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Discovery of O-glycans on atrial natriuretic peptide (ANP) that affect both its proteolytic degradation and potency at its cognate receptor

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Edited by Gerald W. Hart

Atrial natriuretic peptide (ANP) is a peptide hormone that in response to atrial stretch is secreted from atrial myocytes into the circulation, where it stimulates vasodilatation and natriuresis. ANP is an important biomarker of heart failure where low plasma concentrations exclude cardiac dysfunction. ANP is a member of the natriuretic peptide (NP) family, which also includes the B-type natriuretic peptide (BNP) and the C-type natriuretic peptide. The proforms of these hormones undergo processing to mature peptides, and for proBNP, this process has been previously demonstrated to be regulated by O-glycosylation. It has been suggested that proANP also may undergo post-translational modifications. Here, we conducted a targeted O-glycoproteomics approach to characterize O-glycans on NPs and demonstrate that all NP members can carry O-glycans. We identified four O-glycosites in proANP in the porcine heart, and surprisingly, two of these were located on the mature bioactive ANP itself. We found that one of these glycans is located within a conserved sequence motif of the receptor-binding region, suggesting that O-glycans may serve a function beyond intracellular processing and maturation. We also identified an O-glycoform of proANP naturally occurring in human circulation. We demonstrated that site-specific O-glycosylation shields bioactive ANP from proteolytic degradation and modifies potency at its cognate receptor in vitro. Furthermore, we showed that ANP-O-glycosylation attenuates acute renal and cardiovascular ANP actions in vivo. The discovery of novel glycosylated ANP proteoforms reported here significantly improves our understanding of cardiac endocrinology and provides important insight into the etiology of heart failure.

The natriuretic peptide (NP) family comprises atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP), which are peptide hormones involved in maintaining cardiorenal homeostasis. The cardiac NPs, ANP and BNP, possess potent diuretic and natriuretic effects as well as blood pressure-lowering properties that are mediated through the natriuretic peptide receptor A (NPR-A), whereas CNP is a potent anti-fibrotic and anti-remodeling factor acting through natriuretic peptide receptor B (NPR-B) (1–5). All three NPs are synthesized as prohormones and subsequently proteolytically activated by the proprotein convertases corin and furin to form mature receptor-binding peptide hormones. In circulation, NPs have short half-lives (i.e., minutes), presumably owing to processes like receptor-mediated clearance, tissue-specific metabolism, and proteolytic degradation by nephrisin and insulin-degrading enzyme. In an attempt to stabilize ANP, nephrisin inhibitors have recently been introduced for chronic heart failure treatment (6–10), and notably when nephrisin inhibition is combined with an angiotensin II receptor antagonist, cardiovascular mortality is markedly reduced (6), emphasizing the therapeutic potential of NPs in cardiovascular disease.

ProBNP is O-glycosylated, and O-glycans in close proximity to the processing site of BNP have been suggested to co-regulate processing (11). ProBNP, BNP, and the processed N-termini

3 The abbreviations used are: NP, natriuretic peptide; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; CNP, C-type natriuretic peptide; GalNAc-T, N-acetylgalactosaminyltransferase; PNA, peanut agglutinin; HCD, higher-energy collision dissociation; ETD, electron-transfer dissociation; Tn, GalNAcc1-O-Ser/Thr; T, Galj1–3GalNAcc1-O-Ser/Thr; ST, NeuAc2–3Galj1–3GalNAcc1-O-Ser/Thr; dST, NeuAc2–3Galj1–3GalNAcc1-O-Ser/Thr; N, NeuAc2–3GalNAcc1-O-Ser/Thr; NPros, natriuretic peptide receptor type A; NPR-B, natriuretic peptide receptor type B; NPR-C, natriuretic peptide receptor type C; WVA, vicinic viillosa agglutinin; pAb, polyclonal antibody; Fmoc, N-(9-fluorenly)methoxycarbonyl; MAP, mean arterial pressure; BP, blood pressure; UV, urine output; Ne, urinary sodium excretion; RT, room temperature; TRicine, H2O[2-hydroxyethyl]glycine; PE, polyethylene glycol; i.v., intravenously.
nal NT-proBNP are all found in circulation and serve as biomarkers for the diagnosis of heart failure. It is well-established that proBNP and NT-proBNP carry O-glycans of the GalNAc type (11) and that the proportion of O-glycosylated proBNP increases in chronic heart failure (12, 13). O-Glycans are known to co-regulate proprotein processing (14), and studies demonstrate that O-glycans in close proximity to the furin-processing site in proBNP affect activation of the peptide as well as antibody recognition (11, 14–20). It is thus believed that the O-glycosylation of proBNP leads to an increase in the unprocessed form in circulation and corresponding lower biological activity (16, 21). However, comprehensive studies of O-glycosylation of the NPs are missing. O-Glycosylation is the most abundant and complex regulated type of protein glycosylation, and it is estimated that >80% of proteins trafficking the secretory pathway are O-glycosylated (22). O-Glycosylation is initiated in the Golgi by a family of up to 20 polypeptide GalNAc-transferase (GalNAc-T) isoforms at serine and threonine (and possibly tyrosine) residues. The multiple GalNAc-T isoforms are differentially expressed in cells and tissues, which offers a high potential for regulation of specific O-glycosities. This also stresses the need to evaluate different biological sources of proteins to explore potential O-glycosylation (23). It is currently not possible to reliably predict O-glycosities, and technical analytic challenges have long hampered identification of O-glycosities at the proteome level (24). However, recent advances in lectin enrichment and glycoproteomics strategies have facilitated broad screening and discovery of O-glycosities in cells and tissues (25–28).

