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C-type natriuretic peptide decreases soluble guanylate cyclase levels by activating the proteasome pathway

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Abstract

Natriuretic peptides (NP) activate particulate guanylate cyclase (pGC) and nitric oxide (NO) activates soluble guanylate cyclase (sGC). Both guanylate cyclases catalyse the formation of the same second messenger, cyclic guanosine 3\5\-monophosphate (cGMP), which activates the cGMP-dependent protein kinases (PKG). PKG then starts a signalling cascade that mediates many cardiovascular and renal effects, such as smooth muscle relaxation and diuresis. Many cell types possess both sGC and pGC. Because both GC-cGMP systems play complementary roles, an interaction between the two pathways might represent an important physiological control mechanism. In this report we demonstrate an interaction between the two pathways. C-type natriuretic peptide (CNP) decreased the h-subunit of sGC (sGC-h) steady-state protein levels and enzymatic activity in cultured human mesangial cells (HMC) in a time- and dose-dependent manner. This down-regulation was not dependent on changes in sGC-h mRNA levels. Treatment of the cells with the stable cGMP analogue 8-Br-cGMP or the phosphodiesterase type-5 inhibitor Zaprinast produced the same down-regulatory effect. Inhibition of PKG or proteasome activity prevented the CNP-induced reduction of sGC-h protein levels and activity. Taken together, these results demonstrate that pGC activation induces a post-transductional down-regulation of sGC by a mechanism involving PKG and the proteasome pathway.

Keywords: Soluble guanylate cyclase; Particulate guanylate cyclase; Cross-regulation; Protein degradation; Proteasome; cGMP

1. Introduction

Vascular tone is determined by the balance between vasodilators such as nitric oxide (NO), natriuretic peptides (NP), and prostacyclin and vasoconstrictors such as endothelin-1 and angiotensin II [1]. NO and NP initiate multifaceted actions that reduce vascular tone, inhibit platelet aggregation, and promote natriuresis and diuresis [2,3]. NP activates particulate guanylate cyclase (pGC) and NO activates soluble guanylate cyclase (sGC). The pGC is a homodimeric transmembrane receptor and the type B isoform (pGC-B) is activated by the C-type natriuretic peptide (CNP) [4]. CNP and pGC-B comprise a vascular natriuretic peptide paracrine system with ligand and receptor being expressed in endothelial and smooth muscle cells, respectively [5]. The sGC is a cytoplasmic heterodimeric hemoprotein that is composed of two different subunits, a and h. The main sGC isoform, widely expressed in human tissues, is a1h1 [6].

Both GCs catalyse the formation of cyclic guanosine 3\5\-monophosphate (cGMP). The cellular concentration of cGMP is determined by both GC activity and phosphodiesterase (PDE) activity, especially PDE type-5 (PDE-5) [7]. cGMP, acting as a second messenger, activates cGMP-dependent protein kinases (PKG) which initiate a protein phosphorylation cascade that mediates the physiological response [8]. Both GC pathways have feedback regulation. NO-sGC is regulated by the concentration of NO, possibly through a cGMP-dependent pathway [9–11]. NP-pGC also incorporates a self-regulating mechanism [12–15].

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Many cell types possess both sGC and pGC systems that serve complementary functions [16]. Thus, the two pathways probably interact to regulate cGMP levels. Previous reports showed such an interaction, but the observed effects appear to be species- and tissue-specific. NO donors have been reported to increase [17,18], decrease [19], or have no effect [20–22] on the response of blood vessels to ANP, a CNP-related peptide that activates pGC-type A. A lack of effect is also observed in airway smooth muscle cells in culture [23]. Similarly, ANP has been reported to both increase [24] and decrease [25,26] the activity of the NO-sGC pathway in cultured cells. A recent report demonstrated that both GCs cooperatively regulate cGMP-mediated vasorelaxation in human vascular tissue and in eNOS knockout mice that lack endogenous endothelial-derived NO production [27]. In the same murine model, Gyurko et al. [28] demonstrated an up-regulation of prepro-ANP mRNA expression.

The molecular mechanism by which such cross-regulation occurs has not been completely elucidated. In this paper we report a functional interaction between pGC and sGC by analysing the effect of CNP on the levels of the h-subunit of sGC (sGC-h) in cultured human mesangial cells (HMC). We show that CNP decreases sGC-h protein levels and activity by a cGMP-dependent mechanism. This reduction is achieved, at least in part, through increased degradation of sGC-h by proteosomes and occurs without changes in sGC-h mRNA expression.

