HS-142-1, a Novel Non-peptide Antagonist for Atrial Natriuretic Peptide Receptor, Selectively Inhibits Particulate Guanylyl Cyclase and Lowers Cyclic GMP in LLC-PK₁ Cells

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HS-142-1, a novel atrial natriuretic peptide (ANP) antagonist isolated from the culture broth of *Aureobasidium* sp., selectively inhibits ANP-induced cyclic GMP accumulation in porcine kidney epithelial LLC-PK₁ cells. At concentrations from 0.1 to 100 μg/ml (= 2.5 x 10⁻⁸ - 2.5 x 10⁻⁵ M, given the mean molecular weight is 4,000), HS-142-1 prevents intracellular cyclic GMP accumulation initiated by 10⁻⁵ M rat ANP in a dose-dependent manner, but not cyclic GMP accumulation produced by 10⁻⁵ M sodium nitroprusside. HS-142-1 alone has no effects on the basal level of cyclic GMP seen in the absence of ANP. No change of intracellular cyclic AMP was observed upon the treatment of the cells with HS-142-1. Further, the selectivity of HS-142-1 for the guanylyl cyclase-linked receptor was confirmed by affinity labeling studies with bovine adrenocortical membranes. HS-142-1 specifically abolished the labeling of the guanylyl cyclase-linked 135-kDa band in a dose-dependent manner, but not the labeling of the 60-kDa band not coupled to the guanylyl cyclase. These results show that HS-142-1 selectively inhibits ANP-mediated accumulation of cyclic GMP in LLC-PK₁ cells through interacting with guanylyl cyclase-linked receptors.

The family of natriuretic peptides (atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide) produce a variety of biological effects, including diuresis, natriuresis, and vasodilation through interacting with specific receptors in many target tissues.¹⁻⁴ Atrial natriuretic peptide (ANP), the first discovered member of the family, is a circulating hormone mainly secreted from mammalian atria.⁵⁻⁶ At least two functionally and structurally distinct ANP receptors, guanylyl cyclase-linked receptor and guanylyl cyclase-free receptor, are present in various target tissues in different ratios.⁷ Guanylyl cyclase-linked receptor, which is presumed to be the functional ANP receptor, contains a guanylyl cyclase domain in its structure and binding of ANP to an extracellular ligand-binding domain is directly associated with a significant increase in intracellular cyclic GMP accumulation via activation of intracellular guanylyl cyclase.⁸⁻¹⁰ This form of guanylyl cyclase is distinct from soluble guanylyl cyclase, which responds to the vasodilator sodium nitroprusside.¹¹ On the other hand, guanylyl cyclase-free receptor is apparently not coupled to guanylyl cyclase, and thought to be specific for ANP storage or clearance.¹²⁻¹⁴ The cloning and expression of cDNA encoding a mammalian guanylyl cyclase-linked type of natriuretic peptide receptor has shown there are two different subtypes of the receptor.¹⁵ Recently, characterization of the relationship between receptor subtypes and the isopeptides has been reported.¹⁶ However, the true physiological role of ANP is still somewhat uncertain.¹⁷ To discover if endogenous ANP has an actual physiological role, a discovery of a specific and non-peptide ANP antagonist has been awaited for a long time.¹⁸

Recently, we have isolated a novel microbial polysaccharide, β-1,6-glucan esterified with capronic acid.¹⁷,¹⁸ This substance, HS-142-1, competitively and selectively inhibits ANP binding to its guanylyl cyclase-linked receptor from adrenocortical microsomes.¹⁹ In our previous paper,¹⁷ we studied the antagonistic activities of HS-142-1 against ANP-stimulated cyclic GMP production by using a bovine adrenocortical membrane preparation; in that case, however, we could observe only an approximately 1.4-fold increase in cyclic GMP in the presence of ANP along with a stimulant, ATP. In this study, we have explored the biochemical properties of HS-142-1 and established more conclusively that HS-142-1 blocks ANP-induced cyclic GMP production through the interaction with the guanylyl cyclase-linked ANP receptor. For this purpose, we have examined the antagonistic activities of HS-142-1 against ANP-stimulated cyclic GMP accumulation in porcine kidney epithelial LLC-PK₁ cells, which produce cyclic GMP in response to natriuretic peptides.¹⁹,²⁰ We also used photoaffinity labeling and affinity cross-linking to study the specificity of HS-142-1 for the guanylyl cyclase-linked receptor.

