Vol. 126, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1985 Pages 178-184

# STRUCTURE-ACTIVITY RELATIONSHIPS OF ATRIAL NATRIURETIC FACTOR (ANF). II. EFFECT OF CHAIN-LENGTH MODIFICATIONS ON VASCULAR REACTIVITY

R. Garcia[1], G. Thibault, N.G. Seidah, C. Lazure, M. Cantin, J. Genest, and M. Chrétien

Clinical Research Institute of Montreal
110 Pine Avenue West, Montreal, Quebec, Canada H2W 1R7

Received October 22, 1984

The effect of the length of ANF peptides on the inhibition of the norepinephrine-induced contraction was studied. Starting from the 26 residues ANF ($Arg^{101}$-$Tyr^{126}$), shorter N- and/or C-terminal fragments were produced, either by N-terminal chemical cleavage or C-terminal enzymatic digestion of ANF or both respectively. The N-terminal removal of $Arg^{101}$ did not modify the inhibitory response. Further N-terminal truncation up to des-$Arg^{101}$-$Arg^{102}$-$Ser^{103}$-$Ser^{104}$ ANF still produced a marked inhibitory effect on norepinephrine. In contrast C-terminal cleavage had a much more pronounced effect. Since des-$Tyr^{126}$ ANF, des-$Arg^{125}$-$Tyr^{126}$ ANF and des-$Phe^{124}$-$Arg^{125}$-$Tyr^{126}$ ANF exhibit much lower activities than the parent ANF. Finally, when the 5 residues C-terminal to $Cys^{121}$ are removed, the resulting molecule is almost inactive. These data indicate that the C-terminal segment of ANF may modulate the binding of ANF to its receptor(s). Relatively, the N-terminal region seems to be much less important. 

It has been demonstrated by several laboratories that atrial natriuretic factor (ANF), in addition to its natriuretic effect, possess a potent vasorelaxant and vasodilatory activity (1-4). This vasoactivity was further confirmed by the use of a synthetic peptide (5). The mechanism of action is not clear, but because the vasorelaxant effect of ANF is mimicked by sodium nitroprusside, an effect on cGMP has been suggested (3, 6).

Because it seems possible that chain-length may have an importance in the vasoactivity of ANF (7, 8), we have investigated whether N-terminal and/or C-terminal truncation could modify the response of vascular strips to norepinephrine.

## MATERIALS AND METHODS

### Vascular strips

Male New Zealand white rabbits (1.8 – 2.0 kg) were fed Purina rabbit chow and allowed free access to tap water. Under pentobarbital sodium anesthesia

[1] To whom correspondence should be addressed.

0006-291X/85 $1.50

(30 mg/kg i.v.), the thoracic aorta was rapidly excised and gently trimmed of excess fat and connective tissue, and the arterial tissue was helically cut. Each vascular strip, 3 x 30-35 mm, was suspended in a 20-ml tissue bath containing a continuously oxygenated (95% $O_2$ – 5% $CO_2$) Krebs solution at 37°C and pH 7.4. The strips were mounted between a fixed base and a force displacement transducer (Grass, FT-03C). The contractions were registered on a polygraph (Grass, Model 7).

A tension of 2,500-3,000 mg was applied to each strip. The tension was adjusted and bathing fluid changed every 15 min. The strips were allowed to equilibrate for 2 hrs before the experimental procedures began.

The composition of the solution used in this study was (mmol/l): NaCl, 119; KCl, 4.7; $KH_2PO_4$, 1.8; $MgSO_4 \cdot 7H_2O$, 1.17; $CaCl_2 \cdot 6H_2O$, 2.5; $NaHCO_3$, 25.0; and dextrose, 5.5.

For each arterial strip a cumulative dose-response curve to norepinephrine (NE) (L-Norepinephrine bitartrate, Sigma Chemicals) was constructed. Once the standard curve was reproducible, it was repeated 5 min after adding into the bath the peptides assayed to a final concentration of 25 nM for each one. Four to six standard curves were made for each tested peptide.

The contraction elicited for each dose of NE is expressed as a percent of the maximum response.

Results are expressed as means ± SEM. Analysis of covariance and the Dunnet test were used to compare multiple dose-response curves between them.

Peptides

ANF has been found to represent the C-terminal of a much longer chain (pre, pro and connecting peptides) with a total of 152 residues first determined by protein sequencing (9, 10) and confirmed by cloning of rat cDNA (11-13). The signal peptide being made of 24 amino acids, the remaining residues have been numbered from 1 to 128 from N-terminal to C-terminal according to standard peptide nomenclature. Since $Arg^{127}$-$Arg^{128}$ (11-13) are presumably removed by a carboxypeptidase B like enzyme in the granules, the pro-form found is 126 residues long.

