

## **Dissociation of natriuresis and diuresis and heterogeneity of the effector system of atrial natriuretic factor in rats.**

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### **Research Article**

The hypotensive, natriuretic, and diuretic actions of human atrial natriuretic factor-(99-126) (hANF) are accompanied by an elevation of cyclic guanosine monophosphate (cGMP) in plasma and urine. However, the oxidized hANF analogue, human [Met-O<sup>110</sup>]ANF-(99-126) (Met-O-ANF), has been reported to be unable to increase cGMP (Biochem. Biophys. Res. Commun. 128: 538-546). We employed this oxidized peptide to evaluate the relationship between its biological effects and cGMP generation, with cGMP serving as a marker of the recognized property of ANF to stimulate particulate guanylate cyclase. Met-O-ANF appeared to be a partial agonist, exhibiting a decreasing order of relative potency of hypotensive, vasorelaxant, diuretic, and natriuretic functions compared to hANF. A lower degree of cGMP increases was achieved by this analogue in cultured smooth muscle and endothelial cells. Met-O-ANF doses, which led to a significant increase in diuresis, were neither natriuretic nor accompanied by an increase of urinary cGMP. We were thus able to dissociate the diuretic and natriuretic effects of ANF. High doses of the oxidized analogue were required to elevate cGMP levels in plasma and urine. In isolated kidney fractions, Met-O-ANF's action on cGMP was significantly lower in glomeruli (fivefold less), virtually absent in the collecting duct, yet only slightly different (20% less) in thick ascending limb. Our results indicate that the diuretic and natriuretic effects are exerted at distinct sites, with [...]

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# Dissociation of Natriuresis and Diuresis and Heterogeneity of the Effector System of Atrial Natriuretic Factor in Rats

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## Abstract

The hypotensive, natriuretic, and diuretic actions of human atrial natriuretic factor-(99-126) (hANF) are accompanied by an elevation of cyclic guanosine monophosphate (cGMP) in plasma and urine. However, the oxidized hANF analogue, human [Met-O<sup>110</sup>]ANF-(99-126) (Met-O-ANF), has been reported to be unable to increase cGMP (*Biochem. Biophys. Res. Commun.* 128:538-546). We employed this oxidized peptide to evaluate the relationship between its biological effects and cGMP generation, with cGMP serving as a marker of the recognized property of ANF to stimulate particulate guanylate cyclase. Met-O-ANF appeared to be a partial agonist, exhibiting a decreasing order of relative potency of hypotensive, vasorelaxant, diuretic, and natriuretic functions compared to hANF. A lower degree of cGMP increases was achieved by this analogue in cultured smooth muscle and endothelial cells. Met-O-ANF doses, which led to a significant increase in diuresis, were neither natriuretic nor accompanied by an increase of urinary cGMP. We were thus able to dissociate the diuretic and natriuretic effects of ANF. High doses of the oxidized analogue were required to elevate cGMP levels in plasma and urine. In isolated kidney fractions, Met-O-ANF's action on cGMP was significantly lower in glomeruli (fivefold less), virtually absent in the collecting duct, yet only slightly different (20% less) in thick ascending limb. Our results indicate that the diuretic and natriuretic effects are exerted at distinct sites, with only the natriuresis being related to an increase of extracellular cGMP. The variability of differential potency of biological and biochemical effects from tissue to tissue of these two forms of human ANF support the notion of the heterogeneity of the ANF effector system.

## Introduction

The cardiac hormone atrial natriuretic factor (ANF)<sup>1</sup> induces natriuresis and diuresis, possesses vasorelaxant properties,

lowers blood pressure and decreases plasma renin and aldosterone levels (for review, see 1-3). Natriuresis and diuresis are probably produced by complex mechanisms, including a specific sensitivity of the renal artery to ANF, leading to elevated renal blood flow (4, 5) and an increased glomerular filtration rate (6). The latter may be due to enhanced filtration pressure elicited by selective vasodilation of the afferent arterioles and vasoconstriction of the efferent arterioles (7, 8). In addition, ANF apparently inhibits tubular sodium reabsorption, especially at the level of the medullary collecting duct (9-11). Although no receptors have been found on proximal tubules (12, 13), lithium excretion is reported to be increased, suggesting either decreased reabsorption in the proximal tubules (14) or an effect secondary to hemodynamic changes.

Enhanced diuresis and natriuresis after ANF infusions parallel the elevation of plasma, urinary and renal tissue cGMP levels in rats (15), monkeys (16), and humans (17). Particulate guanylate cyclase has been identified as a target enzyme of ANF's action in the glomeruli and collecting ducts (18-20). ANF-induced vasorelaxation has been correlated with a rise in cGMP (21, 22), and vascular smooth muscle cells show heightened intracellular cGMP values (23). Inhibition of renin release by ANF has also been reported to be cGMP-mediated (24). It has been proposed that cGMP is a second messenger for the transmission of ANF's actions (15, 16, 20, 21, 23).