In this study, we performed a comprehensive glycoproteomics analysis of human plasma and mammalian tissues with known endogenous expression of NPs, using a sensitive lectin-enriched glycoproteomics strategy. We discovered novel O-glycans on all three NPs and surprisingly found O-glycans on mature NPs with two glycosites in ANP positioned in the highly conserved receptor-binding region and thus predicted to affect activation by in vitro and in vivo studies in rats.

Results

**ProANP, proBNP, and proCNP are O-glycoproteins**

To explore O-glycans on NPs, we devised a fractionation strategy in which we extracted water-soluble proteins (i.e. cytosolic and secretory granular proteins) from different tissues (neonatal porcine atria, adult porcine ventricle, and human prostate cancer tissue) known to express NPs at high levels. We used a sequential enrichment strategy for O-glycopeptides in combination with sensitive MS-based sequencing as described previously (22, 24, 26). We predicted the most likely O-glycoform in cardiac tissues to be sialylated core 1 structures (ST (NeuAcα2–3Galβ1–3GalNAca1-O-Ser/Thr) and/or diST (NeuAcα2–3Galβ1–3) NeuAcα2–6 GalNAca1-O-Ser/Thr)). We used pretreatment of samples with neuraminidase to remove sialic acid residues to allow for efficient enrichment of core 1 glycopeptides, using peanut agglutinin (PNA) lectin weak affinity chromatography. Subsequent identification of glycosites by tandem MS was done using higher-energy collision dissociation (HCD) and/or electron-transfer dissociation (ETD) fragmentation. Identified glycopeptides were filtered to include only NPs, and 16 NP O-glycopeptides were identified by HCD and/or ETD fragmentation as summarized in Fig. 1A (Fig. S1 and Table S1). We identified both core 1 and monosialylated core 1 structures (Table S1), suggesting that the neuraminidase pretreatment was partially incomplete.

We identified O-glycans on all three NP members, where O-glycosylated proANP and proBNP were identified in porcine atrial cardiac tissues and O-glycosylated proCNP was identified only in human prostate cancer tissue. The NP protein sequences are conserved between humans and pigs, and all identified O-glycosities were within highly conserved sequence motifs between the two species (Fig. S1). Fragmentation analyzes from both HCD and ETD MS/MS of tryptic O-glycopeptides covering proANP113–126, proBNP53–63, and proCNP3–28, respectively, are shown in Fig. S2 (A–F). In proBNP, we confirmed the presence of two O-glycans at Thr48 and Thr58 (position numbers refer to proforms after signal peptide cleavage) in the propeptide (Fig. 1B and Fig. S2 (C and D)), both of which were previously identified in human proBNP (11, 29).

In proCNP, we found a single O-glycan at Thr11 in the propeptide (Fig. S2, E and F), which we previously identified in a SimpleCell line (22). In proANP, we identified four novel O-glycosites with two in the propeptide at Ser6/8 (ambiguously assigned site) and at Ser80. Surprisingly, we identified two O-glycosites in the mature ANP at Ser117 and Ser123 (Fig. 1 (A and B), Table S1, and Fig. S2 (A and B)), and interestingly, Ser117 is conserved in all NPs. This represents the first O-glycosites in mature ANP.

**Probing human ANP O-glycosites by in vitro enzyme glycosylation**

We next sought to test whether particular GalNAc-T isoforms would function with ANP using in vitro glycosylation assays as described previously (30). Natriuretic peptides share a characteristic disulfide bridge in the C-terminal region (Cys105–Cys121 in proANP). We used both 20-mer partly overlapping synthetic peptides covering all potential Ser/Thr acceptor residues in proANP (peptides 1–9), mature ANP with the disulfide bridge (peptide 10) (Fig. S3A), and a full-length recombinant proANP1–126 protein (Fig. S3B). In human proANP, the four glycosites identified in porcine proANP correspond to Ser8 (Ser6 not conserved), Ser26, Ser117, and Ser123 residues, which are covered by peptides 1, 5/6, and 9/10 (Fig. S3A). Most of these peptides did not serve as substrates for any of the 12 tested recombinant GalNAc-T isoforms, although peptide 5, but not 6, was, albeit poorly, utilized by GalNAc-T1 (Fig. S3A). Interestingly, the analysis indicated two other substrate sites, Ser30 and Thr39/Ser40, in peptides 3 and 4 covering the N-terminal propeptide, which served as substrates for several GalNAc-T isoforms, including GalNAc-T1.