Materials and Methods

Materials. Rat ANP ([Arg²,Pro⁵,Pro²]ANP[99-126]) and human ANP [hANP(ANF)[99-126]] were purchased from the Peptide Institute, Inc.,...
Osaka, Japan and Peninsula Laboratories, Inc., respectively. Purified Triton X-100 and disuccinimidyl suberate (DSS) were purchased from Pierce Chemical Co. ([125I]Iodotroxy13]3-[13]IAPN and [125I]-hAPN were purchased from Amersham and New England Nuclear, respectively. Denny-Jaffe reagent (4-[4-azido-3-[13]Iodophenylazoi]benzoyl]-3-aminopropyl-4-oxysuccinimide ester) was from New England Nuclear. Radioimmunoassay kits for cyclic GMP and cyclic AMP measurements were the products of Yamasa Shoyu, Chiba, Japan. Cell culture media were obtained from Nissui, Tokyo, Japan. Reagents for electrophoresis and molecular standards were purchased from Sigma Chemical Co. HS-142-1 was purified from the fermentation broth of *Aureobasidium sp. KAC-2383* in our laboratories as described. All other reagents were of analytical grade.

**Cell culture and cyclic nucleotides measurements.** The LLC-PK1 cells obtained from the American Type Culture Collection (ATCC, CRL-1392) was grown as monolayers in 199 medium with 10% fetal bovine serum in a 5% CO2 water-saturated atmosphere. Confluent cells were trypsinized and subcultured every 3–4 days. For the measurement of cyclic nucleotides, cyclic GMP and cyclic AMP, production, the cells were seeded onto 24-well cluster plates. The confluent cell monolayer was washed twice with DMEM with 20 mM HEPES and were incubated at 37°C for 30 min in a final volume of 0.5 ml of the same medium containing 0.1% bovine serum albumin and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) with varied amounts of ANP or sodium nitroprusside in the presence or absence of different concentrations of HS-142-1. After the incubation, the medium was removed and the extracellular concentrations of cyclic nucleotides were measured directly by radioimmunoassay with Yamasa radioimmunoassay kits. To measure intracellular cyclic nucleotides, 500 μl of chilled 6% trichloroacetic acid solution was added to the cell monolayer after medium removal. The cell suspension was frozen, thawed, and scraped. After agitating on a Vortex mixer, the suspension was immediately centrifuged at 800 × g for 10 min. The supernatants were then extracted three times with water-saturated diethyl ether and lyophilized. Each lyophilized sample was dissolved in water and cyclic nucleotides were measured as described above.

**Affinity labeling studies.** Adrenocortical membranes were prepared and solubilized with Triton X-100 as described except that 50 mM HEPES buffer (pH 7.4) was used in place of Tris-HCl buffer (pH 7.4). A ([125I]-labeled cyclic AMP cAMP affinity probe, the azidoenol ester derivative of hAPN, was prepared by the following procedure using Denny-Jaffe reagent and hAPN. After evaporation of the benzene, 10 μg hAPN dissolved in 100 μl of 50 mM HEPES (pH 7.5) containing 0.1% Triton X-100 was then added, mixed in, and allowed to react for 60 min at room temperature. After the reaction, the reaction product was separated from the unhydrolyzed Denny-Jaffe reagent by purification on a Vydac C18 column (300 A, 4.0 mm x 250 mm) developed with a linear gradient of isopropanol-water (10–70%, v/v) at a flow rate of 1.0 ml/min. For the photolabile labeling study, membrane preparations were incubated in the dark for 60 min at 0°C with the 5 μM [125I]-labeled photolabile probe in 50 mM HEPES buffer, pH 7.4, containing 0.15 M NaCl, 0.1 mM PMSF, 5 mM MgCl2, 0.5 mg/ml bacitracin, and nonradioactive competing ligand when used. Photolysis was done on ice for 20 min, using a 250-W General Electric Sunlamp at a distance of 15 cm. After photolysis, the sample mixtures were dissolved into sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 3% SDS, 10% glycerol, and 0.05 mg/ml bromophenol blue) in the presence of 5% 2-mercaptoethanol. After this was boiled for 4 min, the labeled sample mixtures were put on an SDS-polyacrylamide gel (7.5%) and electrophoresed with prestained markers; the gels were dried and autoradiographed. For the affinity crossing-linking study, membrane preparations or Triton X-100 extracts were incubated with 5 μM [125I]-hAPN for 60 min at 0°C in 50 mM HEPES buffer, pH 7.4, containing the same reagents as those in the photolabile labeling study. After incubation, disuccinimidyl suberate in dimethylsulfoxide was added to a final concentration of 2 mM. After the unreacted disuccinimidyl suberate was quenched by the addition of ammonium acetate to make 2 mM, the labeled samples were processed and analyzed as described above.