Human ANF-(99-126) (hANF) is identical to canine, porcine, and bovine ANF but differs from rat, mouse, and rabbit ANF in that isoleucine in position 110 is replaced by methionine (25, 26). This difference does not influence its glomerular binding characteristics (27). However, hANF can be oxidized at this methionine site (28) and is converted into methoxy-sulf-oxide ANF, human [Met-O<sup>110</sup>]ANF-(99-126) (Met-O-ANF). It is conceivable that this type of oxidation occurs *in vivo*. Met-O-ANF has been reported not to increase cGMP in vascular smooth muscle cells while its binding is only slightly less than that of the parent compound (29). These considerations led us to evaluate the usefulness of Met-O-ANF as a potential tool to relate the specific biological properties of ANF with the cGMP system.

## Methods

**ANF.** Synthetic human ANF-(99-126) (hANF) and human [Met-O<sup>110</sup>]ANF-(99-126) (Met-O-ANF) were purchased from the Institut Armand-Frappier, Laval, Quebec, Canada. They were dissolved at a concentration of 10<sup>-4</sup> M in 0.1 N acetic acid, and kept frozen at -35°C.

**Animals.** Male Wistar rats 300-350 g, purchased from Charles River Canada, St-Constant, Quebec, Canada, were used in all the studies. They were fed normal Purina Rat Chow and allowed free access to tap water.

**Measurements of diuresis, natriuresis, and cGMP excretion.** The jugular vein and urinary bladder were catheterized with PE 50 polyethylene tubing under sodium pentobarbital anesthesia (40 mg/kg body

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1. **Abbreviations used in this paper:** ADH, antidiuretic hormone; ANF, atrial natriuretic factor; hANF, human ANF-(99-126); Met-O-ANF, human [Met-O<sup>110</sup>]ANF-(99-126); MIX, methylisobutylxanthine; PTH, parathormone.

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wt i.p.). Glucose 5% was infused at a flow rate of 3 ml/h throughout the experiment. After a 45-min stabilization period, urine samples were taken during a 20-min preinfusion control period (C). Either hANF or Met-O-ANF was then injected as a bolus via the jugular vein at 20-min intervals in three escalating doses (0.5, 1.0, and 2.0  $\mu$ g). Urine samples were collected for 20 min after each injection and during an additional 20-min recovery period (postinjection, P), starting 20 min after the last treatment. Urinary volume was measured by weight, urinary cGMP levels were determined by radioimmunoassay (30, 31), as previously described (15), and  $\text{Na}^+$  and  $\text{K}^+$  concentrations were assessed by flame photometry.

**Blood pressure measurements.** In an additional group of Wistar rats, blood pressure was monitored continuously via a pressure transducer (Gould Statham P23ID; Statham Instruments, Oxnard, CA) connected to the carotid artery of anesthetized rats and recorded by a polygraph (model 7; Grass Instruments, Inc.). Control blood pressure was measured during an initial 20-min period, followed by 20-min intervals after the injection of the vehicle or of increasing doses (0.5, 1.0, or 2.0  $\mu$ g) of either hANF or Met-O-ANF.

**Determination of plasma cGMP.** Blood samples (500  $\mu$ l) were drawn from the carotid artery into tubes containing EDTA (final concentration 7 mM), centrifuged at 2,500 g for 15 min. The plasma was kept at  $-35^\circ\text{C}$  until the cGMP measurements, when it was deproteinized with perchloric acid (final concentration 0.5 N) and cGMP was extracted with alumina (AG 7) and Dowex (AG 50W-X8) columns before assessment by radioimmunoassay. In one set of experiments, blood for the cGMP determinations was collected at the end of a 20-min control period and 5 min after the injection of the vehicle (acetic acid) or of either 2.0  $\mu$ g of hANF or Met-O-ANF. To eliminate an eventual hemodynamic effect provoked by the blood drawings, we performed a separate experiment using bolus injections of 2.0  $\mu$ g Met-O-ANF without prior blood sampling, and investigated the time course of the cGMP levels by collecting blood either 90 s, or 3, 5, 10, or 30 min after the Met-O-ANF injection. A third set of experiments was undertaken with a cumulative dose of 3.5  $\mu$ g of hANF or Met-O-ANF (0.5, 1.0, and 2.0  $\mu$ g, injected in 20-min intervals). Plasma cGMP levels were measured 5 min after the last injection.