Synthetic ANF (ANF (101-126), previously called ANF 8-33) was kindly provided by Dr. R.F. Nutt (Merck, Sharp and Dohme Research Laboratories, West Point, PA). ANF (103-123) and ANF (103-125) were purchased (Peninsula Laboratories Inc., Belmont, CA). ANF (96-126) and ANF (54-126) were obtained by purification from rat atria (14).

The N-terminal truncated peptides were prepared from synthetic ANF (101-126) by Edman degradation with a 0.3 M Quadrol program on a Beckman 890-C sequencer and purified by HPLC with a $C_{18}$ μBondapak column (15). The following peptides were thus obtained: ANF (102-126), ANF (103-126), ANF (104-126), and ANF (105-126).

The C-terminal truncated peptides were obtained by digestion with several carboxypeptidases from synthetic ANF (101-126), the fragments were purified by HPLC and their amino acid composition determined on a Beckman 120C equipped with a model 126 integrator (15). The following peptides were thus isolated: ANF (101-121), ANF (101-123), ANF (101-124), and ANF (101-125).

RESULTS

Figure 1 shows that all tested peptides with a modified N-terminal produced a significant displacement of the NE dose-response curve to the right

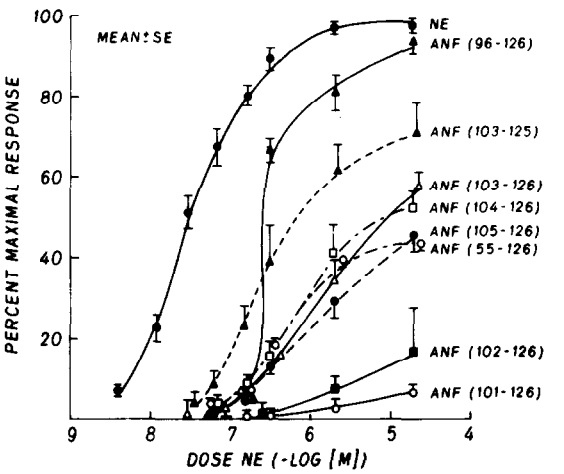


Figure 1: Effect of N-terminal amino acids on ANF vasoactivity to NE on vascular strips.

($p < 0.01$). The most potent peptides were ANF (101-126) and ANF (102-126), no differences were seen between them. Another group of peptides, ANF (105-126), ANF (104-126) and ANF (103-126) produced also a significant shift to the dose-response curve to NE, but significantly less than the previous ones. No differences were seen between the peptides forming this second group. A peptide, ANF (103-125), with two residues less at the N-terminal, and one residue less at the C-terminal than ANF (101-126) still produced a significant shift of the NE curve, but was less active than the previous ones. Of the two native peptides purified from rat atria, with an elongated N-terminal, ANF (54-126), produced a displacement not significantly different from that produced by the peptides ANF (105-126), ANF (104-126) and ANF (103-126). The other natural peptide, ANF (96-126), produced a pronounced displacement at the lower doses, but not at the higher doses of the NE dose-response standard curve.

Figure 2 shows that similar to what was observed with N-terminal modifications, all tested ANF peptides truncated at the C-terminal displace significantly ($p < 0.01$) the NE dose-response curve to the right. However, only ANF (101-125) in which $Tyrosine^{126}$ has been cleaved produced an important shift. ANF (101-123) and ANF (103-123) produced similar displacement curves and not different from ANF (103-125). ANF (101-124) displaces the NE curve mainly at

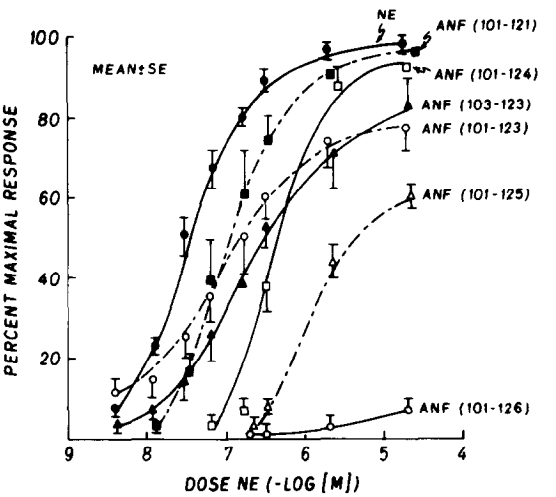


Figure 2: Effect of C-terminal amino acids on ANF vasoactivity to NE on vascular strips.

the lower doses level. ANF (101-121), even if showing a statistically significant displacement of the NE curve, is the less active of all peptides tested.

