR was performed for 40 cycles using primer pairs specific to the β_2 subunit with cDNA from human corpus cavernosum tissue as template. Products were separated on a 1% (w/v) agarose gel and stained with ethidium bromide. St., standard; 1–7, patients 1–7; 8, negative control; 9, human kidney cDNA.

Sequencing of PCR Products

To ensure specificity of the PCR reactions, PCR products of each subunit amplified from human corpus cavernosum were subcloned using the TA Cloning kit® (Invitrogen) and either partially sequenced by ABI PRISM™ dye terminator cycle sequencing (Perkin Elmer) according to the manufacturer's suggestions or analyzed by extensive restriction analysis. The PCR products of the human β_2 subunit (460-, 244-, and 4000-bp PCR products) were cloned into pCR II and sequenced using the M13 reverse and forward primers or internal primers.

RESULTS

By using RT–PCR, we measured expression of guanylyl cyclase mRNAs in human corpus cavernosum. Using α_2/α_{2i}-specific primers, a single PCR product of 392 bp (α_2), but no PCR product of 485 bp (α_{2i}), was amplified from corpus cavernosum cDNA (Fig. 1). Using primers that were specifically designed to exclusively detect the alternatively spliced form α_{2i}, no signal could be detected. PCR products of 488 and 846 bp representing the α_1 and β_1 subunits, respectively, could be detected with approximately equal intensity. Using primers specific to the amplification of the cDNA encoding the β_2 subunit, two faint bands representing PCR products of 460 and 244 bp were amplified (see Fig. 1). After the number of PCR cycles was increased from 35 to 40, these signals became more intense (Fig. 2). No consistent interindividual differences became apparent by the comparative analysis of the cDNAs of all seven patients included in this study. In cDNA from human kidney, the 244-bp variant transcript was absent, while we were able to amplify the 460-bp form (see Fig. 2). Both β_2 PCR products were subcloned into the pCR II vector and sequenced by dye terminator cycle sequencing (Perkin Elmer). The larger 460-bp product was identical to the DNA sequence of the human β_2 subunit that we recently identified in human heart [21]. The 244-bp PCR product lacked interior 216 bp but was otherwise identical to the published human β_2 subunit. Translation of the DNA sequence into the amino acid sequence showed that the lacking DNA sequence did not alter the reading frame. Comparison of the deduced amino acid sequence with the corresponding human and rat β_2 sequences showed that the novel variant lacks 72 amino acids in its central part (Fig. 3). To determine the genomic structure of the gene in the respective region, a 4000-bp band was amplified from human genomic DNA using the same primers as for the amplification of the cDNA fragment, but under different conditions (see Methods). The genomic 4000-bp PCR product was subcloned into the pCR II vector. Subsequent sequencing of this clone and smaller subclones revealed that it contains four exons designated A, B, C, and D (Table 1). Consensus splice sites of the introns obeyed the GT–AG rule. Comparison of the genomic clone with the novel variant β_2 cDNA demonstrated that it is composed of exons A and D while lacking exons B and C.

These findings suggest that the variant cDNA arises by alternative splicing from the *GUCY1B2* gene that we

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RN β2     114 GKKEHVVFLVVQKAHRQIRGAKASRPQGSEDSQADQE
HS β2     114 GKKEHVVFLIVQKAHRKMRKTKPKRLQDSQGMERDQE
HS β2SV   114 GKKEHVVFLIVQKAHRKMRKTKPKRLQDSQGMERDQE

RN β2     151 ALQGTLLRMKERYLNIPVCPGEKSHSTAVRASVLFGK
HS β2     151 ALQAAFLKMKEKYLNVSACPVKKSHWDVVRSIVMFGK
HS β2SV   151 ------------------------------------

RN β2     188 GPLRDTFQPVYPERLWVEEEVFCDAFPFHIVFDEA
HS β2     188 GHLMSTFEPIYPERLWIEEKTFCNAFPFHIVFDES
HS β2SV   151 ------------------------------------

RN β2     223 LRVKQAGVNIQKYVPGILTQKFALDEYFSIIHPQVTFNISSIC
HS β2     223 LQVKQARVNIQKYVPGLQTQNIQLDEYFSIIHPQVTFNIFSIR
HS β2SV   151 LQVKQARVNIQKYVPGLQTQNIQLDEYFSIIHPQVTFNIFSIR
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FIG. 3. Amino acid alignment of both splice forms of the human β_2 subunit. Shown is a comparison of the deduced amino acid sequences of both splice forms detected in human corpus cavernosum (HS β_2 and HS β_{2SV}) with the corresponding sequence of the rat β_2 subunit cloned from rat kidney (RN β_2). Indicated amino acid residue numbers refer to the rat kidney β_2 subunit (SwissProt Acc. No. P22717).

TABLE 1. Exon/intron boundary sequences within the region of the variant β_2 transcript

Exon	Exon size (bp)	3′ splice site*	5′ splice site*
A	>111		. . . CAAGAG/gtactg
B	111	ctgcag/GCCCTC. . .	. . . GAAAAG/gtaagt
C	103	gtccag/GGCATC. . .	. . . GAATCA/gtaaga
D	>132	ggatag/CTACAG. . .	

The gt/ag consensus sequences of splice junctions are underlined.