2. Materials and methods

2.1. Materials

CNP, 8-bromoguanosine 3\5\-cyclic-monophosphate, the antagonist of PKG type I Rp-8-[(4-chlorophenyl)thio]-cGMPs triethylamine (Rp-cGMPs), sodium nitroprusside (SNP), anti-h-tubulin antibody, isobutylmethylxanthine (IBMX), triethanolamine-HCl (TEA), dithiothreitol, GTP, phosphocreatine, creatine kinase, phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin A, aprotinin, ammonium persulfate, Triton X-100 and ponceau red were purchased from Sigma Chemical (St. Louis, MO, USA). Zaprinast was provided by May & Baker Ltd (Dagenham, England). B136 pGC antibody was kindly provided by Suntory Co. (Osaka, Japan). sGC-h antibody and Z-LLF-CHO proteasome inhibitor were purchased from Calbiochem (La Jolla, CA, USA). Spermine NONOate (spNO) was provided by Alexis Biochemicals (Carlsbad, CA, USA), Peroxidase-conjugated goat anti-rabbit Ig was purchased from Chemicon (Temecula, CA, USA). RPMI 1640, foetal calf serum, trypsin-EDTA (0.02%) and penicillin-streptomycin were purchased from Bio Media (Boussens, France). a-[^{32}P]-dCTP, RediPrime radiolabelling system, nitrocellulose membrane, G-Sepharose beads, low and high molecular weight standards were purchased from Amersham-Pharmacia (Piscataway, NJ, USA). BCA protein assay reagent was purchased from Pierce (Rockford, IL, USA). [^{35}S]-methionine/cysteine was provided by Applied Biosystems (Foster City, CA, USA). All the other reagents were of the highest commercially available grade.

2.2. Cell culture

HMC were cultured in RPMI medium containing 10% foetal bovine serum according to previously described procedures [29]. When cells reached confluence, they were subcultured at a ratio of 1:4, using the same incubation medium. The identity of the cells was confirmed by morphological and functional criteria, as previously described [30]. Experiments were performed in passages 3–5. When the cells reached 100% confluence, they were serum-deprived for 24 h before the treatments.

2.3. Protein extraction and Western blot analysis

Following treatment, HMC were washed briefly in PBS and solubilized in lysis buffer (10 mM Tris–HCl pH 7.4, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 500 nM sodium orthovanadate, 50 nM NaF, 1 Ag/ml pepstatin/ leupeptin/aprotinin, 1 mM phenylmethtylsulfonyl fluoride) for 40 min at 4 jC. Protein concentration was determined using the BCA method as recommended by the manufacturers.

To perform Western blot analysis, total cell extracts (30 Ag/lane) were size-fractionated by SDS-PAGE on 7.5% polyacrylamide gels and transferred in 20% methanol to polyvinylidene difluoride membranes (Perkin Elmer, Boston, MA, USA). Ponceau red staining was used to verify equal loading of membranes. The membranes were blocked with Tris-buffered (50 mM) saline solution (pH 7.6) with 0.05% Tween (TBS-T) containing 5% milk powder at room temperature for 1 h. The membranes were incubated with a 1:5000 dilution of a rabbit polyclonal antibody against sGC-h, washed and incubated with a peroxidase-conjugated goat anti-rabbit Ig. The presence of sGC (h) was detected using the SuperSignal system (Pierce) and autoradiography. The films were then scanned and analyzed using appropriate software (NIH Image 1.55 from the National Institutes of Health, Bethesda, MD, USA).

Linearity of sGC-h immunodetection procedure was demonstrated by performing a standard curve with serial dilutions of cellular protein extracts (3.75 to 200 Ag of protein). The optical density of each standard protein was then plotted against its concentration, and regression analysis was used to calculate the concentration of GCs/Ag of total cell lysate. At least two different gels containing increasing concentrations of total cell lysate were analysed, obtaining a linear curve between concentration and optical density (r^2>0.9). To ensure linearity, multiple exposures of each blot were always evaluated. In some experiments, the blots were stripped and reblotted against h-tubulin antibody to assure that GCs protein changes were specific.