As seen in Table I, the most potent peptides in increasing the $ED_{50}$ dose of NE are ANF (101-126) and ANF (102-126), followed by the native form ANF (54-126) and ANF (105-126). Much less inhibitory potency is showed by peptides shortened at the C-terminal, where ANF (101-121) and ANF (101-123), being the peptides which least modified the $ED_{50}$ NE dose.

## DISCUSSION

We have previously shown (16) that the shortest peptide obtained in our laboratories during ANF purification, ANF (101-126), had a powerful natriuretic activity not different from longer forms. The synthetic peptide with similar amino acid sequence was demonstrated to possess similar natriuretic and vasoactive potency (5, 16).

In Figure 1, we have demonstrated that under the present experimental conditions ANF (101-126) is the most potent of all assayed peptides. Furthermore, the removal of $Arg^{101}$ (ANF (102-126)) did not significantly modify its vasoactivity. The cleaving of $Tyr^{126}$ (ANF (101-125)), however, decreased significantly the inhibitory potency of this peptide to inhibit the NE-induced contraction.

TABLE I

EFFECT OF ANF-RELATED PEPTIDES ON THE $ED_{50}$ DOSE-CONTRACTILE RESPONSE TO NE

| Peptide added | NE $ED_{50}$ -log [M] | N |
|---|---|---|
| none | 7.5 | 12 |
| ANF $Glu^{54}$-$Tyr^{126}$ | < 4.7 | 4 |
| ANF $Gly^{96}$-$Tyr^{126}$ | 6.6 | 4 |
| ANF $Arg^{101}$-$Tyr^{126}$ | << 4.7 | 6 |
| ANF $Arg^{102}$-$Tyr^{126}$ | << 4.7 | 4 |
| ANF $Ser^{103}$-$Tyr^{126}$ | 5.0 | 5 |
| ANF $Ser^{104}$-$Tyr^{126}$ | 5.1 | 5 |
| ANF $Cys^{105}$-$Tyr^{126}$ | < 4.7 | 4 |
| ANF $Arg^{101}$-$Arg^{125}$ | 5.4 | 6 |
| ANF $Arg^{101}$-$Phe^{124}$ | 6.4 | 4 |
| ANF $Arg^{101}$-$Ser^{123}$ | 6.8 | 5 |
| ANF $Arg^{101}$-$Cys^{121}$ | 6.9 | 4 |
| ANF $Ser^{103}$-$Ser^{123}$ | 6.5 | 4 |
| ANF $Ser^{103}$-$Arg^{125}$ | 6.2 | 5 |

Further N-terminal cleavage of $Arg^{101}$-$Arg^{102}$ (ANF (103-126), $Arg^{101}$-$Arg^{102}$-$Ser^{123}$ (ANF (104-126)), and $Arg^{101}$-$Arg^{102}$-$Ser^{103}$-$Ser^{104}$ (ANF (105-126)), still produced a marked inhibitory effect on NE.

By contrast with what happens with the N-terminal, modifications in the C-terminal significantly decreased the biological activity of ANF (101-126). The removal of $Arg^{125}$-$Tyr^{126}$ (ANF (101-124)), and $Phe^{124}$-$Arg^{125}$-$Tyr^{126}$ (ANF (101-123)) greatly diminished its inhibitory effect, though still keeping some activity. Removing all residues after $Cys^{121}$ rendered the molecule almost inactive. Currie *et al.* (7) have reported that Atriopeptin I (ANF (103-123)) had no relaxant activity on a NE-contracted strip. However, as seen in Figure 2, ANF (103-123) still has some inhibitory effect on the NE-induced contraction. Sugiyama *et al.* (17), in agreement with our results, demonstrated that ANF (103-123) has still some biological activity in the smooth muscle. They

also show, coinciding with our findings, that the removal of $Arg^{101}$ (ANF (102-126)) does not affect its biological characteristics.

Of the two native peptides which have been used in the present experiments, one, ANF (54-126), the longer form, behaves as the peptides lacking two to four N-terminal amino acids. The other ANF (96-126) is a good inhibitor only with the lower doses of NE. We do not have a clear explanation for those findings, but it could be due to different affinity for the binding sites. A weak compound as determined by activity may bind very tightly with a low Ka but not stimulate the receptor.

