Biological Effects of C-type Natriuretic Peptide in Human Myofibroblastic Hepatic Stellate Cells*

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During chronic liver diseases, hepatic stellate cells (HSC) acquire a myofibroblastic phenotype, proliferate, and synthesize fibrosis components. Myofibroblastic HSC (mHSC) also participate to the regulation of intrahepatic blood flow, because of their contractile properties. Here, we examined whether human mHSC express natriuretic peptide receptors (NPR). Only NPR-B mRNA was identified, which was functional as demonstrated in binding studies and by increased cGMP levels in response to C-type natriuretic peptide (CNP). CNP inhibited mHSC proliferation, an effect blocked by the protein kinase G inhibitor 8-(4 chlorophenylthio)-cGMP and by the NPR antagonist HS-142-1 and reproduced by analogs of cGMP. Growth inhibition was associated with a reduction of extracellular signal-regulated kinase and c-Jun N-terminal kinase and with a blockade of AP-1 DNA binding. CNP and cGMP analogs also blunted mHSC contraction elicited by thrombin, by suppressing calcium influx. The relaxing properties of CNP were mediated by a blockade of store-operated calcium channels, as demonstrated using a calcium-free/calcium re-addition protocol. These results constitute the first evidence for a hepatic effect of CNP and identify mHSC as a target cell. Activation of NPR-B by CNP in human mHSC leads to inhibition of both growth and contraction. These data suggest that during chronic liver diseases, CNP may counteract both liver fibrogenesis and associated portal hypertension.

C-type natriuretic peptide (CNP)1 belongs to the natriuretic peptide family, which also comprises atrial natriuretic peptide (ANP) and brain natriuretic peptide. ANP is mainly synthesized in the atrium, whereas brain natriuretic peptide and CNP are more widely produced (1). The three peptides play an important role in the maintenance of blood pressure and cardio-vascular homeostasis and exert natriuretic and diuretic effects. In addition, natriuretic peptides exhibit relaxing and growth inhibitory responses in parenchymal and mesenchymal cells of various origins (for a review, see Ref. 1). In keeping with the diversity of natriuretic peptide functions, three classes of receptors have been characterized in different tissues, natriuretic peptide receptor (NPR)-A, which is sensitive to ANP and brain natriuretic peptide (2), NPR-B, which is highly specific for CNP (3), and NPR-C, which binds the three natriuretic peptides with similar affinities (2). NPR-A and NPR-B are members of the guanylyl cyclase receptor family and transduce their biological effects via cGMP (2), whereas NPR-C lacks the guanylate cyclase domain and signals through inhibition of cAMP (4). Recent data indicate that the mRNAs for NPR-A, NPR-B, and NPR-C are expressed in human liver (5).

Hepatic stellate cells (HSC) (also known as lipocytes, fat-storing cells, or perisinusoidal cells) are resident cells in the space of Disse that show a pericyte-like orientation, extending long cytoplasmatic processes around hepatic sinusoids. Recent studies have advocated their salient role in the pathogenesis of liver fibrosis and of portal hypertension (6). Following liver injury, HSC undergo phenotypic activation from a quiescent cell containing large retinoid droplets to an activated myofibroblastic-like cell. This state is characterized by intense proliferation, marked synthesis of extracellular matrix, and production of proinflammatory cytokines, which lead to the development of liver fibrosis (6). Proliferation and accumulation of myofibroblastic HSC (mHSC) have largely been documented in experimental models and in culture studies (7, 8). Among several mitogenic growth factors, PDGF-BB, which is highly expressed during chronic hepatic injury, is currently considered as the most potent mitogen (9, 10). Factors that may limit the proliferation of myofibroblastic HSC have also been characterized, such as endothelin-1 (ET-1), cAMP, TNF-α, and prostaglandins (E2 and I2) (11–13). During phenotypic activation, HSC also acquire smooth muscle features, such as the expression of smooth muscle α-actin (6), and contract in response to diverse vasoactive mediators (14–18). Moreover, increasing evidence indicates that enhanced sensitivity of myofibroblastic HSC to contractile peptides elevates intrahepatic resistance and contributes to portal hypertension associated with the development of liver fibrosis (19).

In the present study, we provide the first evidence for a hepatic effect of CNP. In a model of human myofibroblastic HSC that displays the phenotypic characteristics of mHSC found in situ during hepatic fibrosis (20), we show that activation of NPR-B receptor leads to elevation of cGMP levels and results in inhibition of mHSC proliferation and contraction. Analysis of the signaling pathways indicate that growth inhibition is associated with inhibition of MAP kinase (extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase...
(JNK) activations and blunting of AP-1 binding stimulation. Relaxing effects of CNP are consecutive to a blockade of the calcium influx through store-operated calcium channels.