**Results**

**Selective inhibition by HS-142-1 of ANP-induced cyclic GMP accumulation**

The established cell line LLC-PK1 was used to examine the selective inhibition of HS-142-1 for ANP-induced cyclic GMP production. ANP induces, by up to 3-fold, a dose-dependent accumulation of intracellular cyclic GMP in LLC-PK1 cells (Fig. 1A). Since the extracellular appearance of cyclic GMP is used as a marker of ANP’s biological activity, the content of cyclic GMP in the culture medium was also directly measured. ANP stimulates extracellular cyclic GMP accumulation in a dose-dependent fashion as does the intracellular accumulation (Fig. 2A). Sodium nitroprusside, which is an activator of soluble guanylyl cyclase, also increases intracellular and extracellular cyclic GMP level about 10-fold above the basal levels in a dose-dependent manner (Figs. 1B and 2B). The amount of extracellular cyclic GMP produced by ANP or sodium nitroprusside was greater than that of intracellular cyclic GMP (Figs. 1 and 2). ANP more effectively promoted cyclic GMP extrusion and accumulation of extracellular cyclic GMP in the same manner than sodium nitroprusside (Figs. 1 and 2A) as previously reported. We next examined the effects of HS-142-1 on the intracellular and extracellular cGMP accumulations induced by ANP or sodium nitroprusside. HS-142-1 at concentrations from 0.1 to 100 μg/ml (= 2.5 × 10^{-8} – 2.5 × 10^{-5} m, given the mean molecular weight is 4000),
Fig. 2. Dose-dependent Stimulation of Extracellular Accumulation of Cyclic GMP in LLC-PK1 Cells.
LLC-PK1 cells (1.0 × 10^6 cells/dish) were incubated at 37°C with the indicated concentration of rANP (A), sodium nitroprusside (SNP) (B), or HS-142-1 (C) for 30 min. Cyclic GMP contents in the culture medium were directly assayed as described in Materials and Methods. Results are the means ± SEM of these measurements.

inhibited the intracellular and extracellular cyclic GMP accumulations elicited by 10^{-8} M ANP with almost equal potency (Figs. 3A and 3B); IC_{50} values for the two forms of accumulations were 5 and 6 μg/ml (= 1.25 and 1.5 × 10^{-6} M), respectively. HS-142-1 was not as effective in inhibiting the cyclic GMP accumulations caused by a higher concentration, 10^{-6} M, of ANP, since HS-142-1 possibly competes with ANP in binding to a guanylyl cyclase-linked receptor as described. In contrast, HS-142-1 did not inhibit sodium nitroprusside-induced intracellular and extracellular cyclic GMP accumulations at 10 or 100 μg/ml (= 2.5 × 10^{-8} or 2.5 × 10^{-5} M), which are the concentrations that completely blocked the ANP-induced cyclic GMP production (Fig. 4A—D). HS-142-1 alone was without effect on both intracellular and extracellular accumulations of cyclic GMP in LLC-PK1 cells (Figs. 1C and 2C). Basal levels of cyclic AMP in the cells and in the culture supernatant were 2.8 and 1.4 pmol/10^6 cells, respectively. No alteration of these cyclic AMP concentrations was observed upon incubating the cells with HS-142-1, ANP, or sodium nitroprusside at concentrations up to 100 μg/ml (= 2.5 × 10^{-8} M), 10^{-6} M, or 10^{-5} M (data not shown).
Specificity of HS-142-1 for guanylyl cyclase-coupled receptor