**Vasorelaxation of aortic strips.** After sodium pentobarbital anesthesia (40 mg/kg body wt i.p.), the thoracic aorta was rapidly excised, fat and connective tissue were gently trimmed off, and the aorta was helically cut into 1-mm  $\times$  20-mm strips. These were mounted between a fixed base and a force displacement transducer (Grass, FT-03C) in a 20-ml tissue bath containing continuously oxygenated (95%  $\text{O}_2/5\%$   $\text{CO}_2$ ) Krebs solution (in mM: NaCl 123, KCl 2.2,  $\text{MgCl}_2$  0.5,  $\text{NaH}_2\text{O}_4$  0.4,  $\text{CaCl}_2$  5.0, glucose 5.0,  $\text{NaHCO}_3$  25.0) at  $37^\circ\text{C}$  and pH 7.4. A tension of 1.0 g was applied to each strip and a dose-response curve for norepinephrine was constructed. At the half-maximal contraction obtained with norepinephrine ( $36 \pm 3.9$  pmol/liter), relaxation was induced with either hANF or Met-O-ANF, which were added to the tissue in increasing concentrations at 2-min intervals.

**cGMP production in different kidney fractions.** Rat glomeruli were obtained from Wistar rats (280–300 g) which were pentobarbital-anesthetized before removal of the kidneys. The cortex was separated, sliced and placed into Krebs-Henseleit saline (KHS; in mM: NaCl 139, KCl 3.7,  $\text{KH}_2\text{PO}_4$  1.4,  $\text{MgSO}_4$  2.9,  $\text{CaCl}_2$  5.9,  $\text{NaHCO}_3$  58.0) at  $4^\circ\text{C}$ . It was gently grounded through the successive molecular sieves of a 250- and 150- $\mu$ m mesh and passed-through fractions were sieved at 63  $\mu$ m. The retained tissue was washed three times with KHS and then verified under phase-contrast light microscope. It demonstrated to be 90–95% pure intact glomeruli fractions. The rat glomeruli were incubated in the presence of increasing doses of ANF with 500 mmol concentration of methylisobutylxanthine (MIX) for 4 min. The incubation was stopped by addition of 1 ml of 1 N perchloric acid and rapid freezing in liquid nitrogen. Kidneys from anesthetized mongrel dogs were rapidly removed and placed in gassed (95%  $\text{O}_2/5\%$   $\text{CO}_2$ ) KHS at  $4^\circ\text{C}$ . They were decapsulated, cut into slices, and the outer cortex and red and white medulla were carefully dissected. Each fraction was sliced with a Stadie-Riggs microtome, and washed with KHS. The cortex was

digested with collagenase at  $37^\circ\text{C}$  for 45 min, the red and white medulla for 60 min. The cortex suspension was washed with KHS and passed through a 90- $\mu$ m mesh sieve (32). The glomeruli were retained and further purified by successive washings. Thick ascending limbs were obtained directly from digestion of the red medulla (33). Distal collecting ducts were derived from the white medulla. To verify the proper isolation of these nephron segments, microscopic and enzymatic methods were used (34). The usual degree of purity was more than 95% for the glomeruli and 100% for the collecting ducts. For cGMP stimulation, 200–400  $\mu$ g glomeruli, 1.2 mg ascending limb or 300  $\mu$ g collecting duct were preincubated with 250  $\mu$ l KHS at  $37^\circ\text{C}$  for 10 min, then incubated for 4 min with increasing concentrations of hANF or Met-O-ANF in the presence of 100  $\mu$ M MIX (18). The incubation was stopped by the addition of 1 ml of 1 N perchloric acid. After extraction, cGMP was measured by radioimmunoassay, as previously described (31).

**cGMP production by aortic smooth muscle and endothelial cells.** Rat aortic smooth muscle cells were obtained from adult, male Wistar rats by an explant method (35). Bovine aortic endothelial cells were generously donated by Dr. Patricia D'Amore (Harvard Medical School, Boston, MA). Both cell types were cultured at  $37^\circ\text{C}$  in Dulbecco's modified Eagle medium (DME) with 10% calf serum, 50 U/ml penicillin and 50  $\mu$ g/ml streptomycin under 95%  $\text{O}_2/5\%$   $\text{CO}_2$ . When the cells became confluent, they were trypsinized and passaged into 75-cm<sup>2</sup> culture flasks. Cells between the 14th to 20th passages were used for the experiments and passaged into 35-mm petri dishes. When confluence was reached (3–4 d), they were washed twice in DME, without calf serum, and incubated with different concentrations of ANF for 120 min at  $37^\circ\text{C}$  under 95%  $\text{O}_2/5\%$   $\text{CO}_2$ . The 120-min time period was selected since we have previously demonstrated (16) that even very short (1 min) exposure to ANF (which can be either removed or degraded) leads to a continuous accumulation of extracellular cGMP, making the amplification of the response optimal after 2 h. At the end of incubation, the extracellular medium was aspirated and diluted in an equal volume of 1 mM MIX and 20 mM EDTA. Samples were frozen at  $-35^\circ\text{C}$  until cGMP determination by radioimmunoassay.

