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STABLE EXPRESSION OF NATRIURETIC PEPTIDE RECEPTORS: EFFECTS OF HS-142-1, A NON-PEPTIDE ANP ANTAGONIST

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Summary: We established clonal cell lines stably expressing each of two subtypes of membrane bound guanylate cyclases (GC-A and GC-B), which are known as natriuretic peptide receptors. Using these cell lines, we showed that GC-A is an ANP/BNP receptor, whereas GC-B is a specific receptor for CNP. Effects of HS-142-1, a novel non-peptide ANP antagonist, on GC-A and GC-B were examined by using these cells. In cells expressing either GC-A or GC-B, HS-142-1 inhibited cGMP production elicited by ANP or CNP with IC_{50} values of 1.8 μg/ml and 1.5 μg/ml, respectively, and also competitively blocked specific binding of the natriuretic peptides with IC_{50} values of 2.2 μg/ml and 3.3 μg/ml, respectively. These results indicate that HS-142-1 is a potent antagonist of CNP as well as ANP. We also showed that CNP suppressed the growth of cells expressing GC-B by 22% and that HS-142-1 blocked the antiproliferative action of CNP.

Natriuretic peptides constitute a peptide family. Atrial natriuretic peptide (A-type natriuretic peptide, ANP) (1), and brain natriuretic peptide (B-type natriuretic peptide, BNP) (2,3) are primarily accepted as cardiac hormones that regulate salt and water balance and blood pressure. C-type natriuretic peptide (CNP), on the other hand, has been thought to function as a neuropeptide in the central nervous system (4,5). Functions of natriuretic peptides are mediated by the increased production of the second messenger, cGMP, through the activation of particulate guanylate cyclases. Two similar membrane bound guanylate cyclases, termed GC-A and GC-B, have been identified by molecular cloning (6,7). Recently we have shown that GC-B is a specific receptor for CNP whereas GC-A is an ANP/BNP receptor in rat and human natriuretic peptide systems (8,9). Differences in tissue distribution and ligand specificity between GC-A and GC-B suggest distinct physiological functions of these natriuretic peptide receptors.

Abbreviations: ANP, atrial or A-type natriuretic peptide; BNP, brain or B-type natriuretic peptide; CNP, C-type natriuretic peptide; GC-A, membrane bound guanylate cyclase-A; GC-B, membrane bound guanylate cyclase-B; cGMP, cyclic guanosine 3',5'-monophosphate; CHO cell, Chinese hamster ovary cell; PBS(-), Ca^{++}, Mg^{++} -free phosphate buffered saline; DMEM, Dulbecco's modified Eagle's medium; EC_{50}, half-maximal effective concentration; IC_{50}, half-maximal inhibitory concentration; Kd, dissociation constant; Ki, inhibition constant.

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A novel polysaccharide derivative, HS-142-1, was recently isolated from the culture broth of *Aureobasidium* sp. as a non-peptide ANP antagonist (10,11). HS-142-1 competitively blocks specific binding of ^{125}I-rANP to solubilized bovine adrenocortical membrane fractions, effectively inhibits cGMP production elicited by rANP from the membrane fractions, and antagonizes the actions of ANP in vivo and in vitro (10-19). However, it is not yet clear whether HS-142-1 also antagonizes CNP through interacting with its receptor, GC-B. Since most tissues or cultured cells co-express GC-A and GC-B, and a binding protein of natriuretic peptides, the so-called "clearance receptor" is often abundantly expressed in many tissues, it is difficult to elucidate the subtype specificity of HS-142-1 by using tissues or cultured cells.

In this study, we established clonal cell lines that stably expressed either rat GC-A or GC-B by transfection of these genes, and examined the effect of HS-142-1 on GC-B as well as GC-A by using these clonal cell lines. The present study will show that HS-142-1 recognizes both GC-A and GC-B, indicating that HS-142-1 is also a CNP antagonist.

