

## The beta2 subunit inhibits stimulation of the alpha1/beta1 form of soluble guanylyl cyclase by nitric oxide. Potential relevance to regulation of blood pressure.

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### Research Article

Cytosolic guanylyl cyclases (GTP pyrophosphate-lyase [cyclizing; EC 4.6.1.2]), primary receptors for nitric oxide (NO) generated by NO synthases, are obligate heterodimers consisting of an alpha and a beta subunit. The alpha1/beta1 form of guanylyl cyclase has the greatest activity and is considered the universal form. An isomer of the beta1 subunit, i.e., beta2, has been detected in the liver and kidney, however, its role is not known. In this study, we investigated the function of beta2. Immunoprecipitation experiments showed that the beta2 subunit forms a heterodimer with the alpha1 subunit. NO-stimulated cGMP formation in COS 7 cells cotransfected with the alpha1 and beta2 subunits was approximately 1/3 of that when alpha1 and beta1 subunits were cotransfected. The beta2 subunit inhibited NO-stimulated activity of the alpha1/beta1 form of guanylyl cyclase and NO-stimulated cGMP formation in cultured smooth muscle cells. Our results provide the first evidence that the beta2 subunit can regulate NO sensitivity of the alpha1/beta1 form of guanylyl cyclase. Northern analysis for guanylyl cyclase subunits was performed on RNA from kidneys of Dahl salt-sensitive rats, which have been shown to have decreased renal sensitivity to NO. Compared to the Dahl salt-resistant rat, message for beta2 was increased, beta1 was decreased, and alpha1 was unchanged. These results suggest a molecular basis for decreased renal guanylyl cyclase activity, i.e., an increase [...]

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# The $\beta 2$ Subunit Inhibits Stimulation of the $\alpha 1/\beta 1$ Form of Soluble Guanylyl Cyclase by Nitric Oxide

## Potential Relevance to Regulation of Blood Pressure

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### Abstract

Cytosolic guanylyl cyclases (GTP pyrophosphate-lyase [cyclizing; EC 4.6.1.2]), primary receptors for nitric oxide (NO) generated by NO synthases, are obligate heterodimers consisting of an  $\alpha$  and a  $\beta$  subunit. The  $\alpha 1/\beta 1$  form of guanylyl cyclase has the greatest activity and is considered the universal form. An isomer of the  $\beta 1$  subunit, i.e.,  $\beta 2$ , has been detected in the liver and kidney, however, its role is not known. In this study, we investigated the function of  $\beta 2$ . Immunoprecipitation experiments showed that the  $\beta 2$  subunit forms a heterodimer with the  $\alpha 1$  subunit. NO-stimulated cGMP formation in COS 7 cells cotransfected with the  $\alpha 1$  and  $\beta 2$  subunits was  $\sim 1/3$  of that when  $\alpha 1$  and  $\beta 1$  subunits were cotransfected. The  $\beta 2$  subunit inhibited NO-stimulated activity of the  $\alpha 1/\beta 1$  form of guanylyl cyclase and NO-stimulated cGMP formation in cultured smooth muscle cells. Our results provide the first evidence that the  $\beta 2$  subunit can regulate NO sensitivity of the  $\alpha 1/\beta 1$  form of guanylyl cyclase. Northern analysis for guanylyl cyclase subunits was performed on RNA from kidneys of Dahl salt-sensitive rats, which have been shown to have decreased renal sensitivity to NO. Compared to the Dahl salt-resistant rat, message for  $\beta 2$  was increased,  $\beta 1$  was decreased, and  $\alpha 1$  was unchanged. These results suggest a molecular basis for decreased renal guanylyl cyclase activity, i.e., an increase in the  $\alpha 1/\beta 2$  heterodimer, and decrease in the  $\alpha 1/\beta 1$  heterodimer. (*J. Clin. Invest.* 1997; 100:1488–1492.) Key words: guanylyl cyclase • cGMP hypertension • Dahl rat

### Introduction

Cytosolic guanylyl cyclases (GTP pyrophosphate-lyase [cyclizing; EC 4.6.1.2]) are a primary receptor for nitric oxide (NO) generated by NO synthases. These are heme-containing heterodimers, i.e., consisting of one  $\alpha$  and one  $\beta$  subunit (1, 2). NO activates the enzyme by complexing with the central iron in heme (3). Both an  $\alpha$  and a  $\beta$  subunit are required for basal

and NO-stimulated catalytic activity. The  $\alpha 1/\beta 1$  heterodimeric form of guanylyl cyclase is thought to be the universal form. The heterodimer has been purified from lung and brain while message for the  $\alpha 1$  and  $\beta 1$  subunits have been detected by Northern analysis in lung, cerebellum, kidney, heart, liver, skeletal muscle, olfactory mucosa, and tongue (4–6).