2.4. RNA extraction and analysis of mRNA expression by Northern blots

HMC were homogenized using guanidinium isothiocyanate, and total RNA was isolated by repeated phenol–chloroform extractions and isopropanol precipitation as described [31]. Total RNA (10 μg per lane) was denatured by heating in formamide/formaldehyde at 65 °C for 5 min and electrophoresed through a 1% agarose gel containing 0.66 M formaldehyde. To confirm the quantity and quality of total RNA, the 18 S ribosomal RNA concentration in each lane was quantified in the ethidium bromide-stained gels. The RNA was transferred to a nitrocellulose membrane and UV-cross-linked. The membranes were prehybridized at 42 °C for 24 h in 5× SSPE (0.75 M NaCl, 0.05 M $NaPO_4H_2$, 6 mM EDTA, pH 8), 5× Denhardt's solution (0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 5% formamide, 0.5% SDS) and 0.1 mg/ml denatured salmon sperm DNA. The membranes were hybridized at 60 °C in hybridization solution (10% dextran sulfate, 1% SDS, 1 M NaCl, 0.1 mg/ml denatured salmon sperm DNA) with α-^{33}[P]-dCTP 10^6 cpm/ml of radiolabelled sGC cDNA probe. A 408-bp cDNA fragment of sGC was obtained by RT-PCR from HMC total RNA using the following primers based on the human sGC-h1 sequence [32] (forward, base position 350 5′-CGTGTCCTGGGCTCTAA-3′ and reverse, base position 774 5′-ACCACTAGGTCCCGGTC-3′). The nucleotide sequence was determined to confirm that no errors had been introduced by PCR amplification. Membranes were washed twice for 5 min each in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) and 0.5% SDS at 42 °C. Autoradiography was performed at −80 °C for 24–48 h. Densitometric analysis of the exposed films was performed with a scanner and analysed using NIH Image 1.55 software.

2.5. sGC activity assay

The enzimatic assay was performed as described previously [33] with slight modifications. In brief, cells were scrapped in 1 ml of Hanks' balanced salt solution (HBSS; Gibco-BRL, Gaithersburg, MD), and pelleted at 10,000× g for 30 s. The pellet was resuspended in 100 μl of homogenization buffer consisting of 100 mM triethanolamine-HCl (TEA), 5 mM $MnCl_2$, 1.25 mM IBMX, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM PMSF, pH 7.4. Samples were homogenized by sonication and centrifuged at 10,000× g for 10 min at 4°C. The cytosolic fraction was obtained by centrifuging the supernatant fraction at 100,000× g for 1 h at 4 °C. Assays for sGC activity were conducted in a final volume of 50 μl consisting of 40-μl aliquots of each sample and 5 μl of a co-factor solution consisting of 1 M TEA, 50 mM $MnCl_2$, 20 mM dithiothreitol, 1 mM GTP, 50 mM phosphocreatine, 1520 U/l creatine kinase, 10 mM IBMX, 80 μg/ml leupeptin, 80 μg/ml pepstatin A, 8 mM PMSF at pH 7.4. The reaction was started with 5 μl of 10 mM SNP or solvent alone (control). After 15 min at 37°C, assays were terminated by adding 0.9 ml of 50 mM sodium acetate buffer, pH 4.0, and heated for 3 min at 90 °C. cGMP levels were measured using a commercial [^{125}I]-cGMP radioimmunoassay kit (Amersham, Arlington Heights, IL).

2.6. Measurement of cGMP synthesized by sGC in HMC

Control and CNP-treated confluent cells were washed three times with buffer A (Tris 20 mM, NaCl 130 mM, KCl 5 mM, sodium acetate 10 mM, glucose 5 mM, pH 7.45). Cells were then preincubated with the same buffer containing 2.5 mM Ca^{2+} and IBMX 10^{-4} M. Reactions were started with the addition of NO (spNO 10^{-6} M) for 30 min. The medium was aspirated and 1 ml of ice-cold ethanol was added to the plates, which were maintained at 4 °C for 30 min. Cell extracts were centrifuged for 20 min at 2000× g and the supernatant fraction was evaporated to dryness. cGMP levels were determined with the use of a commercial [^{125}I]-cGMP radioimmunoassay kit. Protein concentration in the pellets was determined according to the Bradford method.

2.7. Pulse and chase labelling with [^{35}S]-methionine/cysteine

For metabolic labelling with [^{35}S]-methionine/cysteine, cells were incubated in methionine- and cysteine-free DMEM to deplete intracellular pools. They were then pulsed for 2 h with methionine/cysteine-free DMEM containing 25 μCi/ml of [^{35}S]-methionine/cysteine. After the pulse period, the cells were washed with chase medium (serum-free RPMI), and incubated as detailed below. The incubation medium was then removed, and the cells were scraped off at the indicated time points (0–24 h). The proteins were immunoprecipitated as described below and electrophoresed in a SDS-PAGE gel. Subsequently the dehydrated gels were exposed to autoradiography film.