Next, we tested recombinant proANP1–126 as substrate for the three most widely expressed GalNAc-T isoforms, T1, T2, and T3, and monitored incorporation of GalNAc using VVA lectin (31). In an overnight assay, we detected GalNAc incorporation by GalNAc-T1, but not by GalNAc-T2 and -T3 (Fig. S3B). Incorporation of GalNAc by GalNAc-T1 resulted in...
slower migration of both pAb proANP\textsubscript{1–16} and pAb proANP\textsubscript{99–126}–immunoreactive bands corresponding to a 1–3-kDa increase in mass, suggesting that, at least in vitro, both proANP and N-terminal proANP\textsubscript{1–98} (NT-proANP) are glycosylated by GalNAc-T1.

**Identification of partially O-glycosylated proANP\textsubscript{1–98} in human plasma**

In healthy individuals, proANP forms and ANP\textsubscript{1–28} circulate in low picomolar ranges, which is a likely reason why these low-abundance proANP O-glycopeptides have not been detected in human or animal plasma in our previous O-glyco-proteomics studies (25, 32). To search for O-glycosylated proANP in plasma, we therefore developed a state-of-the-art Western blotting strategy for plasma samples with high endogenous concentrations of proANP in combination with ethanol precipitation (Fig. 2A). We screened 20 plasma samples from our clinical routine laboratory with increased proBNP concentrations and selected the three samples with the highest proANP signal by Western blotting. We used a sensitive antibody directed to the first 16 amino acids of proANP, pAb proANP\textsubscript{1–16} and concluded that plasma proANP migrated as a major band around 9–10 kDa with a variable slower-migrating band at 14 kDa (Fig. 2A). The prominent faster-migrating band at 9–10 kDa was concluded to correspond to NT-proANP. We speculated that the slower-migrating band at 14 kDa could represent an O-glycoform of proANP and explored this by treating the plasma samples with a mixture of neuraminidase and O-glycanase to remove core 1 O-glycans. This resulted in selective loss of the slower-migrating immunoreactive band in all samples (Fig. 2A). We further treated a pool of plasma samples with neuraminidase to remove only terminal sialic acid residues, followed by immunoprecipitation and detection using pAb proANP\textsubscript{1–16} and this resulted in a mobility shift of the slower migrating 14 kDa band by ~1–2 kDa (Fig. 2B). These results demonstrate that proANP in blood exists as

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**Figure 1. Summary of identified O-glycosites on natriuretic peptides.** A, schematic representation of NPs with previously reported O-glycosites (yellow squares with diagonal line) and sites identified in this study (yellow squares with bold, black frame). The NP pro-part is shown in red, Receptor binding involving the cyclic part of the bioactive hormones and their cognate receptors is shown on the right. Scissors indicate corin or furin cleavage sites. A conserved serine in the mature NPs is highlighted in boldface red type and with an asterisk and marked with a white box on BNP and CNP. The six amino acids upstream of the proprotein convertase activation sites are shown with previously identified O-glycosites highlighted in red. B, analysis of NP sequence conservation. The top panel shows conservation among all NPs in five species. The three bottom panels show the conservation tracks for each NP as indicated. The conserved serine in the disulfide ring of ANP is highlighted in red and with an asterisk. Dark gray, complete conservation; medium and light gray, less conservation; white, no conservation. All alignments were performed in ClustalW using the NP proprotein sequences of *Homo sapiens*, *Mus musculus*, *Xenopus laevis*, *O. mykiss*, and *A. japonica*. 

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**Editors’ Pick: O-glycans on atrial natriuretic peptide**

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multiple proteoforms with a minor fraction containing sialylated core 1 O-glycans. Based on band intensities, we estimate that the O-glycoform corresponds to ~10% of total circulating proANP (Fig. 2A).

**O-Glycosylation of ANP impacts proteolytic stability**

ANP has a short half-life in circulation, ascribed to receptor-mediated uptake and degradation by neprilysin and insulin-degrading enzyme (33). Neprilysin inactivates ANP by initial cleavage between Cys105 and Phe106, which opens the disulfide ring, and insulin-degrading enzyme inactivates ANP by initial cleavage between Ser123 and Phe124, truncating the C terminus (34–36). O-Glycans in proximity of cleavage sites can modulate regulated proteolysis by, for example, proprotein convertases and ADAM metalloproteinases (14, 37), so we hypothesized that the O-glycans at Ser117 and Ser123 could impact degradation by neprilysin and insulin-degrading enzyme. To explore this, we used an in vitro proteolysis time-course assay monitoring cleavage of peptides and glycopeptides by semi-quantitative MALDI-TOF MS analysis (38).

