Degradation of Bioactive Substances:

Physiology and Pathophysiology

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CRC Press
Boca Raton Ann Arbor Boston London

Library of Congress Cataloging-in-Publication Data

Degradation of bioactive substances : physiology and pathophysiology /
editor, Jens H. Henriksen.
p. cm.
Includes bibliographical references.
Includes index.
ISBN 0-8493-6858-8
1. Metabolism. 2. Bioactive compounds—Metabolism.
3. Biomolecules—Pathophysiology. [1. Metabolic Diseases.]
I. Henriksen, Jens H.
[DNLM: 1. Blood Chemical Analysis. 2. Catecholamines—metabolism.
3. Peptides—metabolism. QY 450 D3221]
QP171.D44 1991
616.07'561—dc20
DNLM/DLC
for Library of Congress 90-2609
CIP

Direct all inquiries to CRC Press, Inc., 2000 Corporate Blvd., N.W., Boca Raton, Florida, 33431.

International Standard Book Number 0-8493-6858-8

Library of Congress Card Number 90-2609
Printed in the United States

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Chapter 7

PROCESSING AND CLEARANCE OF ATRIAL NATRIURETIC FACTORS (ANF)

Alexander L. Gerbes and Angelika M. Vollmar

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I. INTRODUCTION

Atrial natriuretic factor (ANF) is the first well-defined natriuretic hormone. The best characterized pharmacological and partly physiological actions of ANF are natriuresis, diuresis, and smooth muscle relaxation (for review, see References 1 to 12). Recently, evidence has been provided that ANF might be involved in various other biological actions apart from its role in volume homeostasis such as immune or reproductive functions.[13-17]

ANF is synthesized in the human heart as a 151 amino acid preprohormone and stored as a 126 amino acid prohormone. Upon appropriate stimulation, such as atrial stretch, the prohormone is cleaved into an N-terminal and C-terminal fragment. The latter has been identified as the circulating bioactive ANF 99-126.[18,19] A body of investigations has concentrated on the mechanisms of ANF release and on its biological actions (for review, see References 1 to 14). Thus far, there is much less information on the processing of ANF prohormone as well as on the clearance of bioactive ANF. However, these seem to be topics of considerable interest, since plasma levels and biological activity of ANF are significantly influenced by these processes. This review article will concentrate on the mechanisms of ANF prohormone processing and of degradation and clearance of bioactive ANF, including pathophysiological aspects and possible therapeutic implications. The still poorly understood metabolism of ANF in the central nervous system will not be addressed in this chapter.

II. PROCESSING OF ATRIAL NATRIURETIC PROHORMONE

A. THE HEART

It is well established that the heart atria represent the major site of synthesis for ANF (for review, see References 20 and 21). Atrial muscle cells possess well-developed structures necessary for synthesizing, packaging, and storing this peptide. Initially, a common precursor termed prepro-ANF, which comprises between 149 and 153 amino acids depending on the species (151 amino acids in human), is being synthesized (see Figure 1). This prepro ANF molecule contains a signal peptide sequence at its amino terminus which seems to provide the signal for the co-translational transport of the peptide across the endoplasmatic reticulum. During this transport, the signal sequence is removed and the resulting 126-amino acid peptide is stored in secretory granules. In contrast to other prohormone forms of secreted proteins (e.g., proinsulin, prepromelanocortin, and proenkephalin) which are being cleaved to the mature form during vesicular packaging,[22-24] the ANF prohormone is stored in the atrial granules as the unprocessed prohormone.[25] The only 28-amino acid comprising carboxyterminus ANF(99-126) (see Figure 2) represents the biologically active hormone and is released together with the N-terminal ANF(1-98) into the circulation.[19,26-30] The site and exact mechanism of this proteolytic conversion of ANF(1-126) to ANF(99-126) is not yet known. Two possible mechanisms have to be considered. First, the cleavage occurs during secretional processing; this concept is supported by *in vitro* experiments showing that ANF(99-126) is released from isolated perfused hearts[27,31] and by the fact that plasma is not capable of performing the cleavage.[32] Secondly, data showing that isolated cultured myocytes release the intact pro-ANF provide evidence that the cleavage does occur extracellular of myocytes.[33,34] However, cleavage does not seem to take place in the circulation, because the release of ANF(99-126) from the isolated perfused rat heart seems not to be dependent on serum related enzymes.[32] In this regard, one must be aware of the fact that processing of ANF(1-126) from isolated cultured cells must not necessarily correspond to the *in vivo* conditions. Taken together, the cleavage of pro-ANF may take place during secretion, as well as shortly after secretion from the myocytes.[35-38]