Neither  $\alpha$  or  $\beta$  subunits are catalytically active when expressed alone. Recently, it has been shown that dimerization of the COOH termini from both subunits is required for catalytic activity (7). Although each of the subunits show homologies over the entire sequence, the highest is at the carboxy terminus, which forms the catalytic domain and shares significant homology with that of membrane-bound guanylate cyclases and adenylate cyclases (6). The  $\text{NH}_2$  termini are less homologous and are important in the binding and/or signal transduction of the stimulatory binding signal, i.e., NO, to the catalytic domain (7). The  $\text{NH}_2$  termini of both the  $\alpha 1$  and  $\beta 1$  subunits are required for full regulation of enzyme activity via the heme moiety (7, 8).

Recently, cDNA for isomers of the  $\alpha 1$  and  $\beta 1$  subunits, i.e.,  $\alpha 2$ ,  $\alpha 2i$ , and  $\beta 2$  (9–11) have been identified by homology screening. All of the subunits are  $\sim 70$ – $80$  kD. The  $\alpha$  subunit isoforms are known to form heterodimers with the  $\beta 1$  subunit (9, 10). The  $\alpha 1/\beta 1$  heterodimer has the most basal and stimulated activity. The  $\alpha 2/\beta 1$  heterodimer has less basal and stimulated activity, but appears to be similarly regulated. The  $\alpha 2i/\beta 1$  heterodimer has no basal or stimulated catalytic activity (10). This finding has been attributed to an in-frame insert of 31 amino acids within the catalytic domain of the  $\alpha 2i$  subunit (10). The function of the  $\beta 2$  subunit remains enigmatic.

The  $\beta 2$  subunit is a 76.3-kD protein that most closely resembles the 70-kD  $\beta 1$  subunit of lung guanylyl cyclase (11); it is primarily expressed in the kidney and liver. An 86-amino acid carboxy-terminal region extends beyond the carboxy terminus of the  $\beta 1$  subunit. In this study, we show that the  $\beta 2$  subunit can inhibit activation of  $\alpha 1/\beta 1$  form of the enzyme by NO, and reduce NO-stimulated cGMP levels in smooth muscle cells. Furthermore, Northern analysis shows message for the  $\beta 2$  is increased, and for  $\beta 1$  is decreased in the kidney of Dahl salt-sensitive rat compared to the Dahl salt-resistant rat, suggesting a molecular basis for reduced renal sensitivity to NO in this model.

### Methods

**Materials.** Lipofectin reagent for transient transfection and all cell culture media were purchased from Life Technologies (Gibco BRL, Gaithersburg, MD). The cell lines, i.e., COS 7 (African monkey kidney) and rat aortic smooth muscle cells (A7R5) were purchased from American Type Culture Collection (Rockville, MD). The expression vector pGFP-c1 as well as monoclonal and polyclonal antisera against GFP (green fluorescent protein) were purchased from Clontech (Palo Alto, CA). Poly A<sup>+</sup> RNA isolation kits were from Invitrogen Corp (San Diego, CA).

The rat  $\alpha 1$  and  $\beta 1$  full-length cDNA, subcloned into the expres-

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1. Abbreviations used in this paper: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NO, nitric oxide.

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sion vector pcDNA-neo as 2.2-kb and 1.9-Kb fragments, respectively, were a generous gift from M. Nakane (Abbott Laboratories Diagnostic Division, Chicago, IL). Antibodies to the rat  $\alpha 1$  (monoclonal H6) and  $\beta 1$  (polyclonal A794) subunits were obtained from F. Murad (Molecular Geriatrics, Lake Bluff, IL) and D. Garbers (Howard Hughes, Dallas, TX) respectively. Male Dahl salt-sensitive and salt-resistant rats were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN).