We found that neprilysin degraded ANP completely within 15 min, whereas ANP with the simplest GalNAc (Tn) monosaccharide O-glycan (Tn-ANP_S117 and Tn-ANP_S123) remained at least partially intact after 60 and 15 min, respectively (Fig. 3A). A fraction of intact Tn-ANP_S117,S123 was still detected after a 30-min incubation. In a similar time course with insulin-degrading enzyme, ANP was completely degraded within 15 min, whereas Tn-ANP_S117 and Tn-ANP_S123 remained mainly as full-length glycopeptides after 30–60 min of incubation (Fig. 3B). Interestingly, Tn-ANP_S117,S123 was degraded within 15 min.

**O-Glycosylation of ANP affects receptor activation**

On mature ANP, the two identified glycosites, Ser117 and Ser123, are located in the receptor-binding region and predicted to impact interaction with the atrial natriuretic peptide receptor type A (NPR-A) (Fig. 1A). Ser117 is highly conserved in all NPs, including Xenopus laevis, Oncorhynchus mykiss, and Anguilla japonica (Fig. 1B), suggesting that this site may be particularly important. To address the effect on receptor interaction, we produced a series of ANP glycopeptides corresponding to those identified by a chemoenzymatic approach (39). ANP glycopeptides with GalNAc residues at selected sites were installed by Fmoc solid-phase synthesis, and the sialylated core 1 structures were generated by stepwise enzymatic glycosylation with purified recombinant Drosophila core 1 synthase (dC1GalT1) and human α2,3-sialyltransferase (ST3Gal1) (Fig. 4, A and B).

To evaluate the influence of O-glycans on ANP activation of the cognate receptors, we used HEK293 cells stably expressing full-length coding constructs of human NPR-A or NPR-B (40). Ligand efficacy and potency were evaluated by quantifying accumulation of intracellular cGMP as a measure of receptor activation. First, we tested a panel of ANP peptides and GalNAc-glycopeptides in NPR-A (Fig. 4B) and NPR-B (Fig. S4), and neither of these activated NPR-B. However, whereas non-glycosylated ANP activated NPR-A within the expected EC50 of 0.4–10 nM, the GalNAc-glycosylated ANP glycoforms with O-glycans at either Ser117 or Ser123 reduced the EC50 65–140-fold (Fig. 4, C and D), whereas the presence of O-glycans at both sites completely abolished receptor activation (Fig. S4C). To examine the effects of the elongated natural core 1 O-glycan structures, we tested core 1 (T), ST, and diST glycoforms, and both T-ANP_S117 and ST-ANP_S117 diminished NPR-A activation additionally 3–5-fold compared with the Tn-glycoform, whereas diST-ANP_S117 did not reach full activation even at 10 μM (Fig. 4C). T-ANP_S123 ST-ANP_S123, and diST-ANP_S123 diminished NPR-A activation additionally 2–6-fold compared with the Tn-glycoform (Fig. 4D). Elongated glycoforms of ANP_S123 tended to reach 80% efficacy, whereas ANP_S123 glycoforms reached 120% efficacy compared with ANP. Importantly, treatment of Tn-ANP_S117 with an exo-α-N-acetylgalactosaminidase to remove GalNAc residues (41) restored the EC50, showing that enzymatic treatment and repurification had no effect on the functional state of ANP (Fig. S4D).

**O-Glycosylation of ANP affects in vivo stability and activity**

We further tested in vivo bioavailability and activity of ANP glycoforms in rats. We measured mean arterial pressure (MAP), renal actions, and peptide hormone changes in vivo in normal rats with 60-min intravenous infusion of synthetic ANP, ST-ANP_S117 or ST-ANP_S123, followed by 30-min post-infusion clearance. Overall, ANP induced potent blood pressure (BP)-lowering effect compared with ST-ANP_S117 or ST-ANP_S123 during infusion (Fig. 5A). There is a trend that MAP was sustained or further reduced by ST-ANP_S117 and ST-ANP_S123 after infusion was completed compared with the rebounding effect observed in the ANP group. Both urine output (UV) and urinary sodium excretion (UNaV) were increased by ANP infusion and not by glycosylated ANP (Fig. 5, B and C). The enhanced cardiovascular and renal actions in the ANP

![Figure 2. Identification of circulating O-glycosylated NT-proANP in human plasma.](http://www.jbc.org/Downloaded from)

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**EDITORS’ PICK:** O-glycans on atrial natriuretic peptide

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group were accompanied by significant elevation of plasma and urinary cGMP levels (Fig. S5, A and B). ST-ANPS123 tended to increase cGMP levels as well, and ST-ANPS117 had only a minor effect on cGMP levels. Interestingly, infusion with ST-ANPS123 or ST-ANPS117 presented markedly higher ANP levels in both plasma and urine compared with the ANP infusion group, indicating their exceptional stability in vivo (Fig. S5, C and D).