In this context, the knowledge of the enzyme responsible for selectively cleaving the prohormone and, moreover, of its cellular or subcellular localization would be helpful to

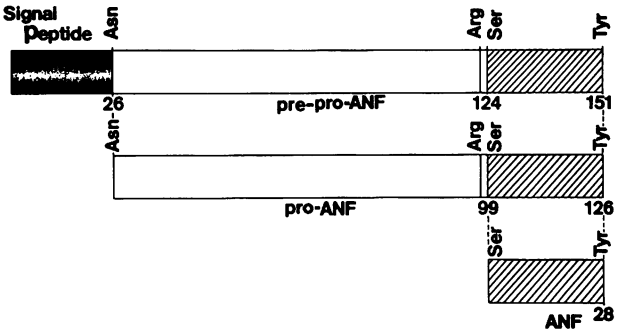

FIGURE 1. Schematic illustration of various ANF forms: synthesized prepro-ANF is stored as ANF(1-126), the prohormone. Upon release and cleavage, ANF(99-126), the circulating C-terminal fragment, exerts biological activities.

Amino acid sequence of human ANF(99-126)

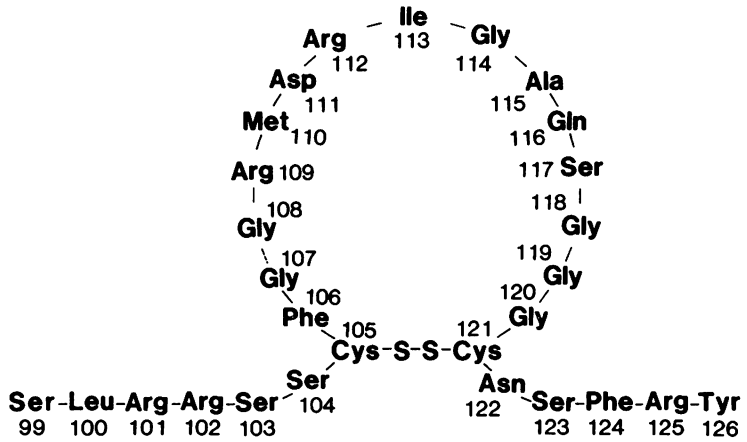

FIGURE 2. Amino acid sequence of the biologically active ANF(99-126).

elucidate this question. Recently, a serylprotease has been isolated from atrial tissue which selectively cleaves the Arg 98-Ser 99 bond of pro-ANF, resulting in ANF(99-126) and ANF(1-98).[39,40] The exact location of this peptide is still controversial. Several possibilities are being proposed:

1. Location of a serine protease with ANF (99-126) substrate specificity was described in the atrial granules containing ANF(1-126).[40] This finding can hardly be reconciled with the absence of ANF(99-126) in these granules.[25]
2. The microsomal fraction,[39] rather than the granule fraction of atrial cells, containing this enzyme activity would be in line with our understanding that pro-ANF is most likely cleaved during exocytotic secretion from its storage granules. Myocytes, but also adjacent atrial mesenchymal cells, may possess such an extragranularly located enzyme.[36]
3. Alternatively, this enzyme might be situated extracellularly;[36] however, this hypothesis has not been backed up by experimental findings thus far.

B. PROCESSING IN VARIOUS OTHER ORGANS

Over the years, evidence has accumulated that the heart is not the only peripheral site of ANF synthesis.[40a] Lung,[41] adrenals,[42] intestine,[17] various lymphoid organs,[15,43] and female reproductive organs[16,44] have been reported to contain ANF prohormone. So far, only the lung and the adrenals have been shown to be able to secrete ANF(99-126).[45,46] This suggests that maturation processes occur in these organs. In cultured chromaffin cells, both the prohormone as well as the mature form of ANF are co-secreted,[45] whereas the lung releases only ANF(99-126).[46]

No information about the exact mechanism of these maturation processes is available, however, a contribution of extraatrial ANF to the circulating ANF pool is being discussed.[41,46]