**Statistics.** The results are expressed as mean  $\pm$  SEM. Comparisons were made by one- or two-way analysis of variance with a posteriori comparisons according to Bonferroni's method. The rank order Wilcoxon test was used to analyze the mean arterial blood pressure and differences in plasma cGMP levels. Differences were considered to be significant at  $P < 0.05$ .

## Results

**Diuresis, natriuresis, and urinary cGMP excretion.** Bolus injections of 0.5, 1.0, and 2.0  $\mu$ g hANF induced a significant ( $P < 0.001$ ), dose-dependent increase of urinary volume (maximal 6-fold), natriuresis (maximal 10-fold) and cGMP excretion (maximal 5-fold) when compared to control values (C, Fig. 1). During the postinjection control period (P), urinary volume and sodium returned to preinjection levels, while urinary cGMP remained slightly elevated. Met-O-ANF induced a significant threefold increase of urinary volume ( $P < 0.05$ ). Though this stimulation was less pronounced than with hANF, it lasted longer throughout the recovery period. In contrast to hANF ( $P < 0.01$ ), there was no increase in urinary sodium excretion after the Met-O-ANF injections, and a 50% decrease (not significant) was even observed at the end of the final control period. Also contrary to the stimulation evoked by hANF, urinary cGMP levels were not influenced by Met-O-ANF at the concentrations mentioned above ( $P < 0.001$ , when compared to hANF). This dissociation appeared to be dose-dependent; with higher doses of Met-O-ANF

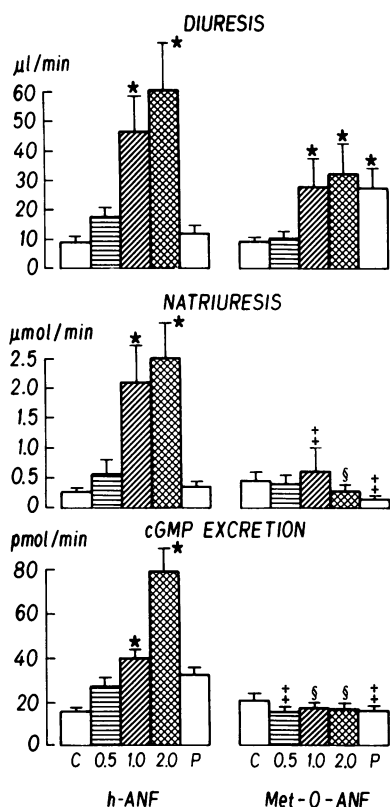


Figure 1. Diuresis, natriuresis and urinary cGMP excretion in Wistar rats after injection of 0.5, 1.0, and 2.0  $\mu$ g of hANF or Met-O-ANF. The values are means $\pm$ SEM,  $n = 8$ . \* $P < 0.05$  when compared to control; ‡ $P < 0.05$  when compared to hANF; § $P < 0.01$  when compared to hANF.

(three times 2  $\mu$ g), we were able to produce a slight increase in natriuresis and cGMP excretion (data not shown). Thus, the diuresis elicited by Met-O-ANF was not paralleled by either natriuresis or enhanced cGMP excretion.

The correlations between doses of hANF and Met-O-ANF and diuresis, natriuresis and cGMP excretion are shown in Table I.

**Arterial blood pressure.** Mean arterial blood pressure did not change significantly upon injection of 0.5  $\mu$ g of either hANF or Met-O-ANF. After doubling the dose, blood pressure was reduced by 17% and 13% by 1.0  $\mu$ g of hANF and Met-O-ANF, respectively (Table II). Subsequent injection of 2.0  $\mu$ g

Table I. Correlation between Doses of ANF, Diuresis, Natriuresis and Urinary cGMP

	hANF	MET-O-ANF
A ANF/cGMP	0.78 <sup>§</sup>	-0.18
ANF/diuresis	0.58 <sup>‡</sup>	0.44*
ANF/natriuresis	0.60 <sup>‡</sup>	-0.11
B cGMP/diuresis	0.41*	0.06
cGMP/natriuresis	0.39*	0.15
Natriuresis/diuresis	0.81 <sup>§</sup>	0.31

In A, either hANF or Met-O-ANF was injected at increasing doses (0.5  $\mu$ g, 1.0  $\mu$ g, 2.0  $\mu$ g). Correlations were calculated between these doses and the resulting urinary cGMP excretion, diuresis, or natriuresis, as reported in Fig. 1 (i.e., for the three doses used in each of eight animals,  $n = 24$ ). In B, the correlations between the natriuretic, diuretic, and cGMP response to either hANF or Met-O-ANF (as shown in Fig. 1) were calculated. \* $P < 0.05$ ; ‡ $P < 0.01$ ; § $P < 0.001$ .