**EXPERIMENTAL PROCEDURES**

**Materials**

Human CNP (32–53) was from Bachem (Voisins-le-Bretonneux, France), and human α-ANP (1–28) was from NeoSystem (Strasbourg, France). PDGF-BB was from Life Technologies, Inc. Fetal calf serum was from Life Technologies, Inc., and pooled human AB positive serum was supplied by the National Transfusion Center. Hybond N+ membrane, rapid hybrid buffer, [3H]thymidine (25 Ci/μmol), and [γ-32P]ATP (5,000 Ci/μmol) were from Amersham Pharmacia Biotech, and 125I-(Tyr)CNP-22 ([125I]CNP) (500 Ci/μmol) was from Peninsula (Mersyside, United Kingdom). AP-1 and NF-κB consensus oligonucleotides, Access RT-PCR kit and Cell Titer 96 Aqueous One Solution cell proliferation assay were from Promega (Charbonnières, France). The plasmid encoding glutathione S-transferase-c-Jun (1–79) fusion protein was a generous gift of Dr. C. Bradham (Chapel Hill, NC). The phospho38 MAP kinase antibody was from New England Biolabs (Ozyme, Montigny le Bretonneux, France), cAMP and cGMP radioimmunoassays were from Immunotech (Marseille, France), and Fura-2/AM was from Molecular Probes (Interchim, Montluçon, France). 8-(4-Chlorophenyl)thio-2′,3′-cyclic monophosphorothioate, Rp-isomer (Rp-8-CPT-cGMP) was from Biolog Life Science Institute (Bremen, Germany).

**Some general protocols for cell culture**

Human mHSC in their active phenotype were obtained by outgrowth of normal liver explants obtained from surgery of benign or malignant liver tumors. This procedure was performed in accordance with ethical regulations imposed by French legislation. Explants were incubated in Dulbecco’s modified Eagle’s medium containing 10% serum (5% fetal calf serum, 5% pooled human serum), and exhaustive incubation of either unconditioned CNP in this medium was stopped by aspirating the medium and rinsing the cells four times with 4 ml of washing buffer (20 mM Hepes, pH 7.4, containing 137 mM NaCl and 1% bovine serum albumin). The cells were then solubilized with 0.4 N NaOH, and cell-associated radioactivity was measured. Nonspecific binding was determined by incubating with 1 μM CNP, and it usually represented 25% of the total radioactivity. Experiments were performed in duplicate, and the protein content was determined by the Bio-Rad protein assay kit in three separate wells.

**cGMP and cAMP Assays**

Confluent mHSC were made quiescent by a 3-day incubation in serum-free Waymouth medium, and then incubated with 0.6 mM isobutylmethylxanthine for 15 min, except otherwise indicated, and then stimulated for various periods of time in phosphate-buffered saline containing varying concentrations of natriuretic peptides. cAMP and cGMP were extracted and assayed as described previously (24), using a commercial radioimmunoassay.

**DNA Synthesis and Cell Proliferation Assays**

DNA synthesis was measured in triplicate wells by incorporation of [3H]thymidine, as described previously (11). Confluent mHSC were made quiescent by a 3-day incubation in serum-free Waymouth medium and then stimulated for 30 h with the indicated effectors, in the presence of 80 μM ZnCl₂, which has been described to enhance the growth inhibitory effects of cGMP analogs (25). [3H]Thymidine (0.5 μCi/well) was added during the last 6 h of incubation. Cell growth assay was performed using the Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega). Human mHSC were seeded in 96-well plates at low density (5000/well) in Dulbecco’s modified Eagle’s medium 5/5, allowed to attach overnight, and made quiescent by a 48 h incubation in serum-free medium. Incubation was performed in Waymouth medium containing 80 μM ZnCl₂ and either 5% human serum or 20 ng/ml PDGF-BB, in the absence or presence of 100 mM CNP, which was added every day for 3 days. The medium was then replaced with or phosphate-buffered saline. Cell growth assay was performed in duplicate, and the protein content was determined by the Bio-Rad protein assay kit in three separate wells.

**Preparation of Whole Cell, Nuclear, and Cytoplasmic Extracts**

Whole Cell Extracts—Whole cell extracts were prepared as described previously (13) with minor modifications. Confluent mHSC were made quiescent by a 3-day incubation in serum-free Waymouth medium and then incubated for various periods of time with the indicated effectors. After a wash in ice-cold phosphate-buffered saline, cells were lysed for 15 min at 4 °C in whole cell extraction buffer (50 mM Hepes, pH 7.4, containing 0.5% Nonidet P-40, 137 mM NaCl, 1 mM EGTA, 10 mM NaF, 1 mM vanadate, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 40 mM β-glycerophosphate, 0.1 mM dithiothreitol). Lysates were centrifuged at 20,000 g for 10 min at 4 °C. Supernatants (whole cell extract) were stored at −80 °C until use.

**Nuclear and Cytoplasmic Extracts**—Nuclear and cytoplasmic extracts were prepared as described previously (13). Confluent mHSC were incubated for various periods of time with the indicated effectors. Cells were then washed two times in ice-cold phosphate-buffered saline and resuspended in 400 μl of Buffer A (10 mM Hepes, pH 7.4, containing 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A). The cells were allowed to swell on ice for 15 min, after which 12.5 μl of 10% Nonidet P-40 was added. The tubes were shaken gently, centrifuged at 2000 × g for 10 min at 4 °C, and supernatants were used as cytoplasmic extracts. The pellet nuclei were resuspended in 40 μl of Buffer C (20 mM Hepes, pH 7.9, containing 1.5 mM MgCl₂, 450 mM...
NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A. After 30 min at 4 °C under constant agitation, nuclear debris were centrifuged at 20000 × g for 15 min. The supernatants (nuclear extract) were frozen in liquid nitrogen and stored at −80 °C.

**Western Blotting Analysis**

Equal amounts of extracts (40 μg) were electrophoresed on a 10% SDS-polyacrylamide gel. Proteins were then electoblotted onto nitrocellulose membranes and blocked in 10 mM Tris, pH 8, containing 150 mM NaCl, 0.05% Tween 20, 5% skim milk. Detection of IkB-α and phospho-p38 MAP kinase was performed after incubation for 2 h with their respective antibodies diluted 1:1000. Immunodetected proteins were visualized by using an enhanced chemiluminescence assay kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Equal loading of proteins in each lane was checked by Ponceau red staining of the membrane.