**Construction of  $\beta 2$  plasmid.** The full-length cDNA for the rat  $\beta 2$  subunit subcloned into the EcoRI sites of Blue script(KS) was a gift from P. Yuen (Memphis, TN). For construction of the GFP- $\beta 2$  fusion protein, cDNA of the  $\beta 2$  subunit was digested with XmnI/BamHI, and the resulting 2.1-kb fragment was ligated at the SmaI/BamHI sites of pGFP-c1 expression vector. The final construct, pGFPc1- $\beta 2$ , was confirmed by restriction analysis and dideoxy sequencing.

**Transient transfections.** The full-length cDNA of the  $\alpha 1$  and  $\beta 1$  subunits inserted into the expression vector pcDNAneo, and GFP-modified  $\beta 2$  in expression vector pGFP-c1 under the CMV promoter, were transfected into COS 7 and A7R5 cells with Lipofectin as previously described (12). Cells were grown to 60% confluence and transfected in six-well plates. 72 h after transfection, cells were harvested, resuspended in buffer containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM EDTA, 10  $\mu$ g/ml Aprotinin, 100  $\mu$ M Leupeptin, 350  $\mu$ M PMSF, and sonicated at 4°C. The soluble fraction was obtained by centrifugation at 20,000  $g$  for 20 min at 4°C. Each set of transfections was performed in triplicate and repeated two times. Control transfections were also performed with the expression vectors.

**Cell cGMP measurement.** Cells grown in six-well plates were washed twice with ice-cold PBS and incubated for 10 min at 37°C with PBS containing 300  $\mu$ M isobutylmethylxanthine with or without 10  $\mu$ M SIN-1. The media was aspirated, and the cells were lysed with 0.1 M acetic acid (pH 3.5) and subsequently neutralized with 0.1 M sodium acetate. cGMP was measured in the supernatant by using a standard radioimmunoassay (Biomedical Technologies, Inc., Stoughton, MA). Levels of cGMP were normalized to protein content.

**Immunoprecipitation and immunoblots.** Aliquots of the cytosolic fraction of cells (prepared as described above) were briefly centrifuged and cleared by incubation with monoclonal antibody against GFP (1:500 dilution) and 50  $\mu$ l protein A/G sepharose for 30 min. Fractions were then reprecipitated, and the supernatants were incubated with the immunoprecipitating antibody (monoclonal anti-GFP) and 50  $\mu$ l of protein A/G sepharose for 12–16 h at 4°C. The complexes were washed twice with lysis buffer and subjected to SDS/PAGE under reducing conditions and Western blotting. The nitrocellulose membrane was blocked with 5% nonfat milk at 4°C for 12 h, and was then incubated with  $\alpha 1$  antibody at room temperature for 60 min. The blot was then washed and incubated with secondary antibody (mouse anti-Ig-horseradish peroxidase) at room temperature for 40 min. Immunodetection was by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). The blot was then stripped and probed with antibodies to  $\beta 1$  and  $\beta 2$  subunits, respectively.

**SDS/PAGE and Western blotting.** 3% stacking and 7.5% resolving gels were cast, samples were boiled at 95°C for 3 min in Laemmli buffer and briefly centrifuged, and the supernatants were loaded on the gel (2–4  $\mu$ g protein/lane). Resolved proteins were blotted on nitrocellulose membranes, and were probed using specific antibodies against the expressed proteins.

**Northern analysis.** A total of 12  $\mu$ g of poly A+ RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde, and was blotted onto nitrocellulose membrane with 20 $\times$  SSC. RNA was fixed by baking in an 80°C vacuum oven for 2 h. Random prime-labeled cDNA probes were used.  $\alpha 1$ ,  $\beta 1$ , and  $\beta 2$  probes were PvuII/EcoRV (550 bp), KpnI/NaeI (650 bp), and EcoRI/EcoRV (330 bp) fragments, respectively, from the corresponding parent plasmids.