Table II. Effect of hANF and Met-O-ANF on Mean Arterial Blood Pressure

	Basal	Dose injected		
		0.5 $\mu$ g	1.0 $\mu$ g	2.0 $\mu$ g
Vehicle	103 $\pm$ 8	95 $\pm$ 9	102 $\pm$ 10	99 $\pm$ 9
hANF	103 $\pm$ 5	100 $\pm$ 4	86 $\pm$ 8*	83 $\pm$ 8*
Met-O-ANF	96 $\pm$ 13	90 $\pm$ 11	84 $\pm$ 10*	81 $\pm$ 10*

Mean arterial pressure of Wistar rats 20 min after injection of vehicle (acetic acid), hANF or Met-O-ANF. \* $P < 0.05$ , rank-order Wilcoxon test, compared to control values. The data are expressed in millimeters of mercury as mean $\pm$ SEM,  $n = 5$ .

lowered it by 19% and 16%, respectively. These decreases in blood pressure were significant ( $P < 0.05$ ) after bolus injection of 1.0 and 2.0  $\mu$ g hANF as well as of Met-O-ANF.

**Plasma cGMP levels.** To investigate whether the absence of urinary cGMP excretion in the presence of a diuretic action of Met-O-ANF was due to a lack of cGMP production or to a difference in cGMP excretion, plasma cGMP levels were determined 5 min after injection of 2.0  $\mu$ g of either Met-O-ANF or hANF. As reported earlier (15), hANF increased plasma cGMP about fivefold ( $P < 0.001$ , Table III) when compared to preinjection values. In contrast to hANF, injection of 2.0  $\mu$ g Met-O-ANF did not elevate plasma cGMP levels. The effect of a single injection of 2.0  $\mu$ g Met-O-ANF on cGMP was then determined at various time intervals without previous blood drawings in order to exclude early or late increases of plasma cGMP levels. No peak of cGMP occurred and plasma levels were even lower 30 min after injection, when compared to control values (Table IV). However, when cumulative doses (0.5, 1.0, 2.0  $\mu$ g) of Met-O-ANF were injected, the increase of cGMP values was twofold, whereas the same dose of hANF induced a sixfold increment ( $P < 0.01$ ). The difference in the cGMP response between hANF and Met-O-ANF was significant ( $P < 0.01$ , Table V).

**Vasorelaxation of aortic strips.** In accordance with previously reported observations (36), hANF produced a 100% relaxation of aortic strips contracted by norepinephrine (Fig. 2). Half-maximal relaxation occurred at  $\sim 100$  nM hANF while Met-O-ANF was less effective as a relaxing agent, pro-

Table III. Plasma cGMP Levels following Single Bolus Injection of ANF

	cGMP levels	
	Prior	Post
	nM	nM
Vehicle	9.8 $\pm$ 1.1	10.6 $\pm$ 2.1
hANF	15.7 $\pm$ 4.0	79.1 $\pm$ 15.2*
Met-O-ANF	14.1 $\pm$ 1.6	15.5 $\pm$ 2.6

Plasma cGMP levels of Wistar rats 5 min after injection of the vehicle (acetic acid) or 2  $\mu$ g of either hANF or Met-O-ANF. \* $P < 0.001$ , compared to control values. The data are expressed as mean $\pm$ SEM,  $n = 4$ .

Table IV. Time Course of Plasma cGMP Levels after Injection of 2.0  $\mu$ g Met-O-ANF

	Time				
	5 min (control)	90 s	3 min	10 min	30 min
cGMP ( $\mu$ M)	8.15	8.08	8.28	7.62	5.77
$\pm$ SEM	$\pm 1.04$	$\pm 1.44$	$\pm 0.40$	$\pm 0.66$	$\pm 0.43$

Plasma cGMP levels of Wistar rats were measured at different time intervals after an injection of 2.0  $\mu$ g of Met-O-ANF. The vehicle (acetic acid) was injected as a control, and a blood sample was taken 5 min later. Data are expressed as means  $\pm$  SEM,  $n = 5$  or 10 (per control).

ducing only  $\sim 14\%$  relaxation at 100 nM. Its onset of action and its time course appeared to be slower than with hANF, but had an almost equivalent maximal effect on vasorelaxation. In fact, a complete relaxation was achieved with Met-O-ANF after an additional two minutes of observation (unpublished data).

**cGMP production by different kidney fractions.** As demonstrated previously (18), hANF elicited a large elevation of cGMP both in rat and canine glomeruli (Figs. 3 and 4). It has to be noticed that in glomeruli, the only renal fraction in which studies can be done both in dog and rat, a similar response was obtained in both species. Met-O-ANF stimulated cGMP production in glomeruli significantly less in both rat and dog kidneys (Figs. 3 and 4 a). To explore the cGMP responsiveness to ANF and its analogues, we have proceeded with tubular fractions from dog kidneys. cGMP levels increased significantly in thick ascending limb both with hANF and Met-O-ANF (Fig. 4 b). In this tissue, the difference between the two analogues was only slight and reached only 20% with the highest dose of ANF. In the collecting duct, ANF increased cGMP two- to threefold while Met-O-ANF did not increase cGMP significantly (Fig. 4 c).