**Extracellular Signal-regulated Kinase, p38 MAP Kinase, and JNK Assays**

Confluent quiescent mHSC were stimulated with the indicated effectors, and whole cell lysates were obtained as described above. ERK activity was assayed in situ, as described previously (11), following electrophoresis of equal amounts of cell lysates (40 μg of proteins) on a 10% SDS-polyacrylamide gel co-polymerized with 0.5 mg/ml myelin basic protein. JNK was assayed in vitro by the phosphorylation of glutathione S-transferase-c-Jun (1–79) fusion protein, followed by SDS-polyacrylamide gel electrophoresis. Immunodetected proteins were visualized by using an enhanced chemiluminescence assay kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Equal loading of proteins in each lane was checked by the Ponceau red staining of the membrane.

**Electrophoretic Mobility Shift Assay (EMSA)**

AP-1 double-stranded consensus oligomer (5′-CCG TGG ATG AGT CAG CCG GAA-3′; 3′-CCG AAT TCA GTC GGC CTT-5′, Promega) and an oligomer (Promega) corresponding to the consensus sequence of NF-kB from the κ light chain enhancer were radiolabeled with T4 polynucleotide kinase and [γ-32P]ATP. Unincorporated nucleotides were removed by filtration through a G50 Fine column. Nuclear extracts (10 μg of protein) were incubated in the binding reaction medium (20 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 0.5 mM dithiothreitol, 0.5 mM PMSF) for 15 min at 4 °C, followed by a 15-min incubation at room temperature with 0.5 ng of the 32P-labeled probe. The DNA-protein complexes were analyzed on a 5% polyacrylamide gel in 0.25 X Tris Borate EDTA electrophoresis buffer. Gels were run at 150 V for 90 min, dried, autoradiographed, and quantified by PhosphorImager analysis.

**Fura-2 Loading and Ca2+ Imaging**

Human mHSC were plated at a density of 15,000 cells/ml in 35-mm dishes, the bottoms of which were replaced by glass coverslips, and allowed to attach in Dulbecco’s modified Eagle’s medium 5/5 for 24 h. Cells were made quiescent in serum-free Waymouth medium for 24 h and the coverslip was mounted on the stage of an inverted microscope. The whole-cell configuration was used to record calcium currents (ICa) with a protocol consisting in a pulse to 0 mV (400-ms duration) preceded by a short pulse of −50 mV (50-ms duration) elicited every 8 s from a holding potential of −100 mV. Time-dependent ICa values were measured as described (28). The cells were voltage-clamped using a patch-clamp amplifier (Biologic, Grenoble, France), and analyzed as described previously (28). The experiments were performed at 25 °C.

**Measurement of Cell Contraction**

Cell areas and cell lengths were determined from the 360 nm fluorescence images recorded to measure the F360/F380 ratios of Fura-2-loaded human mHSC, using the Morphostar II software developed by IMSTAR Co. (Paris, France), as described previously (26). All tracings of cell areas are representative of at least 10 cells and were performed on at least three different cell isolations.

**Electrophysiology**

For patch-clamp experiments, cells were plated on 15-mm coverslips in 24-well plates at a density of 10,000 cells/well and allowed to attach in Dulbecco’s modified Eagle’s medium 5/5 for 24 h. Cells were made quiescent in serum-free Waymouth medium for 24 h and the coverslip was mounted on the stage of an inverted microscope. The whole-cell configuration was used to record calcium currents (ICa) with a protocol consisting in a pulse to 0 mV (400-ms duration) preceded by a short pulse of −50 mV (50-ms duration) elicited every 8 s from a holding potential of −100 mV. Time-dependent ICa values were measured as described (28). The cells were voltage-clamped using a patch-clamp amplifier (Biologic, Grenoble, France), and analyzed as described previously (28). The experiments were performed at 25 °C.

**Solutions**

The external solution contained (in mM) 100 NaCl, 10 HEPES, 26 CaCl2, 5 NaHCO3, 1.2 KH2PO4, 1.2 MgSO4, 2 CaCl2, 10 nM glucose, pH 7.4 adjusted with CaOH. Solutions were applied as described (28). The patch pipettes (3.5–6.0 Mohm) were filled with an internal solution composed of (in mM) 130 CsCl, 5 EGTA (acid form), 0.05 CaCl2, 3 Na2ATP, 2 Na2GTP, 10 HEPES, pH 7.2, adjusted with CsOH, according to Ref. 29.

**Assay of Protein Concentration**

Protein concentration was determined by the Bio-Rad protein assay kit.

**Statistics**

Results are expressed as mean ± S.E. of n experiments and were analyzed by repeated measures ANOVA.