Discussion

Recent advances in O-glycoproteomics have provided a new perspective on the abundance of protein O-glycosylation and how site-specific O-glycosylation regulated by individual GalNAc-T isoforms serves distinct co-regulatory roles with health impact (42). Here, we performed a targeted analysis of NPs motivated partly by previous studies of BNP demonstrating a role of O-glycosylation in processing (16) and partly by our previous indication that antibodies to ANP were being blocked by unexplainable modifications (43). Applying a sensitive targeted glycoproteomics strategy to low-molecular weight proteins and peptides led to the discovery of O-glycans on all NPs and, of particular interest, the presence of O-glycans on the mature NPs. We demonstrated with ANP that a conserved O-glycosite in the receptor-binding region of NPs positively affected circulatory half-life and negatively affected NPR-A receptor activation in rats. The result adds another fundamental function to site-specific O-glycosylation in regulating peptide hormones (42), and this may be more generally applicable...
to short peptide hormones than currently appreciated given the general difficulties with identifying O-glycans. ANP binds to NPR-A through an asymmetric interaction with the receptor homodimer, where the N-terminal part of NPR-A binds to one receptor monomer and the C-terminal part of ANP binds to the other receptor monomer (44). Thus, the entire mature ANP peptide is important for the receptor binding (45). Previous studies of ANP analogs have shown that the highly conserved Ser117 residue in the C-terminal loop is important for NPR-A binding, and substitution of this residue with D-Ser diminishes binding (46). In agreement with this, we found that the identified O-glycan at Ser117 in ANP decreased its activation potency with NPR-A, and this effect was exacerbated by increasing the size of the attached O-glycan (Fig. 4).

Moreover, synthetic ANP glycoforms with O-glycans at both Ser117 and Ser123 completely abolished activation of NPR-A, although this glycoform with two O-glycans was not identified in the studied biological samples. We also explored the interaction of ANP glycoforms with the NPR-B receptor to evaluate whether O-glycans could affect receptor subclass selectivity, but all tested ANP glycoforms remained inactive with NPR-B similarly to unmodified ANP. We did not test for binding to NPR-C, and NPR-C has been suggested to have cardiovascular activity that goes beyond its role as a clearance receptor where...
NPR-C activation leads to lowering of blood pressure (47–49). Future studies on the cellular biology of ANP glycoforms should include relevant primary cells or cell lines expressing endogenous natriuretic peptide receptors.

Peptide hormones, including the NPs, are rapidly degraded in circulation by neprilysin and insulin-degrading enzyme (36), and inhibitors of neprilysin increase the effects of endogenous NPs (50). The catalytic grooves of these enzymes provide for their selective specificities of the small mature forms of NPs, likely resulting from a molecular sieving function in domain 2, restricting access by larger peptides to the active site (51).

O-Glycans are well-known to affect stability of proteins and in many cases directly co-regulate limited proteolysis (14, 37, 52), and the finding that mature ANP is stabilized by one or more O-glycans in in vitro proteolysis assays with neprilysin and insulin-degrading enzyme is in agreement with this. We further found that the proximity of the O-glycosites with the preferred cleavage sites of neprilysin and insulin-degrading enzyme correlated with inhibition of proteolysis (Fig. 3), and the effect of O-glycans on stability was confirmed in in vivo studies with both plasma and urine. The position and size of the O-glycans also affected receptor activation, and, perhaps surprisingly, the presence of a single O-glycan at Ser117 or Ser123 reduced, but did not abrogate, receptor activation (Fig. 4). O-Glycans are uniquely suited to serve fine-tuned regulatory functions because they are usually small and may be accommodated in binding interactions and because they can be differentially regulated by one of the many isoenzymes orchestrating this type of protein glycosylation, in contrast to most other types of protein glycosylation, including N-glycosylation (23). Further studies are needed to address the biosynthetic and genetic regulation of the identified O-glycans on NPs.

ANP and BNP are introduced in the clinical setting for infusion therapy for decompensated heart failure (53–55). However, several studies have failed to demonstrate improved outcome for patients, which, at least in part, appears to be because of hemodynamic effects and the induction of hypotension (56, 57). Thus, current infusion strategies are based on low-dose ANP administration over the course of several days (54). The present results suggest that introduction of O-glycans on these NPs could improve therapeutic use by lowering and extending receptor activation without the noted side effects. Our findings may also, at least partly, explain the “endocrine paradox” in congestive heart failure patients (58). These patients have highly increased plasma NP concentrations concomitant with edema. However, when administered recombinant BNP, they elicit natriuresis, suggesting that endogenous NPs are blunted in their bioactivity, and we hypothesize that this may be due to altered O-glycosylation of proBNP (15, 59). In support of this, it appears that several key glycosyltransferases involved in O-glycosylation initiation and elongation are up-regulated in heart failure in the left ventricle of a rat model of hypertension-induced cardiac hypertrophy (60).