Recently we have shown that CNP has a growth suppressive effect on cultured vascular smooth muscle cells (20), and that CNP is also present in the human monocytic cell line, THP-1 (21), suggesting that CNP may be involved in the hemo-vascular interaction. In this study, we showed that the growth rate of cells stably expressing GC-B was suppressed by CNP and that HS-142-1 was able to abolish the antiproliferative effect of CNP.

Materials and Methods

Materials: Rat α-ANP, BNP-45 and CNP-22 were purchased from Peptide Institute Inc. (Osaka, Japan). ^{125}I-rANP and ^{125}I-[Tyr^0]-CNP were synthesized as described previously (22). HS-142-1 was isolated as previously reported (11).

Transfection and Stable expression of the cDNA clones encoding GC-A and GC-B: The full length cDNAs of rat membrane bound guanylate cyclases (GC-A and GC-B) (9) were subcloned into the eukaryotic expression vector, pSD(X)-dhfr, containing the mouse dihydrofolate reductase gene as a selective marker (23). The resulting expression plasmids, pSD(X)-dhfr-GC-A and pSD(X)-dhfr-GC-B, were transfected into CHO(dhfr$^-$) cells (24) by the Chen-Okayama's method (25). CHO(dhfr$^-$) cells were maintained in α-minimal essential medium containing ribonucleotides and deoxyribonucleotides, supplemented with 10% fetal bovine serum (FCS). Cell populations expressing a rat membrane bound guanylate cyclase together with dihydrofolate reductase were selected in α-minimal essential medium lacking ribonucleotides and deoxyribonucleotides, supplemented with 10% FCS, in the presence of 0.1μM methotrexate. From these selected cell populations, clonal cell lines were isolated by repeated cloning.

Guanylate cyclase assays: CHO cells stably expressing membrane bound guanylate cyclases in 24-well dishes (1.0×10^4 cells per well) were washed twice with PBS(-), preincubated in DMEM + 25mM HEPES (pH 7.4) + 0.1mM isobutyl methylxanthine for 30 min at 37°C, and stimulated for 30 min at 37°C in the same medium containing 0.1% BSA with the various concentrations of natriuretic peptides. After the incubation, the cGMP content released into the medium was determined by a radioimmunoassay.

Binding assays: Receptor-expressing cells in 6-well dishes (1.5×10^5 cells per well) were washed twice with PBS(-), and incubated with various concentrations (for Scatchard analysis experiments) or 200 pM (for displacement experiments) of ^{125}I-labeled natriuretic peptides in DMEM + 25mM HEPES (pH 7.4) + 0.1% BSA for 1 hr at 37°C. After the incubation, the cells were extensively washed with PBS(-) and solubilized with 0.2N NaOH. Cell bound radioactivity was measured by an autogamma counter (Aloka Co. Ltd.). Nonspecific binding was identified as binding activity in the presence of 0.2μM unlabeled natriuretic peptides and was subtracted from total binding activity for determination of specific binding.

Cell growth assays: For determination of cell numbers, CHO cells stably expressing GC-B were seeded into 24-well dishes at 1×10^4 cells per well and maintained in the above selecting medium supplemented with 10% FCS in the presence of various concentrations of CNP for 4

days, with media changes every other day. Then the cells were trypsinized and counted with a hemocytometer (Sysmex).

Results and Discussion

The cDNA clones encoding rat GC-A and GC-B in an expression vector were transfected into CHO(dhfr$^-$) cells. A mouse dihydrofolate reductase gene was used as a selective marker gene which allowed receptor-expressing cell populations to grow in the medium lacking ribonucleotides and deoxyribonucleotides, and containing 0.1μM methotrexate. More than 7 clonal cell lines that stably expressed either GC-A or GC-B at various levels were isolated by repeated cloning. The two cell lines expressing maximal levels of GC-A or GC-B were identified by saturation binding analysis with ^{125}I-labeled either ANP or CNP. The receptor number for ANP or CNP was estimated to be 3600 and 9200 per cell, respectively. Untransfected CHO cells practically expressed neither ANP nor CNP receptors.