**RESULTS**

**Characterization of Natriuretic Peptide Receptor Subtypes in Human Myofibroblastic HSC—Identification of the natriuretic peptide receptor subtypes present in human myofibroblastic HSC was performed by RT-PCR analysis. The mRNA was amplified with specific primers complementary either to the human NPR-A or to the NPR-B or NPR-C DNA sequences, and the PCR product was size-fractionated and blotted. The membrane was hybridized with a labeled oligonucleotide complementary to the respective NPR sequences within the cDNA flanked by the PCR primers. A band of 767 bp corresponding to the size of the NPR-B product was identified in human myofibroblastic HSC (Fig. 1A). In contrast, mHSC did not express the expected 693-bp product or a 379-bp product corresponding to NPR-A or NPR-C, whereas these amplification products were present in human kidney tissue taken as control (Fig. 1A). The functionality of NPR-B in myofibroblastic HSC was assessed in binding experiments. Competition experiments indicated that CNP was more potent than ANP in inhibiting [125I]CNP binding, with IC50 values of 20 and 800 pM, respectively (Fig. 1B). This order of potency and these IC50 values are in agreement with those described for NPR-B (3).**

**In keeping with the presence of NPR-B receptors, and as described in other cells (3), CNP caused a huge (30-fold) increase in cGMP levels in mHSC, whereas ANP elicited a minimal (1,6-fold) effect (Fig. 1C). cGMP levels were also measured in the conditions used in DNA synthesis assays, i.e. in the presence of PDGF-BB (see below). Whereas PDGF-BB alone had no effect, CNP increased cGMP levels to the same extent in the presence of PDGF-BB and in its absence (Fig. 1C).**

**We also determined whether elevation of cGMP increases cAMP levels, via activation of the cGMP-inhibited phosphodiesterase. Blockade of the cGMP-inhibited phosphodiesterase by...
**TABLE I**

cAMP mobilizing agonists do not affect cAMP levels in human myofibroblastic HSC

| Conditions     | cAMP fold over basal levels |
|----------------|-----------------------------|
| Basal          | 1                           |
| CNP (1 μM)     | 0.9 ± 0.1                   |
| CNP (1 μM) (90 min) | 1.0 ± 0.1                   |
| ANP (1 μM)     | 0.9 ± 0.1                   |
| 8-Br cGMP (5 mM) | 1.0 ± 0.2                   |
| Milrinone (10 μM) | 1.1 ± 0.2                   |
| Sarafotoxin S6C (0.1 μM) | 6.4 ± 0.4                   |

Addition of permeant analogs of cGMP reproduced the growth inhibitory effect of CNP, with cGMP 8-Br-cGMP or 8-CPT-cGMP inhibiting thymidine incorporation of serum-stimulated mHSC by 35 and 30%, respectively (Fig. 2C). Finally, the growth inhibitory effect of CNP was markedly reduced by Rp-8-pCPT-cGMP, a protein kinase G inhibitor, as well as by HS-142-1, a guanylyl cyclase-coupled receptor antagonist (31) (Fig. 2D). Taken together, these data indicate that CNP inhibits the proliferation of human myofibroblastic HSC, following binding of the peptide to the guanylyl cyclase receptor NPR-B and the resulting elevation of cGMP.

**CNP Inhibits Both ERK and JNK and Has No Effect on p38 MAPK**—We have previously shown that in human myofibroblastic HSC, antiproliferative effects of ET-1 are associated with inhibition of two enzymes of the MAPK cascade, ERK and JNK (11, 12). Moreover, another enzyme of the MAPK family, p38 MAPK, has recently been associated with growth arrest (32). We therefore investigated the effects of CNP and 8-CPT-cGMP on the activation of ERK, JNK, and p38 MAPK kinase. CNP alone had no effect on either ERK, JNK, or p38 MAPK. PDGF-BB rapidly stimulated ERK, inducing a maximal activation within 10–15 min followed by a decrease thereafter (Fig. 3A). In the presence of CNP, activation of ERK by PDGF-BB was reduced by 30–40% at all time points. JNK was time-dependently activated by PDGF-BB, a maximal 3-fold increase being attained after 10–20 min (Fig. 3B). Addition of CNP to PDGF-BB-stimulated cells reduced JNK activity by 40% (Fig. 3B). CNP also caused a 50% reduction in JNK activity stimulated by human serum (not shown). Finally, whereas serum increased the phosphorylation of p38 MAPK, there was no effect of CNP on serum-stimulated p38 MAPK phosphorylation (Fig. 3C). ANP had no effect on ERK and JNK in cells stimulated by PDGF-BB (not shown). As shown in Fig. 4, the cGMP analog 8-CPT-cGMP (1 μM) reproduced the inhibitory effects of CNP on ERK and JNK activation by PDGF-BB.

**CNP Inhibits AP-1 DNA Binding and Has No Effect on IκB Degradation**—We have recently described that the antiproliferative effects of ET-1 and TNF-α involves activation of NF-κB and reduction of AP-1 proteins activation in human myofibroblastic HSC (12, 13). We therefore investigated the effects of CNP on both transcription factors. Nuclear proteins were isolated from human myofibroblastic HSC treated either with CNP alone, PDGF-BB, or PDGF-BB together with CNP, and analyzed in EMSAs, using a radiolabeled DNA probe containing a consensus AP-1 binding sequence. As shown in Fig. 5A, PDGF-BB increased AP-1 DNA binding time-dependently, with a peak at 30–60 min. CNP markedly decreased AP-1 DNA binding stimulated by PDGF-BB, whereas it did not affect.