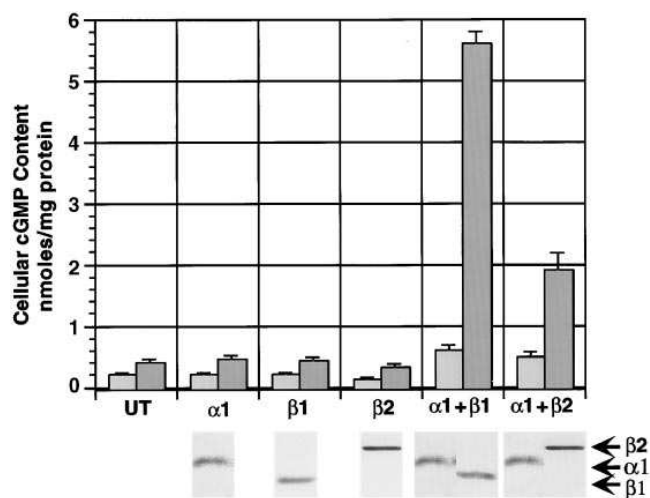
The membrane was prehybridized at 42°C in 5 $\times$  SSC, 5 $\times$  Denhardt's solution, 50% formamide, 0.25% SDS, and 100  $\mu$ g/ml salmon sperm DNA for 3 h, and was hybridized for 16 h with the labeled

probe. For  $\beta 2$  subunit probing, the membrane was washed twice at room temperature with 2 $\times$  SSC and 0.1% SDS for 10 min, and once with 0.1 $\times$  SSC and 0.1% SDS for 15 min. For  $\alpha 1$  and  $\beta 1$  subunit probing, a second wash was performed with 0.5 $\times$  SSC and 0.1% SDS at 65°C for 30 min. Blots were exposed for autoradiography for 10 d. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (13) was probed by rehybridizing the blot at 42°C in the presence of a random prime-labeled 1.2-kb fragment of rat GAPDH cDNA.

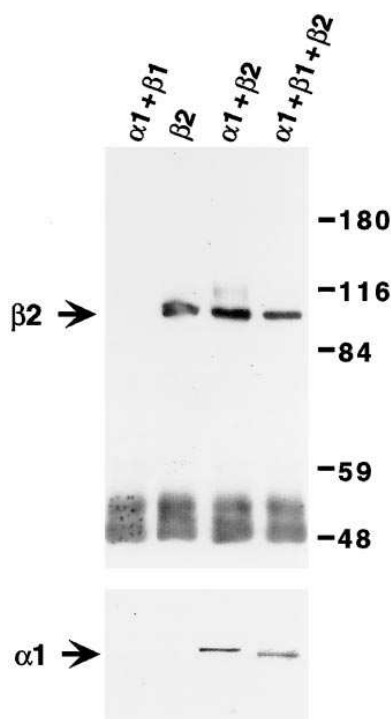
**Statistics.** All results are expressed as means $\pm$ standard error. Differences were assessed by one-way ANOVA or the Student's paired  $t$  test, when appropriate.  $P < 0.05$  was considered significant.

## Results

To determine the interaction of the  $\beta 2$  subunit of soluble guanylyl cyclase with the  $\alpha 1$  subunit and the  $\alpha 1/\beta 1$  heterodimeric form, we coexpressed subunits in COS 7 cells. The  $\alpha 1$ ,  $\beta 1$ , and  $\beta 2$  subunits are not catalytically active when expressed alone. To assess the nature of the activities and interactions of  $\alpha 1$ ,  $\beta 1$ , and  $\beta 2$  subunits of the cyclase, a number of cotransfection studies were performed. In one set of experiments, we coexpressed the  $\beta 2$  subunit with the  $\alpha 1$  subunit. In another set of experiments, the  $\beta 2$  subunit was coexpressed with the  $\alpha 1$  and  $\beta 1$  subunits to determine the effect of  $\beta 2$  on the activity of the  $\alpha 1/\beta 1$  form of guanylyl cyclase. To show that the amount of  $\alpha 1$  and  $\beta 1$  did not change in these experiments, Western blots using antibodies directed against the  $\alpha 1$  and  $\beta 1$  subunits were used. To determine whether the  $\beta 2$  subunit forms a heterodimer with the  $\alpha 1$  subunit and the associations of the  $\alpha 1$ ,  $\beta 1$ , and  $\beta 2$  subunits, immunoprecipitation experiments were