In cells expressing GC-A, both ANP and BNP increased cGMP production in a dose dependent manner with EC_{50} values of 1.2 nM and 1.7 nM, respectively. However, CNP had practically no effect on cGMP production (Fig. 1A). Conversely, in cells expressing GC-B, CNP elicited a dose-dependent increase in cGMP production (EC_{50}: 0.14 nM), whereas both ANP and BNP also showed a dose-dependent increase in cGMP production, though extremely high concentrations were required (Fig. 1B). The results were consistent with the previous data obtained by using COS cells transiently expressing these receptors (9).

We used the above two cell lines to study the effects of HS-142-1. HS-142-1 inhibited the cGMP production stimulated by ANP in cells expressing GC-A in a dose-dependent manner (IC_{50}: 1.8 μg/ml) (Fig. 2A). This result was consistent with the previous data obtained by using neurally derived cell lines (PC12 and NG 108-15) which predominantly express GC-A (19). Similarly, HS-142-1 inhibited the cGMP production induced by CNP in cells

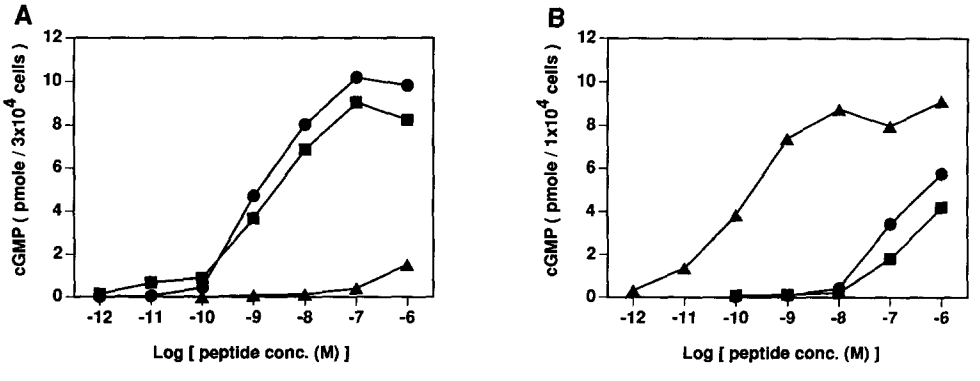

Figure 1. Concentration-dependent stimulation of GC-A and GC-B by three types of natriuretic peptides. CHO cells stably expressing GC-A (A) or GC-B (B) were stimulated with various concentrations of ANP (●), BNP (■) and CNP (▲). The cGMP concentrations released into the medium were determined by a radioimmunoassay.

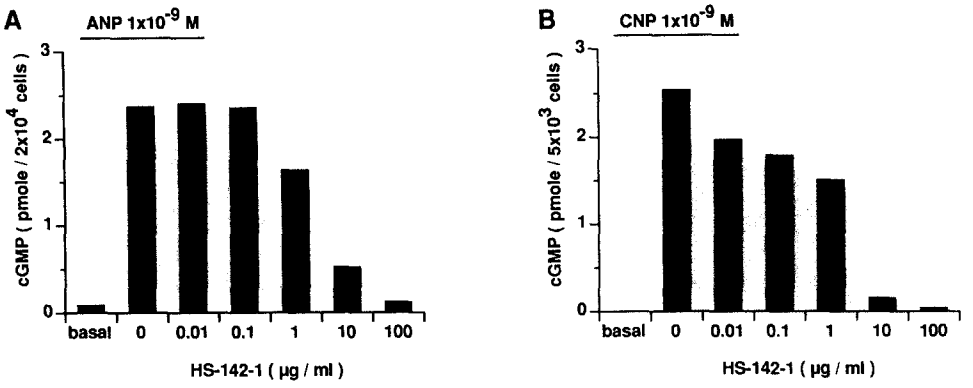

Figure 2. Effects of HS-142-1 on the cGMP production induced by natriuretic peptides in cells stably expressing GC-A (A) or GC-B (B). The cGMP production elicited by $1x10^{-9}$M ANP (A) or $1x10^{-9}$M CNP (B) was measured in the presence of various concentrations of HS-142-1.

expressing GC-B (IC_{50}: 1.5 µg/ml) (Fig. 2B). In both cases, HS-142-1 completely abolished the natriuretic peptide-dependent stimulation of cGMP production at a concentration of 100 µg/ml.