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**FIG. 1.** Characterization of the presence of NPR in human myofibroblastic HSC. (A) detection of NPR mRNAs by RT-PCR. The products of PCR amplification of cDNAs from human myofibroblastic HSC, human heart, and human kidney were prepared as described under “Experimental Procedures,” electrophoresed on a 2% agarose gel, and blotted. The membrane was hybridized with a labeled oligonucleotide complementary to the respective NPR sequences within the cDNA flanked by the PCR primers, as described under “Experimental Procedures.” The arrows point to 693-, 767-, and 379-bp fragments corresponding to NPR-A, NPR-B, and NPR-C cDNAs, respectively. B, characterization of NPR-B by competition experiments. Inhibition of 

**FIG. 2.** Characterization of the presence of NPR in human myofibroblastic HSC. (A). Detection of NPR mRNAs by RT-PCR. The products of PCR amplification of cDNAs from human myofibroblastic HSC, human heart, and human kidney were prepared as described under “Experimental Procedures,” electrophoresed on a 2% agarose gel, and blotted. The membrane was hybridized with a labeled oligonucleotide complementary to the respective NPR sequences within the cDNA flanked by the PCR primers, as described under “Experimental Procedures.” The arrows point to 693-, 767-, and 379-bp fragments corresponding to NPR-A, NPR-B, and NPR-C cDNAs, respectively. B, characterization of NPR-B by competition experiments. Inhibition of 

**FIG. 3.** Characterization of the presence of NPR in human myofibroblastic HSC. (A). Detection of NPR mRNAs by RT-PCR. The products of PCR amplification of cDNAs from human myofibroblastic HSC, human heart, and human kidney were prepared as described under “Experimental Procedures,” electrophoresed on a 2% agarose gel, and blotted. The membrane was hybridized with a labeled oligonucleotide complementary to the respective NPR sequences within the cDNA flanked by the PCR primers, as described under “Experimental Procedures.” The arrows point to 693-, 767-, and 379-bp fragments corresponding to NPR-A, NPR-B, and NPR-C cDNAs, respectively. B, characterization of NPR-B by competition experiments. Inhibition of 

**FIG. 4.** Characterization of the presence of NPR in human myofibroblastic HSC. (A). Detection of NPR mRNAs by RT-PCR. The products of PCR amplification of cDNAs from human myofibroblastic HSC, human heart, and human kidney were prepared as described under “Experimental Procedures,” electrophoresed on a 2% agarose gel, and blotted. The membrane was hybridized with a labeled oligonucleotide complementary to the respective NPR sequences within the cDNA flanked by the PCR primers, as described under “Experimental Procedures.” The arrows point to 693-, 767-, and 379-bp fragments corresponding to NPR-A, NPR-B, and NPR-C cDNAs, respectively. B, characterization of NPR-B by competition experiments. Inhibition of 

**FIG. 5.** Characterization of the presence of NPR in human myofibroblastic HSC. (A). Detection of NPR mRNAs by RT-PCR. The products of PCR amplification of cDNAs from human myofibroblastic HSC, human heart, and human kidney were prepared as described under “Experimental Procedures,” electrophoresed on a 2% agarose gel, and blotted. The membrane was hybridized with a labeled oligonucleotide complementary to the respective NPR sequences within the cDNA flanked by the PCR primers, as described under “Experimental Procedures.” The arrows point to 693-, 767-, and 379-bp fragments corresponding to NPR-A, NPR-B, and NPR-C cDNAs, respectively. B, characterization of NPR-B by competition experiments. Inhibition of 

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10 μM Milrinone (30) did not affect cAMP levels, indicating a negligible participation of the cGMP-inhibited phosphodiesterase in cAMP metabolism in human mHSC. Moreover, when phosphodiesterases were blocked by the nonselective inhibitor isobutylmethylxanthine, neither CNP nor 8-Br-cGMP affected cAMP levels, even after a prolonged 90-min stimulation (Table I); in contrast, as expected (12), the endothelin B receptor agonist sarafotoxin S6C caused a 6-fold increase in cAMP levels. These results indicate that cGMP mobilizing agonists do not signal through cAMP in human myofibroblastic HSC.

Altogether, these data demonstrate the presence of functional NPR-B receptors in human myofibroblastic HSC. The next series of experiments were performed to investigate the biological functions of NPR-B receptors in human myofibroblastic HSC and focused on the effects of CNP on their proliferation and contraction.

**CNP Inhibits the Growth of Human Myofibroblastic HSC via NPR-B Receptor**—DNA synthesis of human myofibroblastic HSC was stimulated with the most potent mitogens for human mHSC, either human serum (5%) or the purified growth factor PDGF-BB (20 ng/ml). (Fig. 2A). DNA synthesis of serum- or PDGF-BB-stimulated cells was reduced dose-dependently by CNP, a maximal 30% inhibition being attained at 10 μM, with an IC₅₀ of 30 pM, in agreement with the IC₅₀ of CNP for NPR-B. In contrast, 10 μM ANP minimally inhibited [³H]thymidine incorporation. Similar results were obtained in cell proliferation assays, indicating that inhibition of [³H]thymidine incorporation is associated with inhibition of cell growth (Fig. 2B).
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CNP analogs 8-CPT-cGMP and 8-Br-cGMP on [3H]thymidine incorporation into DNA. Confluent mHSC were made quiescent in serum-free Waymouth medium over 3 days and were further stimulated for 30 h in the presence of 80 μM ZnCl₂ with 20 ng/ml PDGF or 5% human serum (inset), in the absence or presence of varying concentrations of CNP or ANP. Results are the mean ± S.E. of nine experiments. p < 0.01 for CNP and ANP effects by repeated measures ANOVA.

The effect of CNP and ANP on DNA synthesis and cell growth of human myofibroblastic HSC. A, effect of CNP and ANP on [3H]thymidine incorporation into DNA. Confluent mHSC were made quiescent in serum-free Waymouth medium over 3 days and were further stimulated for 30 h in the presence of 80 μM ZnCl₂ with 20 ng/ml PDGF or 5% human serum (inset), in the absence or presence of varying concentrations of CNP or ANP. Results are the mean ± S.E. of nine experiments. p < 0.01 for CNP and ANP effects by repeated measures ANOVA.

