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# REGULATION OF ATRIAL NATRIURETIC PEPTIDE RECEPTORS IN CULTURED VASCULAR SMOOTH MUSCLE CELLS OF RAT

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SUMMARY: To elucidate the regulation of vascular receptors for atrial natriuretic peptide (ANP), we have studied the binding capacity of $^{125}$I-labeled rat (r) ANP using cultured vascular smooth muscle cells from rat aorta. After preincubation with $3.2\times10^{-8}$M rANP at 37°C, the binding capacity decreased as a function of time; the maximal receptor loss (70-75%) occurred after 4 hrs and persisted for 24 hrs. Pretreatment with cycloheximide (20 μg/ml) and actinomycin D (2 μg/ml) similarly caused a dramatic reduction (∿80%) of the binding capacity after 24 hrs; the half-life ($t_{\frac{1}{2}}$) of the receptor loss was ∿7-8 hrs. Following removal of rANP, the "down-regulated" ANP receptors fully recovered in the presence of 10% fetal calf serum, but not in combination with either actinomycin D or cycloheximide. Concanavalin A dose-dependently inhibited the binding. The binding capacity also decreased with time in the presence of tunicamycin (1 μg/ml) with $t_{\frac{1}{2}}$ of ∿30 hrs.

These data indicate that protein and carbohydrate moieties are essential for the functional integrity of the vascular receptor binding sites for ANP, and suggest that the recovery of the receptor loss by "down-regulation" requires concomitant RNA and protein synthesis. 

Atrial natriuretic peptide (ANP), a newly discovered cardiac hormone, has a potent natriuretic and vasodilatory effect (reviewed in ref. 1-5). The mechanism by which ANP induces such biological actions remains undetermined. The initial biochemical step by which polypeptide hormone acts on the target cells is the binding to its specific receptors on the cell membranes (reviewed in ref. 6,7). We have shown that vascular smooth muscle cells (VSMC) derived from rat aorta have specific receptors for ANP functionally coupled to guanylate cyclase system (8), and that vascular ANP receptors are "down-regulated" following prior

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exposure to ANP (9). Subsequent studies from our laboratory have demonstrated that ANP, after binding to its vascular receptors, is internalized into the cells via receptor-mediated endocytocysis (10). Thus, the modulation of ANP receptor activity by "down-regulation" is most likely due to the internalization of hormone-receptor complexes as demonstrated in other polypeptide hormones, such as insulin, epidermal growth factor (EGF) and so forth (reviewed in ref. 6,7). In the present report, we have attempted to elucidate the regulation of vascular ANP receptors during the modulation of protein synthesis and glycosylation.

## METHODS AND MATERIALS

Rat aortic VSMCs were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (FCS: Flow Laboratories, McLean, VA) and antibiotics as reported (8). Binding studies were performed using $^{125}$I-labeled-rat (r) ANP (specific activity: 100-120 μCi/μg) essentially as described (8,9). In brief, confluent cells (~$5\times10^5$ cells) were incubated with $10^{-9}$M $^{125}$I-labeled-rANP in one-ml Hanks balanced salt solution, pH 7.4, containing 0.1% bovine serum albumin (binding medium) at 24°C for 60 min. After incubation, the cells were washed with ice-cold binding medium, solubilized with 0.5N NaOH, and the cell-bound radioactivity was measured in a γ-spectrometer. Nonspecific binding was determined by adding $3.2\times10^{-7}$M unlabeled rANP simultaneously with radiolabeled rANP.

Drugs used in this study were synthetic rANP$^{1-28}$ (Peptide Institute, Osaka, Japan), cycloheximide, actinomycin D, tunicamycin (Sigma Chemical, St. Louis, MO) and Concanavalin (Con) A (Pharmacia, Uppsala, Sweden).

## RESULTS AND DISCUSSION

### "Down-regulation" of ANP receptors

To study the time course of "down-regulation" of vascular ANP receptors, VSMCs were first incubated with $3.2\times10^{-8}$M unlabeled rANP at 37°C for different time periods, after which the unbound rANP was removed by extensive washing and the cell-bound rANP was allowed to degradade at 37°C for 120 min (10). After completion, the binding capacity of $^{125}$I-rANP was determined by the usual binding studies (8,9). As shown in Fig. 1, the binding activity following prior exposure to unlabeled rANP decreased as a function

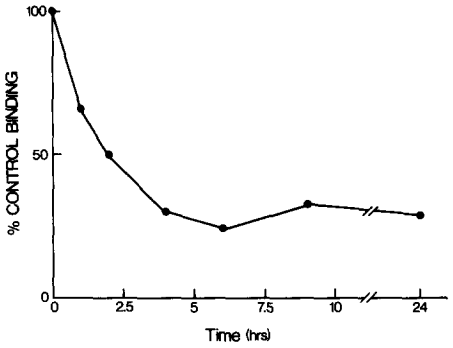


Fig. 1 Time course for down-regulation of vascular ANP receptors. Confluent VSMCs were preincubated with $3.2 \times 10^{-8}$M unlabeled rANP in 1 ml DMEM containing 0.5% FCS for different time periods. At the indicated times, the cells were extensively washed with binding medium to remove the unbound rANP, after which fresh binding medium was readded and the cell were reincubated at 37°C for 2 hrs to permit degradation of the bound rANP. After washing the cells, the binding capacity of $^{125}$I-rANP was measured as described in Methods. The initial binding activity without prior exposure to unlabeled rANP was taken as 100%; each point is the mean of two experiments.