**Figure 1.** Effect of SIN-1 on cyclic GMP content of COS 7 cells transiently transfected with soluble guanylyl cyclase subunits. COS 7 cells ( $1 \times 10^6$ ) were transfected with plasmids containing cDNAs for  $\alpha 1$ ,  $\beta 1$ , and  $\beta 2$  subunits. Cytosolic cGMP levels were determined in the absence (basal) or presence (stimulated) of 10  $\mu$ M SIN-1 (top) (see Methods). In two independent transfections with the expression vectors alone, basal and stimulated cGMP levels were not different from those of untransfected cells (UT) (not shown). Error bars represent standard error;  $n = 3$ . Cytosolic protein (4  $\mu$ g/lane) was separated by SDS-PAGE. After transfer to nitrocellulose membranes, guanylyl cyclase subunits were detected using antibodies to the  $\alpha 1$  and  $\beta 1$  subunits and the GFP-fusion protein (to detect  $\beta 2$ ) (bottom) (see Methods). Light gray bars, minus SIN-1; dark gray bars, plus SIN-1.



**Figure 2.** Detection of heterodimer formation by immunoprecipitation with GFP-specific antibody, and subsequent immunoblotting. Cytosolic fractions of COS 7 cells transfected with GFP-modified  $\beta 2$ , and either  $\alpha 1$  or  $\alpha 1$  plus  $\beta 2$  were immunoprecipitated using an antibody to the GFP-fusion protein and analyzed by Western blotting as described in Methods. An anti- $\alpha 1$  antibody was used to detect  $\alpha 1$ .  $\beta 2$  was detected with an antibody to GFP. No signal was detected with anti- $\beta 1$  antibody (not shown).

performed. In the last experiments, we assessed the effect of  $\beta 2$  on NO-stimulated cGMP formation in cultured rat aortic smooth muscle cells.

cGMP formation in the transfected COS 7 cells was determined under basal and NO-stimulated conditions. cGMP under basal conditions in COS 7 cells was 0.2 nmol/mg protein. Treatment with 10  $\mu$ M SIN-1 for 15 min resulted in a twofold increase in cGMP. Neither basal nor stimulated cGMP formation was altered when cells were transfected with either  $\alpha 1$ ,  $\beta 1$ , or  $\beta 2$  subunits alone.

**$\alpha 1/\beta 1$  and  $\alpha 1/\beta 2$  activity.** In COS 7 cells in which the  $\alpha 1$  and  $\beta 1$  subunits were coexpressed, basal cGMP was increased fourfold, and SIN-1 resulted in a further sixfold increase in cGMP, i.e., to 5.5 nmol/mg protein. COS 7 cells in which both the  $\alpha 1$  and  $\beta 2$  subunits were coexpressed had the same basal

cGMP level as those transfected with the  $\alpha 1/\beta 1$  subunits. SIN-1, however, resulted in an increase in cGMP of approximately twofold, i.e., 1.9 nmol/mg protein, or  $\sim 1/3$  of that seen in  $\alpha 1/\beta 1$  cotransfected cells (Fig. 1).

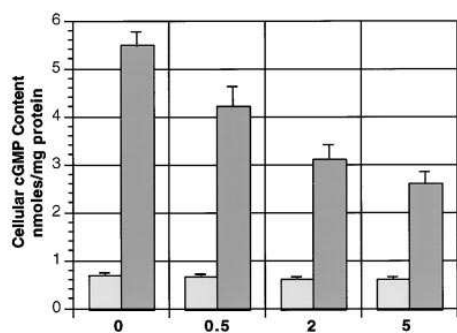
**$\alpha 1/\beta 2$  subunit association.** To determine whether the  $\beta 2$  subunit formed a heterodimer with the  $\alpha 1$  subunit, the latter was coexpressed with the  $\beta 2$ -GFP fusion protein. Immunoprecipitation was performed using a monoclonal antibody to the GFP protein. The immunoprecipitated proteins were analyzed by Western analysis with antibodies against the  $\alpha 1$  and  $\beta 1$  subunits and the GFP protein (to detect the  $\beta 2$  subunit). Both the  $\alpha 1$  and  $\beta 2$  proteins were present in the immunoprecipitated fraction (Fig. 2), indicating that the  $\beta 2$  subunit forms a heterodimer with the  $\alpha 1$  subunit. Control experiments were performed to show (a) that the  $\alpha 1$  subunit did not bind to the GFP protein, i.e.,  $\alpha 1$  did not immunoprecipitate from the lysate of cells transfected with the expression vector and the  $\alpha 1$  subunit, and (b) that  $\alpha 1$  and  $\beta 1$  do not bind nonspecifically to the Sepharose matrix.