2.8. Immunoprecipitation and immunoblot analysis

Cell cultures were chilled on ice and washed three times with cold PBS supplemented with 0.2 mM vanadate. Subsequently the culture dishes were incubated for 30 min with 0.7-ml RIPA lysis buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM tris–ClH pH 7.2, 1 mM PMSF and 0.2 mM sodium vanadate). The resulting solution was spun down (12,000 rpm for 30 min at 4 °C). The protein concentration in each lysate was determined as described. For each immunoprecipitation, 300 μg of extracted cell proteins was used. Incubations with sGC-h antibody were performed overnight at 4 °C with gentle agitation. The antigen–antibody complexes were preabsorbed with 30 μl of swollen protein G-sepharose for 1 h at 4 °C. The beads were pelleted and washed five times with RIPA-buffer. The immunocomplexes were solubilized by boiling in 15 μl of 5× Laemmli sample buffer (0.1 M tris,

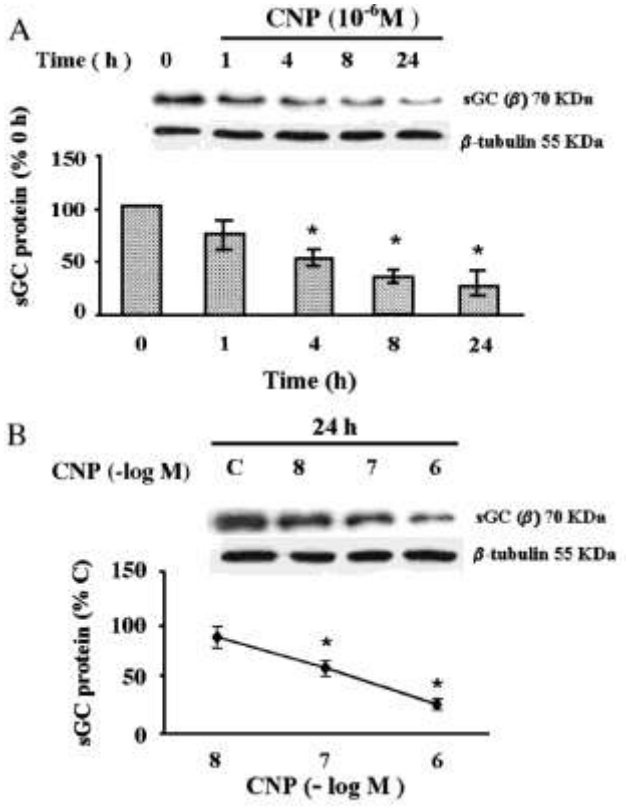

Fig. 1. Effect of CNP treatment on sGC-h protein steady-state levels. (A) Western blot analysis showed that a significant reduction of sGC-h occurred at 8 and 24 h in the presence of 10^{-6} M CNP. (B) The reduction of sGC-h levels at 24 h was dose-dependent. Protein levels of the constitutive protein as h-tubulin did not change. Levels are expressed as percent of control (C), which was assigned a value of 100%. Insets (A) and (B): typical Western blots. n = 6, *P < 0.05 vs. control.

3% SDS, 1M h-mercaptoethanol, 1 M glycerol, bromophenol blue indicator) and analysed by SDS-PAGE under reducing conditions.

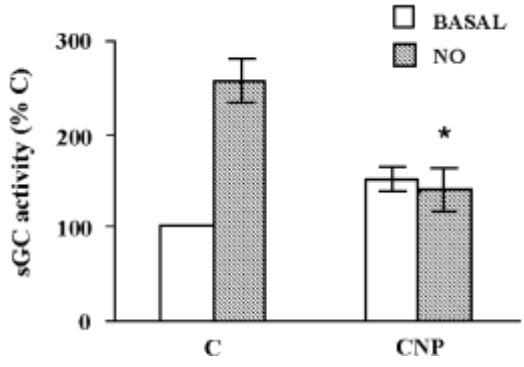

Fig. 2. Effect of CNP treatment on sGC-h activity. 10^{-6} M CNP decreased sGC-h activity during 24 h of exposure. Extracted sGC from control cells (C) or cells treated with CNP (CNP) for 24 h were activated with SNP 10^{-6} M (NO, hatched bars) or not (basal, open bars). cGMP levels produced were expressed as percent of control (C, open bar), which was assigned a value of 100%. Inset: typical radioimmunoanalized cGMP levels; n = 6.