FIG. 2. CNP inhibits DNA synthesis and cell growth of human myofibroblastic HSC. A, effect of CNP and ANP on [3H]thymidine incorporation into DNA. Confluent mHSC were made quiescent in serum-free Waymouth medium over 3 days and were further stimulated for 30 h in the presence of 80 μM ZnCl₂ with 20 ng/ml PDGF or 5% human serum (inset), in the absence or presence of varying concentrations of CNP or ANP. Results are the mean ± S.E. of nine experiments. p < 0.01 for CNP and ANP effects by repeated measures ANOVA. B, effect of CNP on mHSC growth. Human mHSC were seeded in 96-well plates at low density (5000/well) in Dulbecco’s modified Eagle’s medium containing 80 μM ZnCl₂ and either 20 ng/ml PDGF-BB or 5% human serum, in the absence or presence of 100 nM CNP, which was added every day for 3 days. Cell growth was assayed at day 3. Results are the mean ± S.E. of six experiments. p < 0.01 compared with the respective controls by two-way ANOVA for repeated measures. C, effect of the cGMP analogs 8-CPT-cGMP and 8-Br-cGMP on [3H]thymidine incorporation into DNA. Quiescent cells were stimulated over 30 h in medium containing 80 μM ZnCl₂ and 5% human serum in the absence or presence of 1 mM 8-CPT-cGMP or 5 mM 8-Br-cGMP. Results are the mean ± S.E. of six experiments. p < 0.01 by two-way ANOVA for repeated measures. D, effect of the protein kinase G inhibitor Rp-8-pCPT-cGMP and of the NPR-A/B antagonist HS-142-1 on the growth inhibitory effect of CNP. Quiescent cells were preincubated for 30 min either with medium alone or with medium containing 100 μM Rp-8-pCPT-cGMP or 100 μM HS-142-1, and further stimulated over 30 h in medium containing 80 μM ZnCl₂, 20 ng/ml PDGF-BB, and varying concentrations of CNP. Results are the mean ± S.E. of four experiments. ***, p < 0.01 by two-way ANOVA for repeated measures.

FIG. 3. Effect of CNP on ERK, JNK, and p38 MAPK activations in human myofibroblastic HSC. Confluent mHSC were made quiescent in serum-free Waymouth medium over 3 days and were further stimulated with 20 ng/ml PDGF-BB or 5% human serum as indicated, in the absence or the presence of 1 μM CNP. In C, cells were stimulated for 15 min with the indicated factors. Cell lysates were prepared as described under “Experimental Procedures” and assayed for ERK activity by in gel kinase assay, carried out as described under “Experimental Procedures” and quantified by PhosphorImager analysis (A). B, JNK activity, carried out with glutathione S-transferase-c-Jun as substrate as described under “Experimental Procedures” and quantified by PhosphorImager analysis. C, P38 MAPK, analyzed by Western blotting, using an antiserum specific to phospho-p38 MAP kinase. Autoradiograms are representative of two experiments and were quantified by PhosphorImager analysis.

We also examined the effects of CNP on NF-κB by studying the degradation of its inhibitory protein IκB-α, an event that reflects NF-κB activation (33), and on NF-κB DNA-binding (Fig. 5, C and D). In unstimulated cells, a 37-kDa IκB-α protein was detected in cytoplasmic extracts (Fig. 5C). CNP did not affect IκB-α levels, whereas TNF-α caused degradation of IκB-α after 30 min, as expected (Fig. 5C). Moreover, CNP did not affect the DNA binding activity of NF-κB, whereas TNF-α had a strong stimulatory effect (Fig. 5D).

Altogether, these results indicate that the growth inhibitory effects of CNP and cGMP are associated with a reduction of ERK and JNK activation and the blockade of the resulting elevation of AP-1 DNA binding.

CNP Inhibits Thrombin-induced Contraction of Human Myofibroblastic HSC by Blocking Thrombin Stimulation of Calcium Influx through Store-operated Calcium Channels—We investigated the effects of CNP on the contraction of human myofibroblastic HSC by blocking thrombin stimulation of calcium influx through store-operated calcium channels.
myofibroblastic HSC in response to thrombin, one of the most potent contractile agonists for these cells (15). As expected, addition of 1 units/ml thrombin caused a transient contraction of human mHSC, indicated by marked reduction in cell area (14.7 ± 1.5%, n = 10, Fig. 6A) and cell length (11.5 ± 1.3%, n = 10) (not shown), in 100% of cells. Preincubation of mHSC for 10 min with 1 µM CNP totally blunted thrombin-induced mHSC contraction in 100% of cells (Fig. 6B).

Because contraction of human mHSC is associated with elevation of intracellular calcium (15), we investigated the effects of CNP on thrombin-induced increase in [Ca2+]. As shown in Fig. 6A, thrombin caused an initial rapid elevation in [Ca2+], which was followed by a more sustained phase. Incubation of mHSC with CNP shortened the duration of the calcium peak induced by thrombin (Fig. 6B). In thrombin-stimulated cells, the calcium peak lasted 107 ± 7 s and decreased to 76 ± 4 s in thrombin-stimulated cells treated with CNP. In contrast, CNP modified neither the first phase of the calcium response nor the amplitude of the calcium transient induced by thrombin. The effects of CNP were reproduced by addition of the permeant analog of cGMP, 8-CPT-cGMP, which abolished the contractile effect of thrombin and shortened the duration of the calcium peak (Fig. 6C). Like CNP, the cGMP analogs did not affect the first phase of the calcium response, while diminishing the duration of the calcium peak induced by thrombin to 76 ± 8 s (Fig. 6C).