*The exonic and intronic sequences are in uppercase and lowercase letters, respectively.

recently mapped to chromosome 13 by fluorescence *in situ* hybridization [21]. This hypothesis is strengthened by the analysis of a monochromosomal cell hybrid panel where single human chromosomes can be tested separately in PCR (Fig. 4). The PCR product of 4000 bp was amplified from the monochromosomal cell hybrid containing human chromosome 13 and from human genomic DNA as positive control. No specific PCR product could be amplified from either of the other cell hybrids. The same 4000-bp genomic fragment was also amplified by PCR from the genomic PAC clone (LLNLP704E17877Q3, [21]) that was used to map the gene of the β_2 subunit *GUCY1B2* to chromosome 13 (data not shown). Taken together, these data make it very likely that the two cDNA variants are encoded by the same gene on chromosome 13.

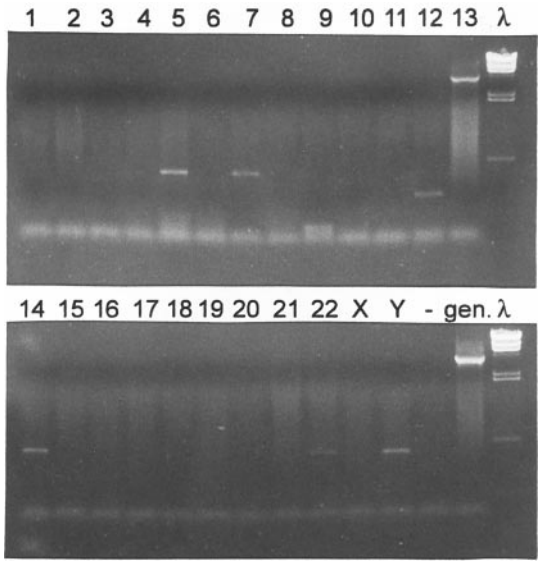

FIG. 4. Assignment of both splice forms of the human β_2 subunit to the same chromosome by PCR amplification of a specific 4000-bp DNA fragment from a somatic cell hybrid mapping panel. PCR was performed with DNA from cell hybrids containing the corresponding human chromosome as indicated: (1–22, X and Y), negative control (−), and human genomic DNA (gen.). Lambda *Hind*III marker (λ) was used as DNA standard. PCR products were separated on a 1% (w/v) agarose gel and stained with ethidium bromide.

DISCUSSION

The present study demonstrates the expression of soluble guanylyl cyclase subunits in human corpus cavernosum on the molecular level. Our data, showing the presence of α_1/β_1 mRNA, suggest that the prototypical α_1/β_1 heterodimeric enzyme is expressed in human corpus cavernosum. Detection of α_2 subunit mRNA in human corpus cavernosum also suggests the presence of the α_2/β_1 heterodimer as a second isoform of soluble guanylyl cyclase. Recently, both of these enzyme isoforms have been detected *in vivo* and have been characterized with respect to their pharmacological properties [16]. Both show sensitivity towards NO-releasing substances and the guanylyl cyclase modulator YC-1.

Recently, Gupta *et al.* demonstrated co-precipitation of the α_1 subunit with the β_2 subunit after co-expression of α_1 cDNA with a β_2 green fluorescence protein fusion construct in COS cells, suggesting the α_1/β_2 subunit heterodimer as another likely isoform of guanylyl cyclase [18]. This notion is strengthened by the finding that co-expression of α_1 cDNA with a β_2 green fluorescence protein fusion construct in COS cells resulted in NO-sensitive guanylyl cyclase activity in the cytosol in this study [18]. The presence of β_2 subunit mRNA in human corpus cavernosum along with α_1 mRNA raises the possibility that this α_1/β_2 heterodimer is also expressed in this tissue. The novel splice variant of the β_2 subunit isolated in this study lacks two exons that are homologous to the N-terminal heme-binding domain of the β_1 subunit. Due to the absence of amino acids that were shown to be critical in heme binding [22], it is likely that a potential enzyme containing the novel splice variant would be NO-insensitive.

The NO–cGMP pathway has been implicated in the pathogenesis of erectile dysfunction, and it has been suggested that biochemical alteration in either production of NO or formation of cyclic GMP could contribute to the disease [2]. We have studied patients with and without erectile dysfunction, and virtually no differences in the expression levels of either of the soluble guanylyl cyclase subunits were apparent. However, we had to do RT–PCR due to the small biopsy specimen obtained, and this method gives qualitative rather than quantitative results. Thus, differences in expression levels may not be completely ruled out. However, data of a functional *in vitro* study by Pickard *et al.* also argue against an abnormality of the cGMP-generating enzyme in human corpus cavernosum in erectile dysfunction [7]. No differences in cyclic GMP content in corpus cavernosum strips from men with vascular impotence compared to controls were found, while there was a diminished formation of NO in the vascular impotence group [7].

In summary, our data suggest the expression of the guanylyl cyclase isoforms α_1/β_1 and α_2/β_1 in human corpus cavernosum which have previously been shown to be sensitive to activation by NO and YC-1. In addition, we

have demonstrated the existence of β_2 subunit mRNA along with a novel variant form of the β_2 subunit that arises by alternative splicing from the β_2 subunit gene *GUCY1B2*. The pharmacological properties of potential enzymes containing these different β_2 subunit forms remain to be elucidated.

The study was supported by the Deutsche Forschungsgemeinschaft (BE 1865/2-1).

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