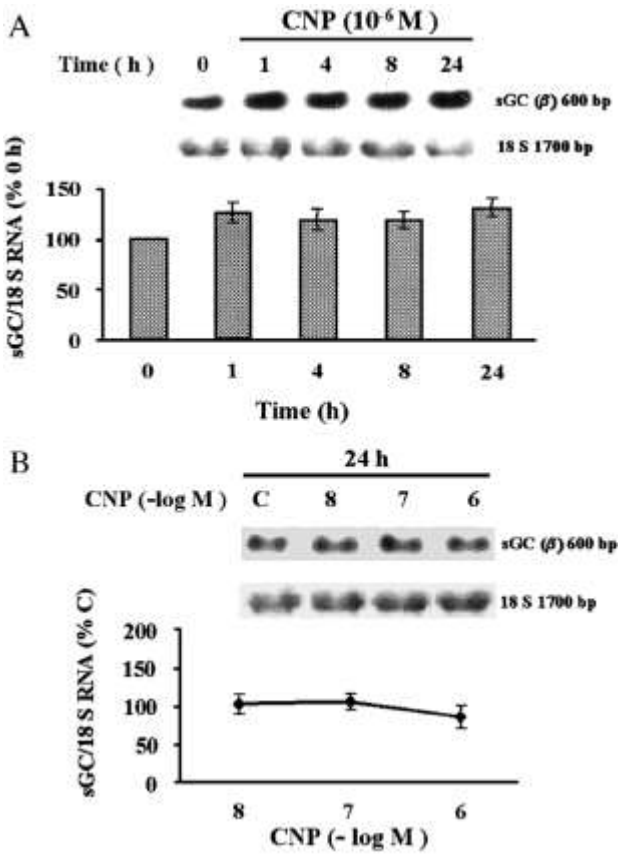

Fig. 3. Effect of CNP treatment on sGC-h mRNA levels. (A) 10^{-6} M CNP did not alter sGC-h mRNA levels during 24 h of exposure. (B) 10^{-8} – 10^{-6} M CNP did not alter sGC-h mRNA levels after 24 h of exposure. Levels were normalized to 18S RNA content and expressed as percent corrected versus control (C), which was assigned a value of 100%. Insets (A) and (B): typical Northern blots. n = 6.

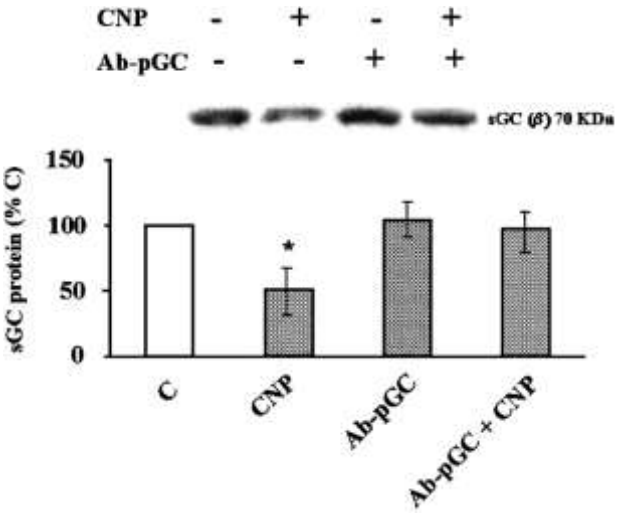

Fig. 4. CNP down-regulation of sGC-h requires pGC activity. Treatment with a specific blocking antibody against pGC (ab-pGC, 5 Ag/ml) for 24 h had no effect on sGC-h levels. However, it prevented the sGC-h down-regulation induced by CNP. Inset: typical Western blot. C, control; n = 6, *P < 0.05 vs. control.

2.9. Statistical analysis

Each experiment was repeated at least three times in duplicate. The data are expressed as means ± S.E. Comparisons were made by analysis of variance followed by Dunnett's modification of the t-test whenever comparisons were followed by a common control. The unpaired two-tailed Student's test was used for other comparisons. The level of statistically significant difference was defined as $P < 0.05$.

3. Results

3.1. CNP decreases sGC protein levels by a pGC/cGMP/pKG-dependent pathway

To demonstrate a possible cross-regulation of GC, HMC were treated with CNP, the agonist of pGC, for 0 to 24 h.