In nonexcitable cells, the initial phase of the calcium response is due to release of calcium from intracellular stores, whereas the second phase of the calcium transient is consecutive to influx of calcium from external medium (34). In order to investigate the importance of calcium influx in mHSC contraction, we examined the response of mHSC to thrombin in the absence of external calcium, i.e. in a calcium-free medium containing 1 mM EGTA. Treatment with EGTA shortened the calcium peak elicited by thrombin to 72 ± 6 s, without affecting either the amplitude of the calcium transient or the first phase of the calcium response (Fig. 6D). Concomitantly, preincubation of mHSC in the calcium-free medium blunted the contractile effects of thrombin (Fig. 6D), thereby reproducing the effects of CNP.

The next series of experiments were designed to characterize the nature of the calcium channel responsible for calcium in-
TABLE II
Calcium currents in human hepatic stellate cells

| Electrophysiological properties | Mean capacitance | Mean amplitude | Mean density |
|-------------------------------|-----------------|---------------|-------------|
| 14                            | 36.9 ± 5.7 pF   | -16.4 ± 21.6 pA | -0.82 ± 0.37 pA/pF |
| 15                            | Not detectable  | Present in 5 cells |             |
| 15                            | Present in 5 cells |             |             |
| 5                             | -16.4 ± 21.6 pA |             |             |

Flux and inhibited by CNP. Voltage-activated calcium currents have been occasionally observed in hepatic stellate cells from rat origin (29, 35). Therefore, we used the patch-clamp technique to examine the presence of low voltage (T-type) and high voltage (L-type) activated calcium channels in human myofibroblastic HSC, with a two-step protocol. High voltage activated calcium currents were present, but only in one-third of the cells studied (Table II), whereas 99% of the cells were positive for smooth-muscle α-actin (20); low voltage activated calcium currents were not detectable. Moreover, thrombin (1 unit/ml) did not stimulate either high voltage or low voltage activated currents (not shown). Thus, the activity of voltage-gated calcium channels cannot provide a mechanism for the transmembrane calcium influx described above. We therefore examined the potential contribution of store-operated calcium channels in calcium influx stimulated by thrombin and used a calcium-free/calcium readdition protocol, which is a sensitive procedure to measure changes in calcium influx through these channels (36, 37). Fura-2-loaded cells were stimulated by thrombin in the absence of extracellular calcium. Once the rapid and transient elevation in [Ca2+]i had returned to basal levels, readdition of calcium was performed and resulted in a rapid rise, indicating that intracellular depletion by thrombin triggers a secondary calcium influx through store-operated calcium channels (Fig. 7A, 8 out of 11 cells). This influx was totally blocked by addition of 10 nM CNP (Fig. 7B, 12 cells over 14). In addition, it was also totally abolished by LaCl3, a store-operated calcium influx blocker (37, 38) (Fig. 7C, 8 cells over 11). In contrast, nitrendipine, a voltage-operated calcium channel inhibitor, had no effect (Fig. 7D, 14 cells over 18). Taken together, these results indicate the essential role of calcium influx in the contractile process and suggest that CNP and 8-CPT-cGMP prevent mHSC contraction by blocking the influx of calcium through store-operated calcium channels.

**DISCUSSION**

We show here that human myofibroblastic HSC express C-type NPR-B. Activation of this receptor leads to inhibition of both growth and contraction of mHSC.

In human liver, mRNAs for the three types of natriuretic peptide receptors, NPR-A, NPR-B, and NPR-C, have been identified by RT-PCR (5). Whereas no information concerning the hepatic cell expressing NPR-B has been provided, biologically active NPR-A and NPR-C have been detected in liver. Thus, binding of ANP to NPR-C inhibits proliferation of hepatoblastoma Hep G2 cells (39). Also, NPR-A and NPR-C are coexpressed in human biliary cells, and NPR-A is coupled to Cl− channels (40). Finally, both NPR-A and NPR-C binding sites are increased during liver regeneration (41). We show that human myofibroblastic HSC exclusively express the NPR-B mRNA, as demonstrated by RT-PCR detection. This receptor is functional, as indicated both in binding experiments and by the dramatic increase in cGMP levels elicited by CNP as compared with the modest effect of ANP, a typical order of potency of natriuretic peptides for NPR-B.

**FIG. 7.** CNP inhibits calcium influx through store-operated calcium channels in human myofibroblastic HSC. Human myofibroblastic HSC were made quiescent by incubation in serum-free medium over 3 days and loaded with Fura-2 as described under “Experimental Procedures.” A, calcium store depletion by thrombin triggers calcium influx through store-operated calcium channels. Fura-2-loaded cells were incubated with 1 unit/ml thrombin. Once the rapid and transient elevation in the [Ca2+]i ratio had fallen down to basal levels, cells were washed, and 10 mM Ca2+ was reintroduced in the medium. B, CNP inhibits store-operated calcium influx. The protocol was as in A, except that 10 nM CNP was added together with EGTA and remained present throughout the experiment. C, LaCl3 inhibits store-operated calcium influx. The protocol was as in A, except that 0.5 mM LaCl3 was introduced together with Ca2+. D, nitrendipine does not affect store-operated calcium influx. The protocol was as in A, except that 1 μM nitrendipine was added together with EGTA and remained present throughout the experiment.