III. CLEARANCE OF BIOACTIVE ANF

A. ORGAN CLEARANCE

Plasma levels of bioactive ANF are determined by both release and clearance. While there is a rich body of information about ANF release, the number of studies on the clearance of this peptide is rather limited.[47–63] The ability of various organs to clear ANF from the circulation can be described by the extraction ratio or by the organ clearance. The extraction ratio is calculated as arterial minus venous blood concentration over arterial blood concentration (%). Multiplication of the extraction ratio with the organ plasma flow results in the organ clearance (liter/minute). Several aspects and possible pitfalls have to be taken into consideration regarding studies on ANF metabolism: since the studies necessarily apply venous and even arterial catheterization, for ethical reasons, many investigations have been done in patients with hypertension or congestive heart failure rather than in healthy subjects. Some of the studies did not determine organ plasma flow, thus restricting their results to extraction ratios. Extraction ratios as well as organ clearance have been determined both under basal conditions and following intravenous ANF administration. For clearance determinations of pharmacologically active ANF doses, specific attention should be paid to the rate of infusion in order to achieve steady-state conditions. Problems may arise by inappropriate intervals of plasma sampling. Furthermore, administration of ANF may decrease blood pressure,[56] affect cardiac output and organ blood flow, and increase glomerular filtration rate; this might result in under- or overestimation of ANF clearance as compared to physiological conditions.

Furthermore, some studies are based on the assumption that there is no pulmonary extraction of ANF. However, there is now evidence, obtained by pulmonary wedge or pulmonary venous sampling,[47,48] that the lung is an important site for ANF extraction.

With these reservations in mind, it seems that there is no major organ specificity for ANF extraction. Lung, liver, kidney, intestine, and lower limb show similar extraction ratios within the respective studies. Values reported, however, differ considerably between various studies (Table 1).[47–51,53,54,56,58–61,63] Extraction ratios were reported to be 0 to 33% for lung, 22 to 75% for liver and intestine, 23 to 67% for kidney, and 15 to 61% for the lower limb. Taken together, it seems that there is a general arterio-venous organ extraction ratio for ANF of about 35%.

Since there are marked differences of organ blood flow, obviously total ANF clearance differs considerably between various organs. Few investigations have determined organ blood flow together with ANF extraction under physiological or steady-state conditions.[47,49,51,56] About 50% of the total body clearance of ANF seems to be furnished by the lung.[47] The renal[47,49,51] (around 15%) and splanchnic-hepatic[47,49,56] (about 20%) vascular beds also contribute significantly to ANF clearance.

Several mechanisms could account for the above-mentioned organ clearance of ANF: receptor-mediated uptake, enzymatic degradation, or organ-specific excretion (urine or bile). However, there are only a few reports of concentrations of ANF in bile[47] or urine.[57,62] The following sections in this chapter will cover enzymatic degradation of ANF, as well as ANF clearance receptors.

TABLE 1
Clearance of ANF by Various Organs

Author/ year	Species	Healthy	(n)	Arterial sampling site	ANF arterio-venous extraction ratio (%) Kidney	Liver/ intestine	Lung	Lower limb	Ref.
Crozier, 1986	Man	No	4—8	Pulmonary/ aortic	50	45	<10	50	58
Henriksen, 1986	Man	Yes	8—14	Femoral	54	28	—	40	54
Schütten, 1987	Man	?	8—14	Pulmonary/ femoral	54	28	0	40	53
Gines, 1988	Man	?	11	Pulmonary	—	71	—	61	63
Vierhapper, 1988	Man	Yes	6	Femoral	—	75	—	—	56
Woods, 1988	Dog	Yes	5	Aortic	55	—	—	—	51
Bates, 1989	Dog	Yes	14	Pulmonary	—	—	32	—	48
Hollister, 1989	Man	No	8—39	Pulmonary/ aortic	35	30	24	38	47
	Dog	Yes	4—7	Pulmonary/ aortic	42	36	19	—	
Krieter, 1989[a]	Rat	Yes	4—7	Femoral	55—61	22—44	—	43—54	49
Kurosawa, 1989	Man	No	6—56	Pulmonary/ femoral/coeliac/ renal	23	24	12	15	59
Masuda, 1989	Dog	Yes	6	Pulmonary/ renal/ hepatic	34	28	33	—	50
Sudhir, 1989	Man	Yes	6	Brachial	67	—	—	—	61
Henriksen, 1990	Man	Yes	6	Femoral	—	53	—	—	60

[a] Data obtained during ANF infusion.

Note: Arteriovenous extraction ratios are calculated as (arterial minus venous) over arterial ANF plasma concentrations and given in percent (%). Sites of sampling for arterial blood are shown in the shown in the table; ideally the arterial plasma concentrations should be determined in the respective organ-supplying arteries. —: not determined.

1. Enzymatic Degradation

Since the kidney is a major target organ for ANF, interest has focused on this organ, not only as a site for receptor mediated clearance of this peptide (see Section III.A.2), but also as a tissue which may be able to enzymatically degrade circulating ANF(99-126).