It has been shown that the two cell lines, the epithelial cell line LLC-PK₁ and the fibroblast cell line NIH3T3 have only guanylyl cyclase-linked and guanylyl cyclase-free receptors, respectively.²⁵ To demonstrate that HS-142-1 specifically recognizes the guanylyl cyclase-linked receptor, we initially tried to examine its effects on the binding of [¹²⁵I]-rANP to the membranes prepared from the two cell lines according to the methods described by Fethiere et al.²⁵ But we failed to detect an obvious and significant specific binding of [¹²⁵I]-rANP to the membranes (data not shown). For this purpose, we therefore did photoaffinity labeling and affinity cross-linking studies in the presence of varying concentrations of HS-142-1 with membranes from bovine adrenal cortex, which is known to have the two types of ANP receptors.²¹ An [¹²⁵I]-labeled photoaffinity probe was synthesized by the reaction of the N-terminal amino group of ANP with [¹²⁵I]-labeled cross-linking agent Denny-Jaffee reagent. When the [¹²⁵I]-labeled photoaffinity probe was incubated in the dark with bovine adrenocortical membranes and the mixture photolyzed and electrophoresed on SDS-polyacrylamide gel, radioactivity was covalently incorporated into several polypeptide bands as shown in the autoradiogram in Fig. 5 (lane 1). The most intense labeling was observed in a band of 135 kDa with lower amounts of non-specific labeling in several peptides, as reported.²¹ No labeling was observed if the sample was not exposed to light (data not shown). The labeling of the 135-kDa band was abolished by unlabeled hANP (Fig. 5, lane 5), but not the non-specifically labeled bands. In contrast, labeling of the 60-kDa band, which corresponds to guanylyl cyclase-free receptor, was weak in the photoaffinity labeling technique as previously reported.²⁶ Based on the agreement with observations reported by Takayanagi et al.,²¹ the 135-kDa band should be derived from guanylyl cyclase-linked ANP receptors and the 60-kDa band should be derived from guanylyl cyclase-free type receptors. Under the identical binding conditions, HS-142-1 specifically abolished the labeling of the 135 kDa band in a concentration-dependent fashion (Fig. 5, lanes 2—4).

Chemical cross-linking techniques were also used to confirm the specificity of HS-142-1 for the ANP functional receptor. Adrenocortical membrane preparations or its Triton X-100 extracts to which [¹²⁵I]-hANP had been bound were treated with the cross-linking agent disuccinimidyl suberate (DSS) and the covalent incorporation of radioactivity into proteins was monitored as before by SDS-gel electrophoresis and autoradiography (Fig. 5, lanes 6—13). In either case, radioactivity was specifically incorporated into two major protein bands under reducing conditions, with apparent molecular weights of 135 kDa and 60 kDa, but to different extents (Fig. 5, lanes 6 and 10). With adrenocortical membranes prepared without using Triton X-100, the 135-kDa polypeptides were intensely labeled, with the 60-kDa peptide labeled less (Fig. 5, lane 10). The labeling of both bands was abolished by unlabeled hANP (Fig. 5, lanes 9 and 13). The 135 and 60-kDa bands should be derived from the labeling of the guanylyl cyclase-linked and -free receptors as previously observed in the experiments with the photoaffinity analog of ANP.²¹ In the absence of a reducing agent, both receptors migrated at 135 kDa as reported²¹ (data not shown). Again, HS-142-1 specifically abolished the labeling of the 135-kDa band in a concentration-dependent manner, but had no effect on the labeling of the 60-kDa band (Fig. 5, lanes 7, 8, 11, 12). Taken together, these results demonstrate by the affinity labeling experiments the specificity of HS-142-1 for the guanylyl cyclase-linked ANP receptor in bovine adrenocortical membranes.

In conclusion, HS-142-1 selectively inhibits particulate guanylyl cyclase and lowers cyclic GMP levels in LLC-PK₁ cells through interacting with the guanylyl cyclase-linked ANP receptors.

**Discussion**

Receptor antagonists have provided powerful tools for better understanding of the physiological and pathophysiological roles of ligands. In the case of ANP, due to the lack of a specific antagonist, the roles of ANP is still being debated.²⁷,²⁸ The availability of a specific antagonist for the functional ANP receptor would be a most useful adjunct in any effort to clarify this point.