In conclusion, our study suggests that all members of the NP family are naturally O-glycosylated and that O-glycans on NPs affect processing, receptor activation, and stability. We hypothesize that O-glycoforms of NPs are produced to fine-tune timing and functions of NPs and posit that O-glycosylated NPs may have potential for therapeutic use.

Materials and methods

Sample preparation and nLC-MS/MS analysis

Atrial appendage tissue from 44 newborn piglets (61), one normal porcine ventricle, and human prostate gland tissue biopsies (pooled) (62) were collected. The local ethics committee approved the use of human tissue (KF 01287197), written informed consent was obtained from all study participants, and the study abides by the Declaration of Helsinki principles. Proteins were extracted by crushing frozen tissue using a CryoPrep tissue extractor (Covaris, Woburn, MA), boiled in water for 20 min, and homogenized with an Ultra-Turrax (IKA, Staufen, Germany). After a 30-min centrifugation at 13,000 × g, the supernatants were collected and pooled, and protein concentration was determined using a bicinchoninic acid assay (Pierce). The prostate gland extract was then adjusted to 0.5 M CH₃COOH, precipitated by −20 °C acetone (67%), incubated for 1 h at −20 °C, and centrifuged at 16,000 × g for 30 min. Subsequently, the supernatant was lyophilized and reconstituted in water.

An aliquot corresponding to 3 mg of protein for the heart samples and 5 mg of protein for the prostate sample was adjusted to 50 mM ammonium bicarbonate, reduced by 5 mM DTT (30 min, 60 °C), and alkylated by adding 10 mM iodoacet-
amidine (30 min, room temperature (RT)). The sample was then digested with 50 μg of trypsin (Roche Applied Science) (37 °C, overnight), purified on C18 Sep-Pak (Waters), and desialylated with 150 units of neuraminidase (P0720, New England Biolabs) in 50 mM sodium citrate (pH 6.0) (37 °C for 2 h). The digest was Sep-Pak-purified, lyophilized, and resuspended in 2 ml of PNA-binding buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM CaCl$_2$, 0.1 mM MgCl$_2$, MnCl$_2$, and ZnCl$_2$) and injected to a pre-equilibrated 2.6-m-long PNA lectin-agarose column (Vector Laboratories). The flow was set to 100 μl min$^{-1}$, and 1-ml fractions were collected. The sample was eluted with 3 × 1 column volume of 0.5 M galactose, 1 M galactose, and 1 M galactose, pH 3, respectively.

All samples were desalted on homemade StageTips (Empore disk-C18, 3 ml) (63, 64). The acidified samples were loaded on the activated StageTips; washed with 0.1% formic acid; eluted with 50% methanol, 0.1% formic acid; lyophilized; and finally dissolved in 0.1% formic acid. Samples were analyzed on an Easy-nLC 1000 LC system (Thermo Fisher Scientific) interfaced via nanoSpray Flex ion source to an LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific). A precursor MS1 scan (m/z 350–1,700) of intact peptides was acquired in the Orbitrap at a nominal resolution setting of 30,000, followed by Orbitrap HCD-MS2 and ETD-MS2 (m/z of 100–2,000) of the five most abundant precursor ions in the MS1 spectrum; a minimum MS1 signal threshold of 50,000 was used for triggering data-dependent fragmentation events; and MS2 spectra were acquired at a resolution of 7,500 for HCD MS2 and 15,000 for ETD MS2. Activation times were 30 and 200 ms for HCD and ETD fragmentation, respectively; isolation width was 4 mass units, and usually 1 microscan was collected for each spectrum. Automatic gain control targets were 1,000,000 ions for Orbitrap MS1 and 100,000 ions for MS2 scans, and the automatic gain control for the fluoranthene ion used for ETD was 300,000. Supplemental activation (20%) of the charge-reduced species was used in the ETD analysis to improve fragmentation. Dynamic exclusion for 60 s was used to prevent repeated analysis of the same components. Polysiloxane ions at m/z 445.12003 were used as a lock mass in all runs.