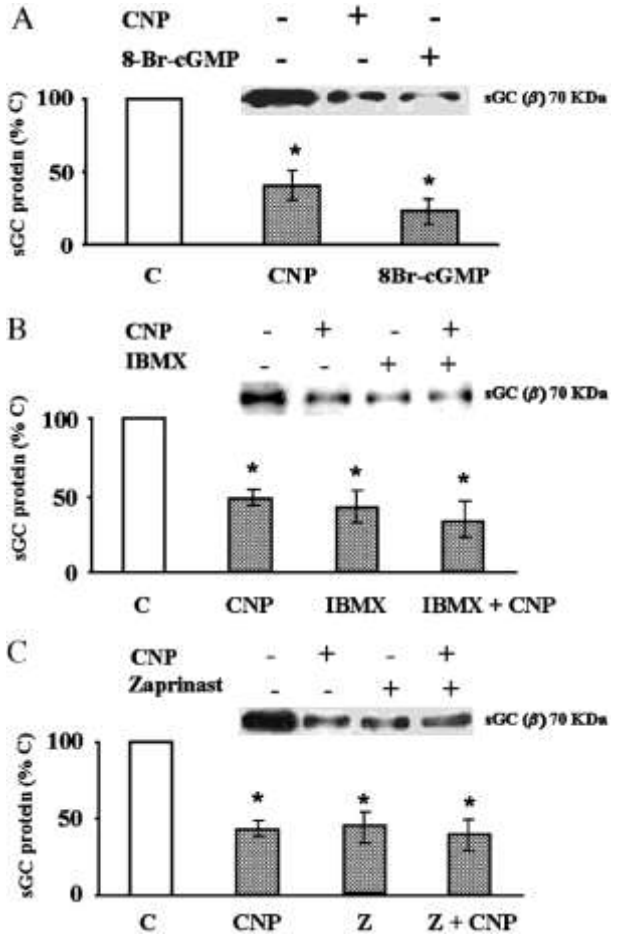

Fig. 5. Both 8-Br-cGMP- and non-CNP-dependent increases of cGMP mimic CNP down-regulation of sGC-h. (A) The stable cGMP analogue 8-Br-cGMP (10^{-6} M) mimicked the effect of CNP-produced cGMP on sGC-h levels during 24 h of exposure. Inhibition of PDE-dependent hydrolysis of cGMP with IBMX (IBMX, 10^{-6} M) (B) or Zaprinast (Z, 10^{-6} M) (C) also mimicked CNP-dependent down-regulation of sGC-h levels. No added reduction was achieved with the combination of Zaprinast and CNP. Insets (A), (B) and (C): typical Western blots. C, control; n = 6, *P < 0.05 vs. control.

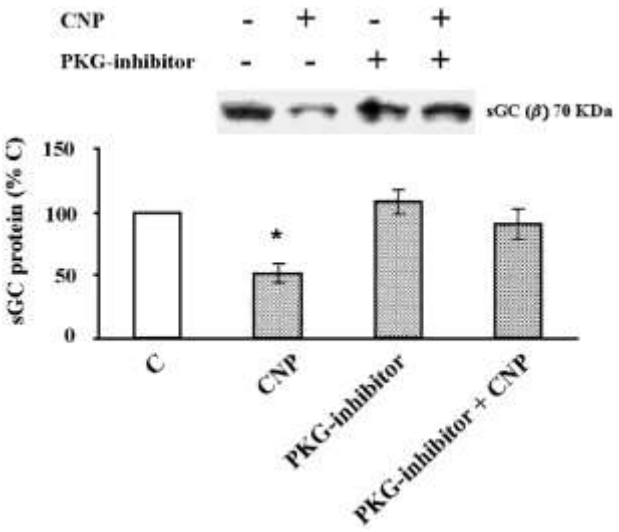

Fig. 6. CNP down-regulates sGC-h levels by activating PKG. Incubation for 24 h with a specific inhibitor of PKG-I (PKG-inhibitor, 25×10^{-6} M) alone had no effect on sGC-h levels. However, it inhibited the CNP-induced sGC-h reduction. Inset: typical Western blot. C, control; n = 6, *P < 0.05 vs. control.

Steady-state sGC protein levels were determined by Western blot using specific sGC-h antibody. CNP decreased the sGC protein levels in a time-dependent manner (Fig. 1A). The decrease was dependent on the concentration of CNP, reaching a maximum effect at 10^{-6} M and 24 h of treatment (Fig. 1B). Therefore, this dose and time were used subsequently to study the regulation of sGC-h steady-state levels.

The next step was to clarify if the CNP treatment could also inhibit the enzyme activity. Thus, we performed a sGC enzyme assay in cells treated with CNP 10^{-6} M for 24 h, extracting the soluble fraction of the cells, which was activated with NO (SNP 10^{-6} M as NO donor) or vehicle (basal) for 15 min, and determining the cGMP levels produced by RIA. sGC activity in CNP-treated cells decreased (Fig. 2) in parallel with the decrease in sGC protein levels.

We next performed Northern blot analysis for specific sGC-h mRNA to determine if the inhibitory effect of CNP was due to differences in mRNA expression. No changes in the sGC-h mRNA levels occurred up to 24 h (Fig. 3A) or with any concentration of CNP up to 10^{-6} M (Fig. 3B).