Peptidase degradation of ANF is considered to play an important role among possible clearance mechanisms[64,65] (see also Chapter 4). The brush border of the renal proximal tubule is known to be very rich in peptidases. These enzymes are membrane bound and their active sites face the lumen of the tubule.[66] Incubating ANF(99-126) with these kidney microvilli results in a major degradation product cleaved between cysteine and phenylalanine (Cys 105-Phe 106).[64,65,67-69] Since the intact ring structure of ANF(99-126) is essential for its biological activity,[70,71] ANF is effectively inactivated by this cleavage process.

The properties of the ANF degrading activity have been investigated by several groups. The responsible substance is a glycoprotein[64,67,69] with a molecular weight of approximately 94 kDa, sensitive to metalloendoprotease inhibitors such as phosphoramidon and thiorphan. In view of these findings, the major proteolytic enzyme responsible for the ANF cleavage seems to be the endoprotease EC 3.4.24.11.[63,67,69] EC 3.4.24.11 has been reported also to possess enkephalin degrading activity.[72] However, there may be various forms of EC 3.4.24.11 differing in cleavage site specificity and tissue distribution.[67,73-77] Thus, one may speculate that the kidney possesses a specific type of endoprotease EC 3.4.24.11.

Apart from the major cleavage site within the loop structure, secondary attack by aminopeptidases has been hypothesized.[64] These enzymes are not initially responsible for degradation because specific inhibitors like captopril or amastatin have no significant effect.[64] After the major cleavage process at the Cys-Phe bond of the ring structure, more complex degradation processes seem to follow, producing smaller ANF fragments.[68] The major sites of renal ANF degradation by the endopeptidase seem to be the proximal tubule and the glomerulus.[78]

Prompted by these observations, efforts have been made to develop ANF analogues which are resistant to this enzyme, as well as specific enzyme inhibitors which could be therapeutically employed (see Section III.C).

Recent reports indicate ANF degrading activity by the vasculature[79] and the adrenal capsule.[73] No information is available about the enzymatic degradation activity of other organs reported to contribute to ANF clearance (see Section III.A).

2. Clearance Receptors

Specific binding sites for ANF have been identified on various cells such as endothelial cells, vascular smooth muscle cells, inner medullary collecting duct cells, and in various organs such as lung, kidney, adrenal gland, liver, and intestine.[80-82] Several observations demonstrate that there are at least two different types of ANF receptors with different molecular size and different biological properties.[83-88] The development of truncated ANF analogues which selectively bind to one receptor type such as des[(18-22)]-rANF(4-23)-NH_2,[89,90] recently has allowed for better quantitative discrimination of both receptors.

The current concept of both receptors is as follows: the 130-kDa receptor exhibits a higher affinity for circulating biologically active ANF, is coupled to cyclic guanylate cyclase, and mediates the biological effects. Thus, it has been called B- (biological) or R_1-receptor. The other receptor type shows a molecular weight of 65 kDa, exhibits a lower affinity to biologically active ANF, and binds truncated ANF analogues; it has thus been called C- (clearance) or R_2-receptor.[91-94] C-receptors might mediate suppression of adenylate cyclase activity by ANF.[95,96] Interestingly, it has been shown that the C-receptor may regulate the biologic activity of endogenously released ANF,[94] and there is evidence that this receptor type indeed has a clearance function.[91]

In anesthetized rats, administration of an ANF analogue selectively binding to C-receptors induced marked dose-dependent increases of ANF(99-126) plasma concentration and decreases of its volume of distribution and metabolic clearance rate. Furthermore, there is

evidence that a major part of ANF mertabolism is due to an internalization following the binding of circulating ANF to the C-receptor and subsequent intralysomal hydrolysis.[91] The regulation of the C-receptor is poorly understood as yet. There is evidence that maneuvers decreasing intravascular volume decrease ANF plasma levels and, in turn, increase total ANF receptor density.[97-99] Surprisingly, this increase of ANF receptor density is coupled to a reduced biological response to ANF. The observation that C-receptors, but not B-receptors, are augmented following dehydration[100] could explain this seeming contradiction and indicates that regulation of C-receptor density might be involved in volume homeostasis. There are few data on the quantitative relationship between B- and C-receptors in various tissues. In the kidney, in particular on glomeruli, the majority of ANF receptors seems to be of the clearance type.[100,101] The same has been observed on cultured bovine aorta endothelial cells,[83] on adrenal zona glomerulosa cells,[101] and on lung membranes.[88]

These recent observations are compatible with the concept of a functional importance for the C-receptors, which are distinctly different from the B-receptors, in the clearance of ANF — a rather unique mechanism in the metabolism of peptides.