We further investigated whether  $\beta 2$  could bind to the  $\alpha 1/\beta 1$  form of the enzyme to form a heterotrimer. The  $\alpha 1$ ,  $\beta 1$ , and modified  $\beta 2$  subunits were coexpressed in COS 7 cells. Immunoprecipitation was performed with a monoclonal antibody to the GFP protein. The  $\alpha 1$  and  $\beta 2$  subunits were present in the immunoprecipitated fraction (Fig. 2). The  $\beta 1$  subunit was not detected, implying that a heterodimer was formed consisting of the  $\alpha 1$  and  $\beta 2$  subunits, and that the  $\beta 1$  subunit did not bind to the complex, i.e., a heterotrimer was not formed.

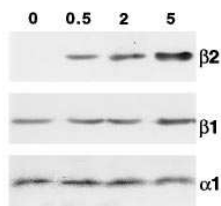
**Effect of  $\beta 2$  on the activity of the  $\alpha 1/\beta 1$  form of guanylyl cyclase.** We investigated whether the  $\beta 2$  subunit could inhibit the activity of  $\alpha 1/\beta 1$  heterodimer. Coexpression of increasing amounts of the  $\beta 2$  subunit with constant amounts of the  $\alpha 1$  and  $\beta 1$  subunits in COS 7 cells resulted in a decrease in SIN-1-stimulated cGMP without significant decreases in basal levels (Fig. 3 A). To rule out that inhibition was due to a decrease in the expression level of the  $\alpha 1$  or  $\beta 1$  subunits, Western analysis was performed. The amount of  $\alpha 1$  and  $\beta 1$  was unaffected by the expression of  $\beta 2$  (Fig. 3 B).

**Effect of  $\beta 2$  on NO-stimulated cGMP formation in smooth muscle cells.** In these experiments, cultured aortic smooth muscle cells were transiently transfected with the GFP-modified  $\beta 2$  in the same manner as COS 7 cells. SIN-1 stimulated

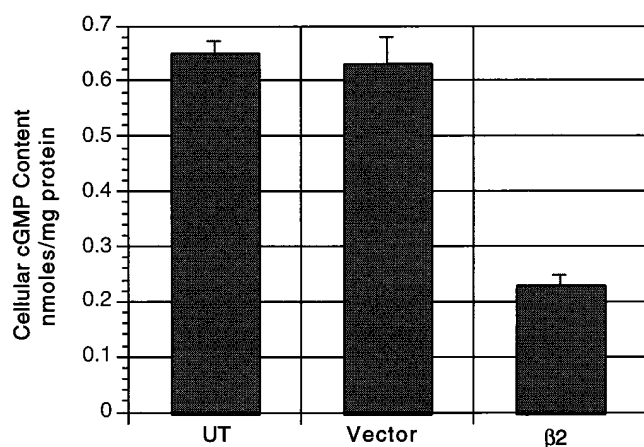
**A**



**B**



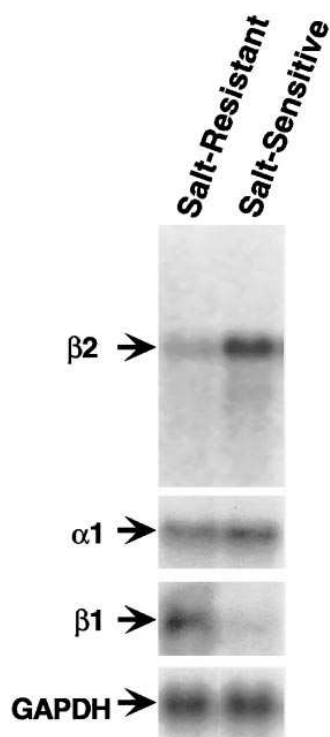
**Figure 3.** Dose response effect of the  $\beta 2$  subunit on the NO-stimulated cGMP responses of the  $\alpha 1/\beta 1$  form of soluble guanylyl cyclase. (A) COS 7 cells ( $2 \times 10^6$  per well) were cotransfected with 5  $\mu$ g each of both the  $\alpha 1$  and  $\beta 1$  plasmids and increasing concentrations of the  $\beta 2$  plasmid (0.5–5.0  $\mu$ g). Cell cGMP content was determined in the presence and absence of 10  $\mu$ M SIN-1 (see Methods). Endogenous cGMP levels ranged from 0.20–0.65 nmol/mg protein. Error bars represent Mean  $\pm$  SE of triplicate determinations from one representative experiment. Light gray bars, minus SIN-1; dark gray bars, plus SIN-1. (B) Western analysis of cytosolic protein extracts from the same cells in which cGMP content was measured. Cytosolic proteins (4  $\mu$ g/lane) were separated by SDS-PAGE, blotted onto nitrocellulose, and probed separately with antibodies to the  $\alpha 1$ ,  $\beta 1$ , and  $\beta 2$  subunits.