of time; the maximal receptor loss (70-75%) occurred after 4 hrs and persisted for 24 hrs. This result is consistent with our previous data (9) in which pretreatment with unlabeled human (h) ANP ($3.2 \times 10^{-9}$ and $3.2 \times 10^{-8}$M) for 24 hrs leads to a substantial reduction (55 and 75%) of total receptor number without changing the affinity of vascular ANP receptors. These data indicate that the modulation of vascular ANP receptors by ANP, i.e. "down-regulation" mechanism, is a time- and dose-dependent process.

Degradation of ANP receptors

To investigate the rate of disappearance of vascular ANP receptors, VSMCs were preincubated with either cycloheximide (20 µg/ml) or actinomycin D (2 µg/ml) at 37°C for different time periods and $^{125}$I-rANP binding capacity was determined (Fig. 2). Pretreatment of protein synthesis inhibitors similarly caused a dramatic reduction (∿80%) of the binding activity after 24 hrs; the half-life ($t_{\frac{1}{2}}$) of the receptor loss induced by either drug was ∿7-8 hrs. While the cellular protein content as measured by Lowry's method was reduced by 55-60% after 24 hrs, the viability

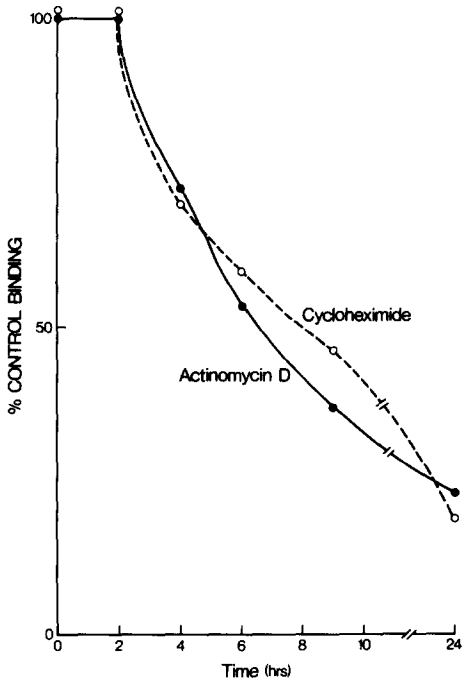


Fig. 2 Effects of cycloheximide and actinomycin D on $^{125}$I-rANP binding activity in VSMCs.
Confluent VSMCs were incubated at 37°C in 1 ml DMEM containing 0.5% FCS in the presence of either (o) cycloheximide (20 μg/ml) or (●) actinomycin D (2 μg/ml) for different time periods. At the indicated times, the cells were washed and $^{125}$I-rANP binding capacity was determined as described in Methods. The initial binding activity without pretreatment was taken as 100%; each point is the mean of two experiments.

of the cells was more than 90% as assayed by trypan blue exclusion (data not shown). The apparent $t_{\frac{1}{2}}$ for degradation of vascular ANP receptors in the present study is comparable to that for EGF receptor (∿6 hrs) (11) and growth hormone receptor (∿10 hrs) (12).

Recovery of ANP receptors

To examine the metabolic requirements for the recovery of vascular ANP receptors after "down-regulation", the capacity of rANP ($3.2\times10^{-8}$ M) treated cells to recover $^{125}$I-rANP binding activity during the modulation of protein synthesis was studied (Fig. 3). In the presence of 10% FCS, the cells whose ANP receptors had been "down-regulated" fully recovered the binding activity within 24 hrs, whereas they restored only about 60% of the initial

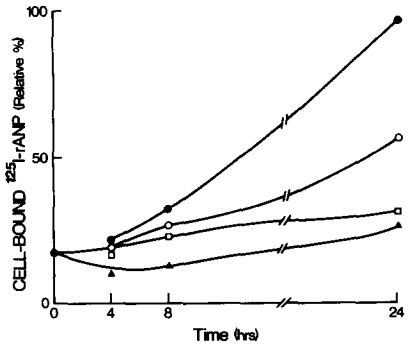


Fig. 3 Recovery of vascular ANP receptors after "down-regulation".