To clarify the mechanism involved in this sGC down-regulation, we co-treated the cells with a specific pGC-blocking antibody (5 Ag/ml) and 10^{-6} M CNP. The antibody itself had no effect on sGC-h levels (Fig. 4). However, it did abrogate CNP reduction of sGC-h protein steady-state levels (Fig. 4). Thus, the reduction of sGC-h levels in response to CNP required the activity of the natural NP receptor, pGC.

A soluble cGMP analogue, 8-Br-cGMP (10^{-5} M), mimicked the CNP effects on the sGC-h levels (Fig. 5A). Furthermore, treatment with a general PDE inhibitor (IBMX, 10^{-6} M) and the specific PDE-5 inhibitor Zapri-

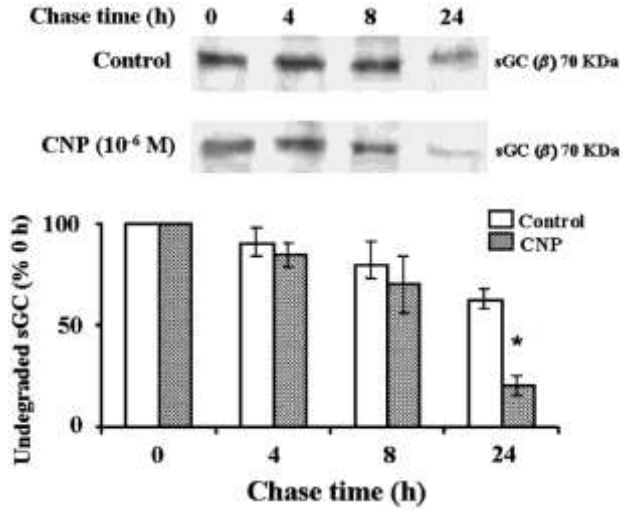

Fig. 7. Time-dependent loss of newly synthesized sGC-h. HMC were pulse-labelled with [^{35}S]-methionine/cysteine for 2 h. After the washout period, cells were incubated with 10^{-6} M CNP and harvested at the times indicated. CNP significantly increased the loss of sGC-h at 24 h. Undegraded sGC-h levels are expressed as percent of the control (time = 0 h) value. Inset: typical autoradiographs of immunoprecipitates from control and CNP-treated cells. n = 6, *P < 0.05 vs. control.

nast (10^{-6} M) had similar effects as CNP (Fig. 5B and C). Taken together, these results show that the down-regulatory effect of CNP is dependent on pGC activation and cGMP production.

We next looked at the participation of PKG, the main kinase activated by cGMP. Rp-cGMPs (25×10^{-6} M), a PKG type I specific inhibitor, prevented the CNP-induced decrease in sGC-h protein levels (Fig. 6). Thus, PKG

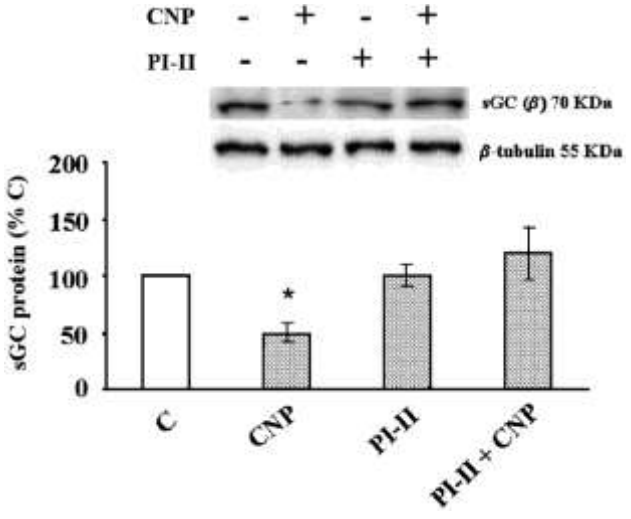

Fig. 8. CNP-induced reduction of sGC-h levels requires proteasome activity. HMC were incubated with the specific proteasome inhibitor 10^{-5} M PI-II for 24 h. PI-II alone did not alter sGC-h levels. However, it did inhibit the CNP-induced down-regulation while protein levels of the constitutive protein h-tubulin did not change. Inset: typical Western blot. C, control; n = 6, *P < 0.05 vs. control.

Table 1
Effect of PI-II on cGMP production by CNP-treated cells

Treatment	cGMP (%)[a]
Control	100
Control + NO	264 F 12
CNP-treated + NO	147 F 11[b]
CNP-treated + NO + PI-II	278 F 18

[a] NO-stimulated values are mean F S.E.; n = 5.
[b] P < 0.05 vs. other NO-stimulated cells.

activity mediates the sGC-h down-regulation elicited by CNP-pGC activation.