HS-142-1, a novel β-1,6-glucan isolated from the culture broth of Aureobasidium sp., has been found to be such an antagonist.²⁷,²⁸,²⁹ Because of our previous findings,³⁰,³¹ that HS-142-1 competitively and selectively inhibits ANP binding to the purified guanylyl cyclase-linked receptor, we were interested in exploring the biochemical properties of HS-142-1 as an ANP antagonist. In this study, HS-142-1 showed a dose-dependent inhibition of ANP-induced cyclic GMP production by porcine kidney epithelial LLC-PK₁ cells. Further, HS-142-1 appears to be quite selective for the actions of ANP. Alterations in accumulations of cyclic GMP in the LLC-PK₁ cells or in the culture

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**Fig. 5.** Specific Recognition by HS-142-1 of Guanylyl Cyclase-linked ANP Receptor.

Photoaffinity labeling of the ANP receptors in the bovine adrenocortical membranes (lanes 1-5). Non-solubilized membranes (200 μg of protein) were incubated with 5 nM [¹²⁵I]-labeled photoaffinity probe in the absence of competing ligand (lane 1) or in the presence of 3 x 10⁻⁷ M non-labeled hANP (lane 5) or varied amounts of HS-142-1 (0.2 μg/ml, lane 2; 1.0 μg/ml, lane 3; 5.0 μg/ml, lane 4) for 1 hr at 0°C, photolyzed, electrophoresed on SDS-polyacrylamide gel (7.5%) under reduced conditions, and autoradiographed as described in Materials and Methods. Affinity cross-linking of [¹²⁵I]-hANP to bovine adrenocortical membranes (lanes 6-13). Solubilized membranes (400 μg) (lanes 6-9) or non-solubilized membranes (400 μg) (lanes 10-13) were incubated with 5 nM [¹²⁵I]-hANP in the absence of competing ligand (lanes 6 and 10) or in the presence of 3 x 10⁻⁷ M non-labeled hANP (lanes 9 and 13) or different concentrations of HS-142-1 (1.0 μg/ml, lanes 7 and 11; 5.0 μg/ml, lanes 8 and 12) for 1 hr at 0°C. The cross-linking reaction was done by the incubation of the membranes with disuccinimidyl suberate (DSS) for 20 min at 5°C. After which, the reaction was quenched and the membranes were centrifuged. The washed membranes were solubilized in the sample buffer containing 5% 2-mercaptoethanol. The covalent incorporation of radioactivity into protein was monitored by SDS-gel electrophoresis and autoradiography as described in Materials and Methods. Standard molecular weight marker proteins are myosin (205,000), β-galactosidase (116,000), phosphorylase b (97,400), bovine serum albumin (66,000), and ovalbumin (45,000).
medium may be explained through effects on synthesis and/or metabolism of the cyclic nucleotide. A general effect of HS-142-1 on cyclic nucleotide phosphodiesterase seems unlikely, since the effects of HS-142-1 are inhibitory with respect to cyclic GMP and of no effect with respect to cyclic AMP. Nitroprusside elicits marked accumulation of cyclic GMP in many types of cells through the activation of a cytosolic soluble guanylyl cyclase. HS-142-1 does not inhibit the cyclic GMP accumulation elicited by nitroprusside, indicating that a general effect on cyclic GMP metabolism is not involved in the HS-142-1-elicted inhibition of ANP-mediated accumulation of cyclic GMP formation.

The selectivity of HS-142-1 for the guanylyl cyclase-linked receptor was confirmed by two different affinity labeling techniques. Although the reason why the two ANP receptors were labeled to different degrees in the two different labeling methods remains unclear, HS-142-1 specifically abolished the labeling of the 135-kDa band, which corresponds to the guanylyl cyclase-linked receptor. These observations are consistent with our previous results that HS-142-1 selectively inhibits $[^{125}]$-rANP binding to the guanylyl cyclase-linked receptor purified from adrenocortical membranes. The further, it is worthy to comment that HS-142-1 blocks not only ANP-induced renal responses such as diuresis and natriuresis but also the hypotensive effect in vivo. In conclusion, HS-142-1 selectively interacts with the guanylyl cyclase-linked ANP receptor and lowers the cyclic GMP level in LLC-PK$_1$ cells. HS-142-1 then provides a powerful new tool with which to elucidate the physiological and pathophysiological roles of ANP.

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References