In addition, HS-142-1 inhibited ^{125}I-ANP binding to cells expressing GC-A in a dose-dependent manner (IC_{50}: 2.2 µg/ml). Similarly, HS-142-1 inhibited ^{125}I-[Tyr^0]-CNP binding to cells expressing GC-B (IC_{50}: 3.3 µg/ml) (Fig. 3). At a concentration of 1 mg/ml, HS-142-1 completely displaced the specific binding of natriuretic peptides to both GC-A and GC-B. As shown in Fig. 4, HS-142-1 apparently altered dissociation constants (Kd) without changing the maximum number of GC-A or GC-B per cells. This indicates that HS-142-1 is a competitive inhibitor of both GC-A and GC-B. Inhibition constants (Ki) of HS-142-1 for GC-A and GC-B were calculated to be 0.39 µM and 0.24 µM, respectively, indicating that antagonistic

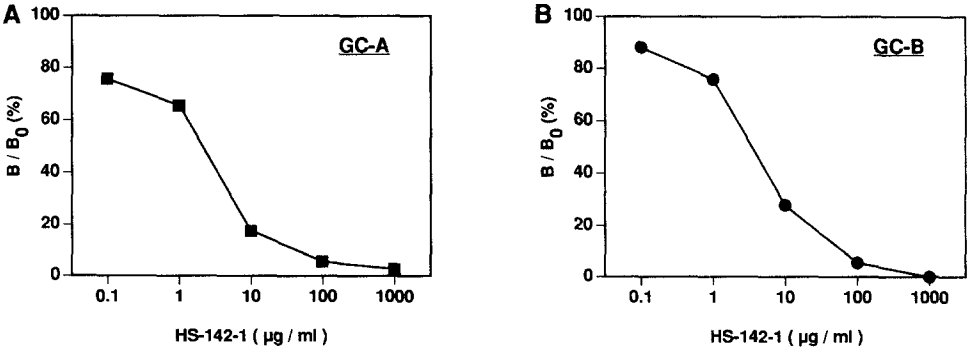

Figure 3. Effects of HS-142-1 on the specific binding of ^{125}I-labeled natriuretic peptides to cells stably expressing GC-A (A) or GC-B (B). Concentration-dependent inhibition of the specific binding of ^{125}I-ANP (A) or ^{125}I-[Tyr^0]-CNP (B) by HS-142-1 is shown. Inhibitory effects are expressed as percentages of the specific binding of ^{125}I-labeled natriuretic peptides.

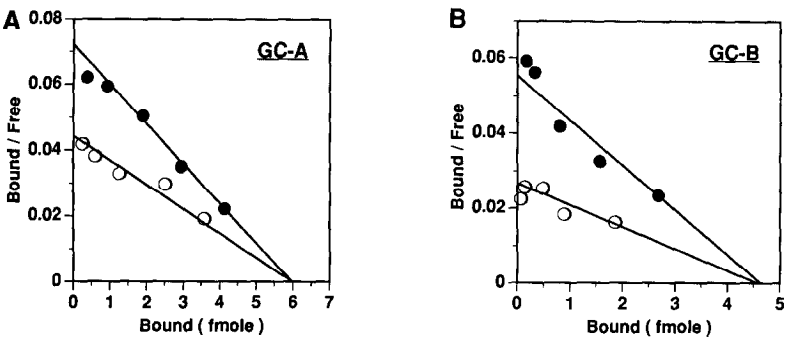

Figure 4. Scatchard analyses of effects of HS-142-1 on the specific binding of ^{125}I-labeled natriuretic peptides to cells stably expressing GC-A (A) or GC-B (B). The specific binding of ^{125}I-ANP (A) or ^{125}I-[Tyr^{0}]-CNP (B) was measured in the absence of HS-142-1 (●, Kd; 167 pM and 167 pM, respectively) or the presence of 1 μg/ml of HS-142-1 (○, Kd; 273 pM and 340 pM, respectively).