3.2. CNP-stimulated decrease of sGC-b requires proteasome activity

The observation that CNP reduced sGC-h protein levels without a change in sGC-h mRNA expression prompted us to investigate the stability of sGC. After pulse labelling for 2 h with ^{35}S-methionine/cysteine, immunoprecipitated labelled-sGC-h from untreated cells decreased over time, and 25 + 5% was lost in control cells after 24 h of chase (Fig. 7). After 24 h of CNP treatment, 70 + 5% of the labelled-sGC-h protein was lost, indicating a more rapid degradation induced by CNP.

To study the mechanism of sGC-h degradation, we treated the cultures with the cell-permeable proteasome inhibitor, Z-LLF-CHO (PI-II, 10^{-5} M). PI-II prevented the CNP effect on sGC-h protein steady-state levels (Fig. 8). Protein levels of the constitutive protein h-tubulin did not change on PI-II-treated cells. Thus, cGMP released after pGC activation by CNP reduced the level of sGC-h by a specific mechanism dependent on the proteasome pathway.

To confirm this result, the effect of NO (SNP or spNO 10^{-6} M as NO donors, 15 min) on cGMP synthesis was tested in cultured cells pretreated with vehicle (control) or CNP (10^{-6} M, 24 h), with or without PI-II (10^{-5} M). cGMP levels were measured by RIA. CNP blunted the significant stimulation cGMP production induced by NO in control cells (Table 1). Proteasome inhibition by PI-II prevented this down-regulation.

4. Discussion

Our results clearly show that CNP decreased the levels of sGC-h protein and enzymatic activity, but this effect is not dependent on changes of sGC-h mRNA expression. Blocking pGC activity with a specific antibody prevented the reduction of sGC steady-state levels by CNP. The pGC blocking antibody used here (B136, kindly provided by Suntory) is a competitive and selective antagonist for the CNP receptor, and its neutralizing efficiency has been described [34]. The inhibitory effect of CNP was mimicked by 8-Br-cGMP, a cell-permeable analogue of cGMP

that is resistant to hydrolysis by PDE-5 but activates PKG. These results are further supported by the prevention of native cGMP degradation by inhibition of PDE-5 with Zaprinast.

In endothelial, mesangial and smooth muscle cells, PKG type I (PKG-I) begins the biochemical cascade in which selected serine and threonine residues on target proteins are phosphorylated [35–37]. The specific antagonist for PKG-I, Rp-cGMPs, blocked the CNP-stimulated down-regulation of sGC protein levels. Thus, the down-regulatory effect is mediated by PKG in response to cGMP produced by pGC.

Dephosphorylation has been implicated as the mechanism of sGC desensitisation in the sea urchin and in mammals [38]. Ferrero et al. [39] showed that PKG indirectly inhibits sGC activity through dephosphorylation in bovine chromaffin cells, and pretreatment with CNP decreases the activity of sGC. One of the target proteins of PKG-I is PDE-5, the major enzyme that degrades cGMP in vivo and in vitro [40,41]. Thus, activation of PDE-5 by PKG-I may provide negative feedback regulation of the sGC/cGMP signalling pathway. While our results are compatible with the possibility that PKG activates PDE by phosphorylation, we clearly show here that in cultured HMC, CNP regulates the absolute levels of sGC.

The down-regulation of sGC levels was achieved through CNP promotion of proteasome activity. Proteasomes are large multisubunit proteases that are found in the cytosol. They recognize, unfold and digest protein substrates that have been marked for degradation by the attachment of a ubiquitin moiety [42]. Blockage of proteasome activity with PI-II, a cell-permeable, irreversible inhibitor of the proteasomes [43], inhibited the accelerated rate of protein degradation induced by CNP. This effect was specific for sGC, because the constitutive protein h-tubulin was not changed by the proteasome activation. Indeed, the cGMP levels produced in NO-stimulated cells previously treated with CNP and PI-II were increased almost two times compared to NO-stimulated cells treated with CNP alone, where the increase in cGMP levels due to NO stimulation was inhibited by the down-regulation of sGC. Mason et al. [44] demonstrated that phosphorylated proteasomes have significantly higher activities when compared to non-phosphorylated ones in rat fibroblasts. Therefore, CNP-activated PKG could start a phosphorylation cascade leading to increased proteasome activity.

Because both GC-cGMP systems have complementary roles to play, an interaction between the two pathways to regulate cGMP levels might represent an important physiological control mechanism. In this way, an excess or deficiency in one mediator could be compensated by the other. Conversely, the interaction may constitute a negative-feedback system that prevents overactivation of cGMP signaling in one cell type by NO and/or NP. Our results support this hypothesis, but it remains to be determined if NO down-regulates pGC levels or activity.

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