**cGMP levels in cultured aortic smooth muscle and endothelial cells.** As reported previously (16), hANF increased intra- and extracellular cGMP levels in cultured smooth muscle cells in a dose-dependent manner, with up to 75-fold stimulation of extracellular cGMP occurring at a concentration of 1  $\mu$ M hANF (Fig. 5 a). Met-O-ANF was less potent than hANF in

Table V. Plasma cGMP Levels Determined 5 Min after a Cumulative Dose of 3.5  $\mu$ g of ANF

	cGMP (nM)	
Vehicle	6.0 $\pm$ 1.3	*
hANF	33.7 $\pm$ 4.5	*
Met-O-ANF	11.9 $\pm$ 2.4	*

Successive cumulative doses (0.5, 1.0, 2.0  $\mu$ g) of hANF or Met-O-ANF were injected into Wistar rats at 20-min intervals. Blood samples for the cGMP measurements were taken 5 min after the last injection. \*  $P < 0.01$ , † Not significant by analysis of variance, but significant when analyzed by the rank-order Wilcoxon test. The data are expressed as mean  $\pm$  SEM,  $n = 4$ .

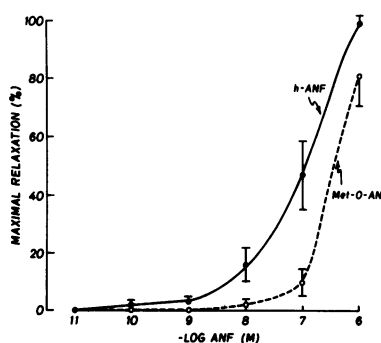


Figure 2. Dose-response curve of relaxation evoked by hANF and Met-O-ANF in rat aortic strips previously contracted by norepinephrine. The values are means  $\pm$  SEM,  $n = 6$ .

augmenting cGMP in these cells, evoking only a 25-fold increase at a concentration of 1  $\mu$ M. The results demonstrated in Fig. 5 a include the means of 16 different experiments obtained in cells at the passages between 14 to 20 derived from different stocks of original cells. As could be expected from the higher variability observed in response to hANF, the response to this parent compound varied from undistinguishable from that to Met-O-ANF to fivefold higher. These results suggest that the heterogeneity of the response between ANF and Met-O-ANF may also be due to the difference in the cell stock, cell growth, origin and/or passages.

Stimulation of cultured bovine endothelial cells with hANF (1  $\mu$ M) resulted in a 15-fold rise in extracellular cGMP levels but only an 8-fold increase was observed after incubation with Met-O-ANF ( $P < 0.05$ ; Fig. 5 b).

## Discussion

Natriuresis and diuresis were the first actions of ANF to be reported in the literature (37). Since then, many studies have been undertaken to elucidate the mechanisms of its natriuretic action, which was always coupled to the diuretic effect.

We report here a dissociation of natriuresis and diuresis. An analogue of human ANF-(99–126), Met-O-ANF, which is oxidized at methionine position 110, completely loses its natriuretic properties at doses that produce a significant ( $P < 0.05$ ) increase in diuresis (Fig. 1). In contrast to escalating doses of hANF, which correlate most significantly with diuresis ( $r = 0.58$ ) and natriuresis ( $r = 0.60$ ), the same doses of Met-O-ANF correlate only with diuresis ( $r = 0.44$ ) but not with natriuresis ( $r = -0.11$ ; Table I). Injection of Met-O-ANF results in a loss of the highly-significant correlation between natriuresis and diuresis that is observed with hANF. It can be seen in

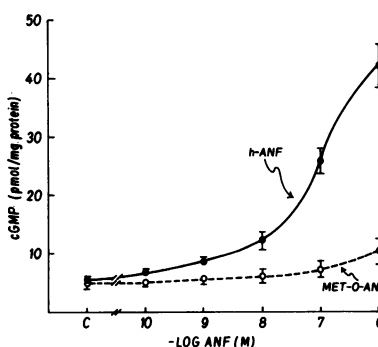


Figure 3. Effect of hANF and Met-O-ANF on cGMP levels in rat glomeruli. The glomeruli have been isolated as described in Methods. The incubation of glomeruli in presence of increasing concentration of ANF was performed in the presence of 0.5 mmol MIX for a period of 5 min.