**Figure 4.** NO-stimulated cGMP content in rat aortic smooth muscle cells transiently transfected with the GFP-modified  $\beta 2$  plasmid. Cultured rat aortic smooth muscle cells ( $2 \times 10^6$  cells/well) were transfected with 5  $\mu$ g of GFP-modified  $\beta 2$  plasmid. Smooth muscle cell cGMP levels were determined as described in the Methods, in the presence of 10  $\mu$ M SIN-1 concentration. Control transfection with the expression vector pGFP-c1 did not change basal or SIN-1 stimulated cGMP levels. Basal levels of cGMP ranged from 0.35–0.42 nmoles/mg protein. Bars represent mean  $\pm$  standard error. UT, untransfected.

(10  $\mu$ M, 10 min, 37°C) cGMP levels were reduced by  $\sim 33\%$  compared to untransfected cells, i.e., 0.26 nmol/mg protein versus 0.65 nmol/mg protein ( $P < 0.05$ ) (Fig. 4).

**Northern analysis for guanylyl cyclase subunits in the Dahl salt-sensitive rat.** Finally, we compared guanylyl cyclase subunit message in the kidney of Dahl salt-sensitive and salt-resistant rats by Northern analysis. Message for the  $\beta 2$  subunit was significantly more abundant in kidney from the Dahl salt-sen-



**Figure 5.** Northern analysis for soluble guanylyl cyclase subunits in Dahl salt-sensitive and salt-resistant rat kidneys. 12  $\mu$ g poly A<sup>+</sup> RNA was prepared from Dahl salt-sensitive and salt-resistant rat kidney and electrophoresed, blotted, fixed to nitrocellulose membrane, and hybridized to random prime labeled probes for  $\alpha 1$ ,  $\beta 1$ , and  $\beta 2$  (see Methods). GAPDH was used to confirm equal RNA loading in both lanes.

sitive rats while the  $\beta 1$  subunit message was more highly expressed in the kidney from the Dahl salt-resistant rat (Fig. 5). Approximately equal levels of  $\alpha 1$  message were detected in both Dahl salt-sensitive and salt-resistant rats.

## Discussion

This study indicates that the  $\beta 2$  subunit complexes with the  $\alpha 1$  subunit to form a heterodimer with reduced sensitivity to NO, and can inhibit the NO-stimulated cGMP accumulation in cells containing the  $\alpha 1/\beta 1$  form of guanylyl cyclase by exchanging with the  $\beta 1$  subunit. The ability of  $\beta 2$  to inhibit guanylyl cyclase activity may be important in the pathogenesis of hypertension in the Dahl rat.