Growth inhibitory properties of natriuretic peptides have been described in various cells, but the receptor involved is clearly cell-specific. Thus, the antiproliferative effects of natriuretic peptides are mediated by NPR-B in chondrocytes and NIH3T3 fibroblasts (42, 43), NPR-A in cardiac fibroblasts (44), and NPR-C in mesangial, vascular smooth muscle, osteoblastic, and astroglial cells (45–48). The molecular mechanisms involved include inhibition of ERK by NPR-C and NPR-A (49) and inhibition of JNK and of AP-1 binding via NPR-A, as recently described in mesangial cells (50). In human myofibroblastic HSC, CNP is far more potent than ANP in inhibiting mHSC growth, and the IC50 of CNP for inhibiting mHSC growth is similar to that of CNP for its receptors. The antiproliferative effects of CNP are reproduced by cGMP analogs and blocked by the protein kinase G inhibitor Rp-8-pCPT-cGMP, as well as by HS-142–1, an antagonist of guanylyl cyclase-coupled receptor (31). Growth inhibitory effects of CNP are associated with inhibition of ERK and JNK and blockade of DNA binding activity of AP-1; these effects are reproduced by permeant analogs of cGMP. This suggests that inhibition of ERK and JNK by CNP are crucial events in the blockade of AP-1-activated genes. The mechanism by which CNP inhibits ERK and JNK in human mHSC is unknown but may involve induction of the MAPK phosphatase MKP-1, as reported for ANP in mesangial cells (51), and/or inhibition of receptor tyrosine kinase
by CNP, as observed for the PDGF receptor in smooth muscle cells (52).

In human myofibroblastic HSC, a part of the signaling pathways stimulated by cGMP-elevating agonists is common to those stimulated by cAMP-dependent agonists. Thus, CNP, which increases cGMP, and ET-1, which activates a prostaglandin/cAMP pathway (12), both cause mHSC growth arrest by blocking ERK and JNK activations and the resulting AP-1 DNA binding activity. In contrast, whereas ET-1 stimulates NF-κB and activates cyclooxygenase-2 (13), CNP has no effect on either NF-κB (Fig. 5) or cyclooxygenase-2 (not shown). However, the question arises of whether CNP may increase cAMP via cGMP. This is unlikely, based on two lines of evidence: (i) CNP and 8-Br-cGMP do not increase cAMP, even after prolonged incubation; (ii) the activity of cGMP-inhibited phosphodiesterase, which is inhibited by cGMP and hydrolyzes cAMP (30) is negligible in human myofibroblastic HSC (Table I). These results demonstrate that blockade of both the ERK/JNK cascade and AP-1 DNA binding are common crucial steps in the growth inhibitory effects of both cGMP-elevating factors and cAMP-mobilizing agonists.

Contraction of myofibroblastic HSC in culture has been reported in response to ET-1 via endothelin-A receptors, thrombin, angiotensin II, substance P, thromboxane A2, and vasopressin (14–17), and contractility is counteracted by relaxing agents, such as cGMP-elevating factors (nitric oxide or interleukin-1 (53, 54)), and cAMP-elevating agonists (PGE2, PGI2, and adrenomedullin (16, 55)). Although vasodilating properties of natriuretic peptides have been found in diverse tissues (1), few studies have investigated their effects on hepatic hemodynamics. In normal liver, ANP regulates intrahepatic resistance because it antagonizes the increase in portal pressure elicited by α1-adrenergic (56). In rats with experimental cirrhosis, systemic administration of CNP reduces portal pressure (57), but whether this effect relates to a decrease in intrahepatic resistivity is not yet clear. In the present study, modulation of voltage-gated calcium currents in glomerulosa cells have been recorded with the high voltage gated calcium channels (L-type) recorded with the patch clamp technique were only occasionally detected, as described by others in rat HSC (29, 35); and (iii) low voltage activated calcium channels (T-type) were undetectable. Therefore, we hypothesized that store-operated calcium channels may be the CNP target and used a calcium-free/calcium read-in protocol. This protocol has been used in several cell types (see, for example, Refs. 36 and 37) and relies on the fact that either calcium mobilization or depletion of calcium from endogenous stores constitutes an essential step for stimulation of store-operated calcium channels (SOC). We observed that thrombin stimulation of SOC in human mHSC was totally blocked by CNP as well as by a SOC inhibitor, LaCl3. Taken together, these data strongly suggest that CNP relaxes myofibroblastic HSC following blockade of calcium influx through store-operated calcium channels. These results, which constitute the first report of store-operated calcium channels as a target for CNP, provide a new mechanism for the relaxing effects of CNP.

Recent evidence supports a role for myofibroblastic HSC in the regulation of sinusoidal tone, and thereby of intrahepatic resistance (19). Modulation of sinusoidal tone results from the fine tuning between contractile and relaxing activities of diverse mediators produced locally. Little is known regarding hepatic production of CNP. CNP mRNA is expressed in human liver (5), suggesting the existence of a local natriuretic peptide system, but cellular origin of the peptides remains to be determined. Our preliminary data suggest that hepatocytes are a major source of CNP in human liver and that its expression is increased during chronic liver diseases. These results suggest that during chronic liver injury, CNP could play a key role in counteracting liver fibrosis and associated portal hypertension by inhibiting mHSC proliferation and antagonizing the contractile response of these cells to vasoactive mediators.

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