B. PATHOPHYSIOLOGY

Elevated plasma concentrations of ANF have been found in various pathophysiological states, such as congestive heart failure, renal failure, and in some patients with liver disease (see References 102 to 105). An increased release of ANF has been suggested in these diseases.[63,106-109] However, elevated plasma concentrations might also be caused by decreased clearance. To date, little is known about clearance in pathophysiological states. Recently, it has been reported that the fractional extraction of various organs is unchanged in patients with liver cirrhosis.[54] In an experimental model, it has been shown that the renal clearance of ANF varies considerably, depending upon glomerular filtration rate;[51] thus, augmented ANF plasma concentrations in renal diseases might be partly due to reduced ANF elimination. The proportion of B- and C-receptors for ANF in aorta, adrenal, and kidney seems unchanged in experimental congestive heart failure.[101] In an experimental model of cirrhosis, C-receptor density on glomeruli was increased, whereas B-receptors tended to be decreased;[110] however, these animals were not investigated for ANF clearance. Clearly, there is a need for further investigations on whether and how metabolic clearance and degradation of ANF is affected by pathophysiological states.

C. THERAPEUTIC IMPLICATIONS

Beneficial effects of ANF application have been reported in congestive heart failure, hypertension, renal failure, chronic obstructive lung disease, and liver disease.[111-118] Augmented circulating levels of ANF may be achieved by exogenous administration of ANF. Due to the peptide character of the substance, ANF can be administered only intravenously. The rapid metabolic clearance[119-121] makes repeated bolus injections or continuous infusions necessary. Alternatively, circulating ANF concentrations might be elevated by blocking its clearance. This might be achieved by administering truncated analogues, binding to clearance receptors only and, thus, augmenting the concentration of ANF reaching the B-receptors.[91,94,122]

Furthermore, inhibitors of the ANF degrading peptidase might show the same results. Indeed, several investigations employing endopeptidase EC 3.4.24.11 inhibitors[122-132] showed that the circulating ANF immunoreactivity was elevated and this was accompanied by an augmented biological activity, such as increased urinary volume and sodium excretion. Interestingly, these chemicals seem to be especially useful in pathophysiological conditions. They have been shown to enhance the biological activities (natriuretic and depressor responses) of ANF in experimental models of hypertension[124,130-132] and volume expansion.[128] Further investigations will evaluate the potential of this rather elegant therapeutic approach.

IV. SUMMARY

Atrial natriuretic factor, the first well-defined natriuretic hormone is synthesized in the human heart as 151 amino acid (AA) preprohormone and stored as 126 AA prohormone in atrial granules. Upon appropriate stimulation, the prohormone is cleaved into a 98 AA N-terminal fragment and a 28 AA C-terminal fragment, the biological active ANF(99-126), both circulating in plasma. The cleavage occurs most likely either during or shortly after the release from the myocyte granules. The respective enzyme, a serylprotease, is discussed as being bound to plasma membranes of myocytes or of atrial mesenchymal cells. There is increasing evidence for ANF synthesis and processing in peripheral organs other than the heart.

Circulating ANF(99-126) is cleared by various organs such as lung, liver, intestine, kidney, and upper and lower limbs. Reported arterio-venous extraction ratios vary greatly, but do not differ much between organs, the average extraction ratio being bout 35%. Due to marked differences of organ blood flow, the contribution of various organs to total body ANF clearance differs considerably.

Major mechanisms for ANF clearance are uptake by clearance receptors and degradation by an endoprotease EC 3.4.24.11. Clearance receptors, distinctly different from the receptors mediating the biological actions of ANF, have been demonstrated in various organs. Characterization of the ANF degrading enzyme activity has been performed in kidney tissue. Whether and how pathophysiological states affect ANF clearance is still poorly understood. Inhibition of clearance by ANF analogues binding to clearance receptors and by inhibitors of degrading peptidase can increase the biological action of circulating ANF. This may prove to be a therapeutic approach in diseases with smooth muscle contraction or volume overload.

ACKNOWLEDGMENTS

Jens H. Henriksen, M.D., Ph.D., University of Copenhagen, Denmark, and William R. Samson, Ph.D., University of Missouri–Columbia, are thanked for critical reading of the manuscript. M. Hummel is thanked for preparation of the manuscript. Alexander L. Gerbes, M.D., has been supported by the Deutsche Ferschungsgemeinschaft (Ge 576).

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