**Data analysis**

Data processing was performed using Proteome Discoverer version 1.4 software (Thermo Fisher Scientific), using Sequest HT node as described previously with small changes (22). All spectra were initially searched with the full cleavage specifictiy, filtered according to the confidence level (medium, low, and unassigned), and further searched with the semi-specific enzymatic cleavage. In all cases, the precursor mass tolerance was set to 6 ppm, and fragment ion mass tolerance was set to 50 milli-mass units. Carbamidomethylation on cysteine residues was used as a fixed modification. Methionine oxidation and HexNAc and HexHexNAc, HexNAAcNeuAc, and HexHexNAcNeuAc attachment to serine, threonine, and tyrosine were used as variable modifications for ETD MS2. All HCD MS2 scans were preprocessed as described (22) and searched under the same conditions mentioned above using only methionine oxidation as a variable modification. All spectra were searched against a concatenated forward/reverse Sus scrofa (pig)-specific database (UniProt, February 2014, containing 33,652 canonical entries) or human-specific database (UniProt, January 2013, containing 20,232 canonical entries) using a target false discovery rate of 1%. FDR was calculated using target decoy PSM validator node, a part of the Proteome Discoverer workflow. The resulting list was filtered to include only NPs with glycosylation as a modification. The full data set will be published elsewhere and made available at the GlycocDomainViewer (https://glycnodomain.glycomics.ku.dk) (65).

**Peptide synthesis, in vitro glycosylation, and glycan elongation**

Synthetic ANP and CNP peptides (Phoenix Pharmaceuticals), synthetic 20-mer ANP peptides (NeoBioLab), and ANP-Tn peptides (Synpeptide) were confirmed to elicit correct mass by MALDI-TOF analysis, and selected peptides were analyzed for purity by reverse-phase HPLC. Recombinant proANP (Immundiagnostik) was produced in Escherichia coli.

In vitro glycosylation of peptides was performed in 25 μl of 25 mM cacodylic acid sodium, pH 7.4, 10 mM MnCl$_2$, 0.25% Triton X-100, 4 mM UDP-GalNAc (Sigma), 10 μg of acceptor peptides, and 0.2 μg of purified recombinant glycosyltransferase enzyme. All reactions were incubated at 37 °C, and product development was evaluated by a Bruker Autoflex MALDI-TOF instrument with accompanying Compass version 1.4 FlexC series software. Glycosylation of recombinant proANP was performed as above using 1 μg of acceptor protein and 0.6 μg of purified recombinant glycosyltransferase with complete EDTA-free protease inhibitor mixture (Roche Applied Science).

ANP-Tn peptides were elongated to T by enzymatic treatment with recombinant purified Drosophila core 1 synthase in 100 mM MES, 0.1% Triton X-100, 20 mM MnCl$_2$, and 0.5 mM UDP-Gal at 37 °C (39). After purification by reverse-phase HPLC (C18), the glycan was further elongated to ST by enzymatic treatment with recombinant purified o2,3-sialyltransferase in 25 mM Tris (pH 6.5) and 2 mM CMP-Neu5Ac at 37 °C and purified by reverse-phase HPLC (C18). GalNAc from Tn-ANP$_{117}$ was removed by enzymatic treatment byexo-α-N-acetylgalactosaminidase (41) in 100 mM Tris (pH 7.4) and purified by reverse-phase HPLC (C18). Chemoenzymatically elongated glycopeptides and Azyme-treated Tn-ANP$_{5119}$ were quantified by reverse-phase HPLC (C18) using ANP (≥95%, Phoenix Pharmaceuticals) as standard peptide.

**Deglycosylation of plasma and Western blotting**

For the human samples, the department has a general approval for using human material for analytical purposes without specific applications to the local ethics committee. All samples were anonymized prior to analyses without any possible traceability to the patient. Plasma from patients with elevated endogenous proBNP (>100 pm) was diluted 1:1 with 50 mM phosphate buffer, pH 6, and incubated with 10 milliunits of broad neuraminidase (Merck Millipore, catalogue no. 480716) and 2 milliunits of O-glycanase (Merck Millipore, catalogue no. 324716) for 16 h at 37 °C and subsequently precipitated by eth-

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anol (64%), vortexed, and centrifuged at 4,000 \times g for 20 min at 4 °C. The pellet was discarded, and the supernatant was dried in a SpeedVac and resuspended in water. For specific removal of sialic acids, the sample was treated with 100 units of α2-3,6,8-neuraminidase (New England Biolabs) for 1 h at 37 °C on 330 μl of supernatant in a total volume of 1 ml in 50 mM sodium acetate, pH 5. For immunoprecipitation, samples were precipitated with rabbit pAb NT-proANP\textsubscript{1–16} (66) using Dynabeads protein A (Thermo Fisher Scientific) according to the manufacturer’s instructions.