That the  $\beta 2$  subunit forms a heterodimer with the  $\alpha 1$  subunit is consistent with the prevailing view that  $\alpha 1$  complexes to a  $\beta$  subunit to form a heterodimer (14), and generalizes the hypothesis that both  $\alpha$  and  $\beta$  subunits are interchangeable. There is no evidence for the formation of heterotrimers, i.e.,  $\alpha 1$ ,  $\beta 1$ , and  $\beta 2$ , suggesting that  $\beta 2$  can exchange with the  $\beta 1$  subunit. Our data also indicates that, like other heterodimeric forms of guanylyl cyclase, the  $\alpha 1/\beta 2$  heterodimer contains a heme group, since its activity is regulated by NO. The histidine 105 of the  $\beta 1$  subunit has been shown to be a likely residue forming a linkage to the central iron of the heme moiety, since substitution of the histidine yielded an NO-insensitive enzyme lacking the prosthetic heme group (15). Interestingly, the  $\beta 2$  subunit does not have a histidine between residues 86 and 129, suggesting that the heme–ligand complex interaction with the subunits is different in heterodimers involving the  $\beta 2$  subunit. We found less NO-stimulated cGMP formation in COS cells transfected with the  $\alpha 1/\beta 2$  heterodimer versus the  $\alpha 1/\beta 1$ , indicating that the  $\alpha 1/\beta 2$  heterodimer is less NO-sensitive. Whether this result is due to reduced complexation of NO to the heme iron or to activation of the enzyme by the NO–heme complex cannot be determined from these experiments. Reduced activity of heterodimers containing the  $\beta 2$  subunit, however, is consistent with deletion mutant experiments that have shown that the 64 NH<sub>2</sub>-terminal amino acids of the  $\beta 1$  subunit are necessary for regulation by NO, since these are not present in the  $\beta 2$  subunit (7).

We also show that the  $\beta 2$  subunit inhibits stimulation of cGMP formation in COS cells transfected with the  $\alpha 1/\beta 1$  form of guanylyl cyclase and endogenous NO-sensitive guanylyl cyclase activity in smooth muscle cells. This study suggests that the mechanism is an exchange of  $\beta$  subunits and formation of the  $\alpha 1/\beta 2$  form of the enzyme. We have previously shown that adrenal smooth muscle cells contain message for the  $\alpha 1$  and  $\beta 1$  subunits (16), however, whether aortic smooth muscle cells contain the same subunits is not known. Interactions of the  $\beta 2$  subunit with other subunits, i.e.,  $\alpha 2$  and  $\alpha 2i$ , have not been characterized.

Our results suggest that  $\beta 2$  may play a role in normal physiology and pathophysiology by regulating NO-sensitivity. These results extend the proposal that differential expression of  $\alpha$  isoforms ( $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 2i$ ) modulates NO-sensitive guanylyl cyclase activity (10, 17) by suggesting that differential regulation of the  $\beta$  subunits ( $\beta 1$  and  $\beta 2$ ) may also regulate NO-sensitive guanylyl cyclase activity in cells. Whether expression of the  $\beta 2$  subunit is constitutive or regulated is not known. In a study by Ujiie et al. (5) in which the differential expression of mRNA for the subunits was investigated in the rat kidney, the

$\beta 2$  subunit was detected only 29% of the time in the interlobular artery/afferent arteriole as opposed to the detection of message for  $\alpha 1$ ,  $\alpha 2$ , and  $\beta 1$  subunits 100% of the time. This result may provide evidence not only that  $\alpha 1$ ,  $\beta 1$ , and  $\beta 2$  subunits may be present in the same cell, but also that the expression of  $\beta 2$  is variable, and therefore, regulated in cells containing  $\alpha 1$  and  $\beta 1$  subunits.

The Dahl salt-sensitive rat develops hypertension when fed a high-salt diet (18, 19). Increased renal vasculature resistance and reduced salt excretion have been determined in the Dahl salt-sensitive rat, and have been postulated to play a central role in the pathogenesis of hypertension (20, 21). NO reduces renal vascular resistance, and increases sodium excretion (22, 23). It has recently been shown that nitroprusside causes less of an increase in cGMP excretion in Dahl salt-sensitive versus salt-resistant rats (24), suggesting that there is a reduction in the sensitivity of renal guanylyl cyclase to NO. Our results may provide a molecular explanation for reduced stimulation of cGMP by NO in the Dahl salt-sensitive rat. Message for  $\beta 2$  is increased, and  $\beta 1$  is decreased in the kidney of the Dahl salt-sensitive compared to the Dahl salt-resistant rat, suggesting that reduced sensitivity to NO may be linked to an increase in the less NO-sensitive  $\alpha 1/\beta 2$  form, and/or decrease in the more active  $\alpha 1/\beta 1$  form of guanylyl cyclase in the kidney. The association between  $\beta 1$  and  $\beta 2$  expression remains to be determined. It is possible that expression of the  $\beta$  subunits is interdependent, i.e., increased  $\beta 2$  expression reduces  $\beta 1$  expression, or decreased  $\beta 2$  expression leads to increased  $\beta 1$  expression, or that they are independent of each other, suggesting two primary changes.

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