ability of HS-142-1 against CNP is similar to that against ANP. Accordingly, we clearly showed here that HS-142-1 is also a potent antagonist of CNP, hence it is a functional antagonist for all natriuretic peptides identified so far. Furthermore, it is interesting that HS-142-1 had little or no effect on ^{125}I-ANP binding to clearance receptors which bind all natriuretic peptides but lack guanylate cyclase activity (12,14).

Recently, we have found that CNP is produced by THP-1 cell line of monocyte origin and elicits antimitogenic and antiproliferative action in cultured vascular smooth muscle cells (20,21). This suggests that CNP may be involved in the hemo-vascular interaction, especially in initial steps of atherosclerosis, because invasion and foaming of macrophages in the subendothelial space and excessive vascular smooth muscle proliferation are considered to be a key event for initiation of atherosclerosis. In this study, we examined the effects of CNP in the absence or presence of HS-142-1 on serum-induced proliferation of cells stably expressing GC-B. As shown in Fig. 5A, CNP suppressed the growth of cells expressing GC-B by 22% in a dose-dependent manner. The antiproliferative action of CNP was completely blocked by HS-142-1 (Fig. 5B). Effective concentrations of HS-142-1 were similar to those obtained by the experiment in which HS-142-1 inhibited the cGMP production elicited by CNP. This clearly shows that the antiproliferative action of CNP is mediated by the increased production of the second messenger, cGMP, through the activation of GC-B. In addition, HS-142-1 will be a useful tool to examine physiological functions of CNP in the hemo-vascular system as well as in the central nervous system.

In conclusion, we established the clonal cell lines that stably express each of two subtypes of receptors for natriuretic peptides (GC-A and GC-B). Using these cell lines, we showed here that HS-142-1 antagonizes CNP by inhibiting cGMP production from the specific receptor, GC-B, in a way indistinguishable from that in which ANP is antagonized by HS-142-1. Moreover, CNP suppresses the growth of cells expressing GC-B and HS-142-1 is able to block the antiproliferative action of CNP.

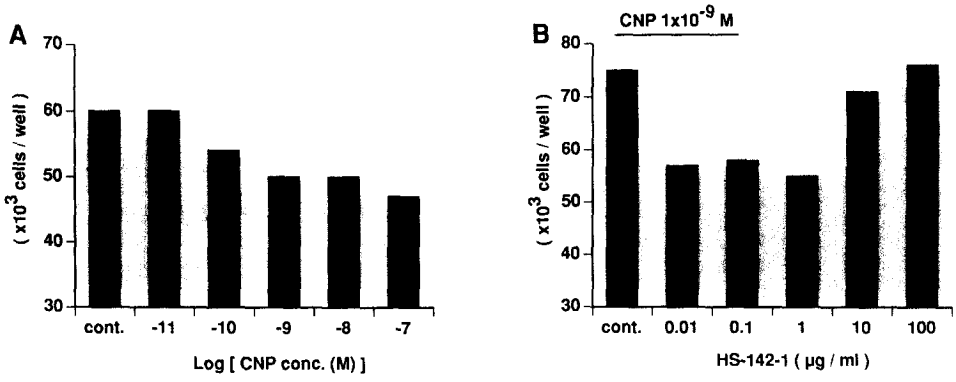

Figure 5. Effects of CNP in the absence (A) or presence (B) of HS-142-1 on serum-induced proliferation of cells stably expressing GC-B. (A) Inhibition of serum-induced proliferation of cells stably expressing GC-B by CNP. (B) Effects of HS-142-1 on the antiproliferative action of $1x10^{-9}$M CNP. Cell growth assays were performed as described in Materials and Methods.

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