Our results seem to indicate that the C-terminal may modulate the binding of these peptides to their receptors, since small modifications greatly reduced their activity. By contrast, the N-terminal seems to be much less important in this modulation.

Immunoreactive ANF has been found in rat plasma (18), but its circulating form is not known. According to our present results, it is to be expected that circulating ANF should be closely similar to its more active forms.

ACKNOWLEDGEMENTS

This work was supported by the MRC to the Hypertension Group and to R.G. and G.T. (MA-7665), and by a Program Grant (PG-2) from the MRC to M.C., N.G.S. and C.L.. C.L. is a "Chercheur-boursier" from the F.R.S.Q. We thank Mrs. S. Diebold, Mrs. S. Olivieri and Ms. J. Bilodeau for their skillful technical work and Ms. F. De Coste for her secretarial assistance.

REFERENCES

1. Deth, R.C., Hong, K., Fukagawa, S., Rocco, R., Smart, J.L., Lynch, C.J., and Wawad, R. (1982) Fed. Proc. 41, 983a.
2. Currie, M.G., Geller, D.M., Cole, B.R., Boylan, J.G., YuSheng, W., Hollenberg, S.W., and Needleman, P. (1983) Science 221, 71-73.
3. Garcia, R., Thibault, G., Cantin, M., and Genest, J. (1984) Am. J. Physiol. 234, R34-R39.
4. Kleinert, H.D., Maack, T., Atlas, S.A., Januszewicz, A., Sealey, J.E., and Laragh, J.H. (1984) Hypertension 6 (Suppl. I), I-143-I-147.
5. Garcia, R., Thibault, G., Nutt, R.F., Cantin, M., and Genest, J. (1984) Biochem. Biophys. Res. Comm. 119, 685-688.
6. Hamet, P., Tremblay, J., Pang, S.C., Garcia, R., Thibault, G., Gutkowska, J., Cantin, M., and Genest, J. (1984) Biochem. Biophys. Res. Comm. 123, 515-527.
7. Currie, M.G., Geller, D.M., Cole, B.R., Siegel, N.R., Fok, K.F., Adams, S.P., Eubanks, S.R., Gallupi, G.R., and Needleman, P. (1984) Science 223, 67-69.
8. Misono, K.S., Fukumi, H., Grammer, R.T., and Inagami, T. (1984) Biochem. Biophys. Res. Comm. 119, 524-529.

9. Thibault, G., Garcia, R., Cantin, M., Genest, J., Lazure, C., Seidah, N.G., and Chrétien, M. (1984) FEBS Lett. 167, 352-356.
10. Lazure, C., Seidah, N.G., Chrétien, M., Thibault, G., Garcia, R., Cantin, M., and Genest, J. (1984) FEBS Lett. 172, 80-86.
11. Yamanaka, M., Greenberg, B., Johnson, L., Sielhamer, J., Brewer, M., Friedemann, T., Miller, J., Atlas, S., Laragh, J., Lewicki, J., and Fiddes, J. (1984) Nature 309, 719-722.
12. Maki, M., Takayanagi, R., Misono, K.S., Pandey, K.N., Tibbetts, C., and Inagami, T. (1984) Nature 309, 722-724.
13. Zivin, R.A., Condra, J.H., Dixon, R.A.F., Seidah, N.G., Chrétien, M., Nemer, M., Chamberland, M., and Drouin, J. Proc. Natl. Acad. Sci. USA, in press.
14. Thibault, G., Garcia, R., Seidah, N.G., Lazure, C., Cantin, M., Chrétien, M., and Genest, J. (1983) FEBS Lett. 164, 286-290.
15. Thibault, G., Garcia, R., Carrier, F., Seidah, N.G., Lazure, C., Chrétien, M., Cantin, M., and Genest, J. Biochem. Biophys. Res. Comm., in press.
16. Seidah, N.G., Lazure, C., Chrétien, M., Thibault, G., Garcia, R., Cantin, M., Genest, J., Nutt, R.F., Brady, S.F., Lyle, T.A., Paleveda, W.J., Colton, C.D., Ciccerone, T.M., and Veber, D.F. (1984) Proc. Natl. Acad. Sci. USA 81, 2640-2644.
17. Sugiyama, M., Fukumi, H., Grammer, R.T., Misono, K.S., Yabe, Y., Morisawa, Y., and Inagami, T. (1984) Biochem. Biophys. Res. Comm. 123, 338-344.
18. Gutkowska, J., Horký, K., Thibault, G., Januszewicz, P., Cantin, M., and Genest, J. Biochem. Biophys. Res. Comm., in press.