Confluent VSMCs were preincubated with $3.2 \times 10^{-8}$M unlabeled rANP at 37°C for 24 hrs, after which the cells were washed to remove the unbound rANP and further incubated at 37°C for 2 hrs to permit degradation of the bound rANP as described in Fig. 1. Then the medium was replaced with DMEM supplemented with (●) 10% FCS or (○) 0.5% FCS, (□) 10% FCS plus cycloheximide (20 µg/ml), and (▲) 10% FCS plus actinomycin D (2 µg/ml). At the indicated times, the cells were washed and $^{125}$I-rANP binding capacity was determined as described in Methods. The initial binding activity without prior exposure to unlabeled rANP was taken as 100%, and the results are expressed as the relative percentage of cell-bound radioactivity; each point is the mean of two experiments.

binding activity in low concentration (0.5%) of FCS. Furthermore, the capacity to recover the "down-regulated" receptors upon the addition of 10% FCS was blocked by either actinomycin D or cycloheximide. The restoration of ANP binding activity in the presence of serum and the inhibition of this recovery by protein synthesis inhibitors following receptor "down-regulation" as demonstrated in this study appear to be similar to the regulation of EGF receptors (11,13,14), and suggest that the recovery of vascular ANP receptors after "down-regulation" may involve either the synthesis of new receptor molecules or the recycling of internalized receptors in a manner that requires concomitant RNA and protein synthesis.

Glycosylation of ANP receptors

It has been shown that lectins which interact with carbohydrate moieties of the cell membrane inhibit the receptor binding of polypeptide hormones, such as insulin (15) and EGF (16).

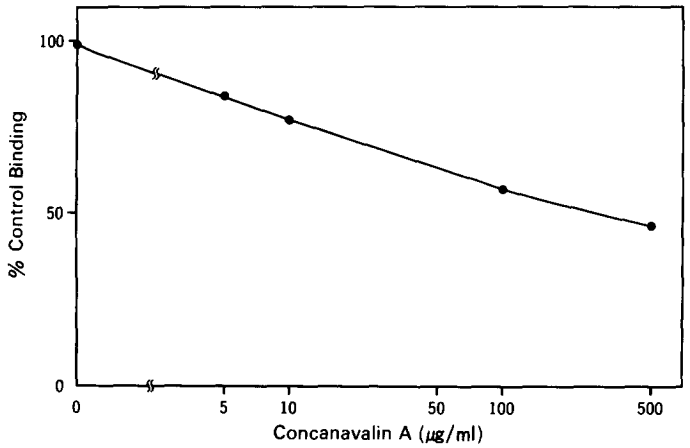


Fig. 4 Effects of concanavalin (Con) A on $^{125}$I-rANP binding activity in VSMCs.
Confluent VSMCs were incubated at 37°C for 2 hrs in 1 ml DMEM containing 0.5% FCS in the presence of Con A in concentrations as indicated. The cells were then washed free of unbound Con A and $^{125}$I-rANP binding capacity was determined as described in Methods; each point is the mean of duplicate dishes.

Therefore, the effect of Con A, a lectin specific for mannose, on $^{125}$I-rANP binding activity was studied (Fig. 4). In VSMCs which were preincubated with Con A in various concentrations (5-500 μg/ml) at 37°C for 2 hrs and washed free of unbound Con A, there was a dose-related decrease of $^{125}$I-rANP binding activity. These data suggest that vascular ANP receptors are, or closely associated with, mannose-containing glycoprotein.

To determine the role of carbohydrates in the functional properties of vascular ANP receptors, the effect of tunicamycin, an antibiotic that specifically inhibits N-glycosylation of oligosaccharides in glycoprotein biosynthesis (17,18), on $^{125}$I-rANP binding activity was studied (Fig. 5). The binding capacity of the cells treated with tunicamycin (1 μg/ml) decreased as a function of time with $t_{\frac{1}{2}}$ of ∿30 hrs. While the binding capacity decreased ∿85% of the initial binding activity after 48 hrs (Fig. 5), the cell viability was more than 90% and the cellular protein content was not affected by tunicamycin treatment (data not shown). These data suggest that N-glycosylation of the membrane glyco-

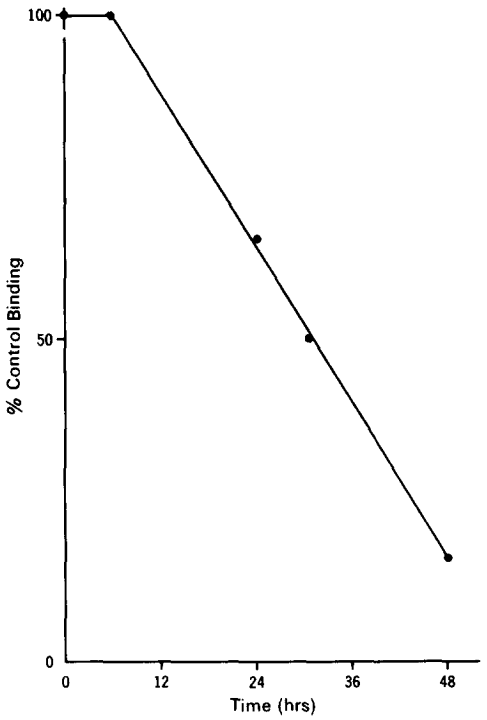


Fig. 5 Effects of tunicamycin on $^{125}$I-rANP binding activity in VSMCs.

Confluent VSMCs were incubated at 37°C in 1 ml DMEM containing 0.5% FCS in the presence of tunicamycin (1 μg/ml) for different time periods. At the indicated times, the cells were washed and $^{125}$I-rANP binding capacity was determined as described in Methods; each point is the mean of two experiments.

protein is required for the functional integrity of the binding component in vascular ANP receptors.

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