All samples were run on 16%/10%/6% Tris-Tricine gels at 125 V for 90 min and transferred to nitrocellulose membranes, and nonspecific binding was blocked using 5% skim milk in TBST. The blocked membranes were incubated with primary antibody pAb NT-proANP\textsubscript{1–16} or pAb proANP\textsubscript{99–126} diluted 1:1,000 overnight at 4 °C followed by secondary antibody goat anti-rabbit IgG coupled to horseradish peroxidase (Cell Signaling) diluted 1:2,000 for 1 h at RT. For lectin blots, 1% polyvinylpyrrolidone was used as blocking reagent, and lectin used was VVA-biotin (Vector Laboratories) diluted 1:2,000 overnight at 4 °C followed by secondary antibody streptavidin coupled to horseradish peroxidase (Dako, Agilent) diluted 1:4,000 for 1 h at RT. For development, SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used on an Odyssey Fc Imaging System (LI-COR Biosciences). Band intensities were calculated using Image Studio version 2.0.38 (LI-COR Biosciences).

### ANP receptor cGMP activation assay

The cGMP content in HEK293 cells stimulated with peptide was measured using the cGMP kit (Cisbio) according to the manufacturer’s instructions. Briefly, HEK293 cells stably expressing either human NPR-A or human NPR-B (40) were resuspended in Dulbecco’s modified Eagle’s medium buffer supplemented with 25 mM HEPES, pH 7.4, 0.1% BSA, and 1 mM 3-isobutyl-1-methylxanthine (Sigma) to achieve a cell density of 0.4 million cells/ml. Stimulated cells (2,000 cells/well) were incubated at 37 °C for 30 min before the cells were lysed, anti-cGMP cryptate conjugate and cGMP-\textsubscript{d2} conjugate were added, and the plate was incubated for 1 h at room temperature. The plate was read on an Enspire Multilabel Reader (PerkinElmer Life Sciences) with excitation at 340 nm and measurements of emission at 615 and 665 nm. The FRET ratios (665/615 nm) were converted into cGMP concentrations by interpolating values from a cGMP standard curve.

### Proteolytic degradation assay

**In vitro** cleavage activity was assayed by adding 1 ng of nephrilysin (R&D Systems) or 50 ng of insulin-degrading enzyme (R&D Systems) to 325 pmol of peptide or glycopeptide substrate in a total volume of 10 μl. Reactions were performed in 50 mM Tris, 0.05% Brij-35, pH 9 (nephrilysin), or 50 mM Tris, 20 mM NaCl, pH 7.5 (insulin-degrading enzyme), and incubated at 37 °C. Protein development was monitored after 15 min, 30 min, 60 min, and 24 h by MALDI-TOF analysis.

**In vivo evaluation of glycosylated ANP**

We investigated the BP actions of equimolar doses of (600 pmol/kg/min) ANP, ST-ANP\textsubscript{117}, or ST-ANP\textsubscript{123} (n = 4/group) in normal male Sprague–Dawley rats (250–350 g; Charles River Laboratories, Wilmington, MA). Studies were performed in accordance with the Animal Welfare Act and with approval of the Mayo Clinic Institutional Animal Care and Use Committee.

Anesthesia in rats was induced with 133 mg/kg intraperitoneal inactin (Sigma), and rats were maintained on a heating pad for 1 h until complete anesthesia was achieved. With oxygen flow, the rats were then subjected to catherizations and blood and urine collections (2). A polyethylene (PE)-50 tube catheter was placed into the jugular vein for intravenous (i.v.) saline and peptide infusion. The carotid artery was cannulated with a PE-50 tube catheter for BP measurement (Sonometrics, London, Canada). After completion of the above procedural setup, a 45-min equilibration period was performed, which included continuous i.v. saline infusion. After the 45-min equilibration period, baseline (0 min) BP was recorded. The inulin and saline infusion was replaced by continuous i.v. infusion of either ANP, ST-ANP\textsubscript{117} or ST-ANP\textsubscript{123} for 60 min. The infusion rate was weight-adjusted and equals the weight \times 0.7/6,000 ml/min. A post-infusion clearance (time 60–90 min) was performed for 30 min. At the end of the study, blood was collected to determine plasma ANP and cGMP levels by radioimmunoassay and ELISA. Glycosylation on Ser-117 of ANP underestimates the actual ST-ANPS117 concentration (data not shown). Urine was collected at the end of the infusion and post-infusion clearances (time = 60 and 90 min). Urinary sodium was measured with pHox Ultra (Nova Biomedical, Waltham, MA). UV and UNaV were calculated as urine volume or sodium clearance per min. Urinary cGMP and ANP excretion rate were calculated based on raw concentrations obtained in the urine and UV.

### Statistical analysis

**In vitro** receptor activation data are represented as mean ± S.E. In vivo data are represented as mean ± S.D. One-way ANOVA was used for comparison of more than two groups, followed by Tukey’s post hoc tests, and two-way ANOVA was used when multiple time points were involved. Unpaired \( t \) test was used for comparison of two groups. Statistical significance is defined as \( p < 0.05 \) (two-tailed). Data were analyzed with GraphPad Prism version 8 software.

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Discovery of O-glycans on atrial natriuretic peptide (ANP) that affect both its proteolytic degradation and potency at its cognate receptor

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