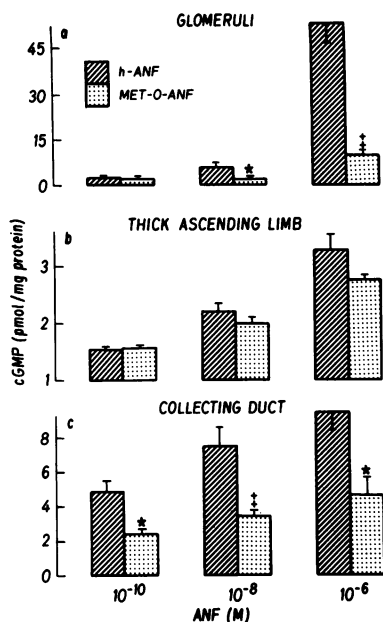


Figure 4. Effect of Met-O-ANF and hANF on cGMP production in different kidney fractions. cGMP generation by (a) glomeruli, (b) thick ascending limb, and (c) collecting duct after stimulation with either hANF (hatched columns) or Met-O-ANF (dotted columns). The data are the means of four to five ( $\pm$ SEM) experiments. \* $P < 0.05$ ; † $P < 0.01$  when compared to hANF.

Fig. 1 that the profile of the dose response of diuresis is different between hANF and Met-O-ANF. The diuresis with Met-O-ANF is slower in its onset and persists in the postinjection control period, suggesting a delayed onset and prolonged action of Met-O-ANF. It cannot be ascertained at the present whether or not this delayed and prolonged action of Met-O-ANF is related to receptor heterogeneity, as discussed below, or due to a prolonged stimulation of particulate guanylate cyclase which we have observed previously (38, 39).

To the best of our knowledge, this is the first report of a diuretic action of ANF without simultaneous natriuresis. It suggests that the diuretic mechanism is at least partially independent of sodium excretion. On the other hand, this analogue is as effective as ANF in lowering blood pressure and is only slightly less vasorelaxing.

cGMP has been shown to be a marker of the biological activity of ANF and may mediate its effects (16). We have

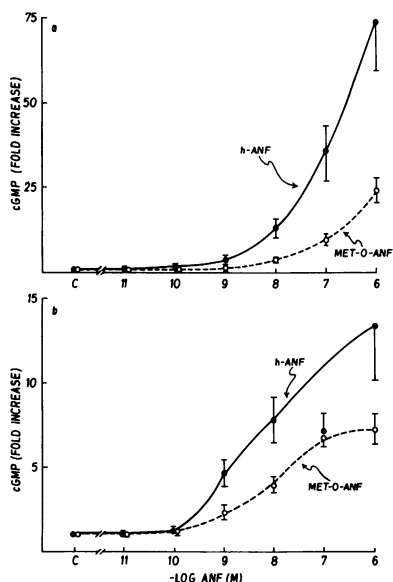


Figure 5. cGMP levels in (a) rat aortic smooth muscle cells ( $n = 16$ ) and (b) bovine aortic endothelial cells ( $n = 8$ ) after stimulation by either hANF or Met-O-ANF. The values are means  $\pm$  SEM.

reported previously that bolus injections and infusions of ANF in rats, monkeys and humans increase plasma and urinary cGMP (15–17). Actually, the minimum dose of ANF needed to elevate cGMP in plasma is even lower than that required to stimulate diuresis and natriuresis (40). Met-O-ANF enhanced diuresis at doses that did not affect plasma or urinary cGMP levels. At first glance, these results are compatible with the notion that ANF uses another second messenger system to express its diuretic function. ANF has been reported to inhibit adenylate cyclase activity (41). However, we did not observe any significant changes of cAMP levels in our previous investigations (17), or in pilot experiments using Met-O-ANF (unpublished data). LY83583, an inhibitor of particulate guanylate cyclase, has no effect on the biological potency of ANF in vascular strips, yet only partial inhibition of this enzyme was achieved with this agent in that study (42).

Nevertheless, it is conceivable that the diuretic effect of ANF is exerted via its interaction with particulate guanylate cyclase at a tubular site where a rise in intracellular cGMP does not lead to its increase in urinary excretion. Such a situation would be analogous to the long-known difference between the effects of parathormone (PTH) and antidiuretic hormone (ADH) on urinary cAMP levels. Although both hormones are agonists of renal adenylate cyclase, with PTH stimulating it in the cortex and ADH doing likewise in the medulla, only the actions of PTH are reflected by urinary cAMP increases (43, 44). Moreover, the diuretic potency of Met-O-ANF dissociated from natriuresis is suggestive of an effect on free water clearance. This component of ANF's diuretic activity could be mediated via an inhibition of ADH secretion, which has been reported (45–48), or via its tubular effect (49). Receptors for ANF have been described in various sections of the medulla (12, 50). Our results indicate that such an anti-ADH action of ANF, whether or not related to the stimulation of particulate guanylate cyclase, is not reflected by urinary or plasma cGMP levels. Analyzing different dog nephron segments (Fig. 4 b), we showed that the potency of ANF and Met-O-ANF is similar in one part of the tubule, the thick ascending loop. It would appear that although quantitatively this tissue does not contribute to urinary increases of cGMP, it is compatible with the possibility that at least part of the diuretic action of ANF is exerted at this part of the tubule.

The dissociation of natriuresis and diuresis in this study indicates that ANF's renal effects are exerted at several levels. In addition to its action on glomeruli, where we demonstrated a much lesser effect of Met-O-ANF, both substances appear to be equally potent at the level of diuresis not accompanied by natriuresis, i.e., free water clearance. The natriuretic effect of ANF, which appears to be faithfully reflected by cGMP in urine, is apparently exerted at a level of the tubule where Met-O-ANF is devoid of any significant potency (Fig. 4 a and c). This is compatible with the data of other investigators and suggests that the natriuretic action depends on the effect on glomeruli as well as on the action at the lowest level of the tubule (10, 27).

This report illustrates that a similar decrease of blood pressure can be achieved by Met-O-ANF and hANF. Whether this hypotensive action is mediated via a fall in cardiac output or vasodilation is still not entirely resolved; however, it is not accompanied by an increase of plasma cGMP in the case of Met-O-ANF. Again, this observation could give the impression that cGMP generation is not related to this biological

function of ANF. These in vitro experiments have demonstrated that ANF and Met-O-ANF have an ability to stimulate particulate guanylate cyclase in vascular smooth muscle cells, resulting in an increase of cGMP levels. It has been previously suggested that Met-O-ANF does not increase cGMP levels at all in cultured vascular smooth muscle cells. In the experiments by Hirata et al. (29), the maximal doses used were lower than in this publication. In addition, we have observed in our own experiments high variability in the degree of the increases of cGMP observed between ANF and Met-O-ANF, as mentioned in Results. This variability can be attributed to the degree of passage of the cells or their growth state, although the exact mechanisms for this heterogeneity of response are not understood at the present time. The effect of the cell synchronization and cell growth on the expression of the particulate guanylate cyclase is at the present time under active investigation in our laboratory. It has been recently reported that different tissues as well as subcloning of the same cell-type result in a differential expression of ANF receptors and the responsiveness to ANF in its ability to increase cGMP levels. This differential expression of ANF receptors (51) may also be involved in the observed variability of the response to hANF and Met-O-ANF. The in vivo effects could be also affected by differential modification of levels and/or clearance of endogenous ANF by the two compounds (52), but the in vitro studies reported here support the notion of real heterogeneity of cGMP effector system.

The effects of hANF and Met-O-ANF are also distinct in cultured cells, where almost one order of magnitude of lower potency is observed with Met-O-ANF. Met-O-ANF is able to raise plasma cGMP levels but only with higher doses. We have recently observed that sodium nitroprusside, a known vasodilator, at doses that induce vasodilation and a reduction of blood pressure equivalent to that caused by hANF, does not elicit an increase of plasma cGMP in humans (39, 53) although its mechanism of action is clearly related to the stimulation of soluble guanylate cyclase in smooth muscle cells (54–56). We have already shown that sodium nitroprusside, although able to increase intracellular and extracellular cGMP levels in smooth muscle cells in vitro, does not modify cGMP levels in endothelial cells (16). These data suggest that the origin of augmentation of plasma cGMP is in endothelial rather than in smooth muscle cells. cGMP, which is mediating vasodilation in smooth muscle cells, could then not egress in sufficient quantities to alter plasma cGMP levels. Thus, an agonist such as hANF could lead to only an apparent parallelism between plasma cGMP levels and its vasodilatory potency. This parallelism, in fact, could be secondary to its endothelial site of action and could explain why Met-O-ANF has similar vasorelaxant and hypotensive potencies as hANF without changing plasma cGMP levels.

The presence of two or three types of ANF-receptors has been demonstrated (57–61), with only one of them being apparently linked to particulate guanylate cyclase. The relative density of these two receptors apparently varies from tissue to tissue (62). It is also conceivable that the coupling of the receptor to the guanylate cyclase itself may vary. The observations in the present study support the notion of the heterogeneity of the effector system of ANF in different tissues. At the level of cGMP generation as well as at the level of biological effects, the oxidized analogue of ANF and the parent compound vary in activity from very similar to clearly distinct

degrees of potency. The data also show that urinary and plasma cGMP levels strongly correlate with natriuresis induced by ANF, but do not reflect its hypotensive and diuretic potency. These results also indicate that the diuretic and natriuretic actions of ANF are exerted at distinct sites of the nephron. The relationship between the heterogeneity of the effector system and the biological effects demonstrated in the present study deserves further investigation.

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