Dendroaspis natriuretic peptide relaxes isolated human arteries and veins

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Abstract

Background: Dendroaspis natriuretic peptide (DNP) is the newest member of the natriuretic peptide family and is a circulating peptide in humans. The effects of DNP on the human vasculature are unknown. Since other natriuretic peptides are known to cause vasorelaxation, we determined the response to DNP on human blood vessels in vitro. We also investigated the mechanism of DNP mediated vasorelaxation.

Methods: Rings of human internal mammary artery and saphenous vein were suspended in an organ bath. The response to cumulative concentrations of DNP was obtained. Inhibiting agents were used to determine the mechanism of this vasorelaxation.

Results: DNP caused dose-dependent relaxation, with a greater effect on the internal mammary arteries (relaxation from \(2.7 \times 10^{-7}\) mol/l DNP: 80.6 ± 4.1%) than the saphenous veins (33.4 ± 4.1%). At \(10^{-7}\) mol/l, DNP resulted in less arterial relaxation compared with atrial and C-type natriuretic peptides and similar relaxation to brain natriuretic peptide. In veins, DNP caused the greatest relaxation of the natriuretic peptides. DNP increased tissue cyclic guanosine monophosphate (cGMP) determined by radioimmunoassay by over 7-fold. Barium chloride and indomethacin attenuated DNP mediated vasorelaxation. However, glibenclamide, charydotoxin, apamin, tetraethyl-ammonium chloride and diisothiocyanato-stilbene-2,2'-disulfonic acid did not. DNP mediated vasorelaxation was mildly attenuated with removal of the endothelium. DNP immunoreactivity was identified in both arteries and veins.

Conclusions: The current study demonstrates that DNP is an endogenous human natriuretic peptide that relaxes human arteries more than veins. Furthermore, DNP mediated vasorelaxation involves the inward rectifying potassium channels, prostaglandins, and cGMP. This newest member of the natriuretic peptide family may have an important physiologic role in the human vasculature.

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1. Introduction

The natriuretic peptide family consists of structurally homologous peptides that regulate vascular tone. These include atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) which activate the natriuretic peptide receptor A (NPR\(_A\)), and C-type natriuretic peptide (CNP) which activates the natriuretic peptide receptor B (NPR\(_B\)) [1,2]. This family of peptides modulates vascular tone and sodium excretion under basal conditions and in pathophysiologic states such as congestive heart failure [3].

Dendroaspis natriuretic peptide (DNP) is a new member of the natriuretic peptide family that has 38 amino acids and a 17-amino acid disulfide ring structure resembling ANP, BNP, and CNP [4]. This novel peptide was originally isolated from the venom of the green mamba (Dendroaspis angusticeps), but was found to have in vitro vasorelaxing properties in experimental animal preparations [4,5]. Like ANP and BNP, DNP activates the NPR\(_A\). However, little is known regarding the effects of this peptide on the human vasculature.

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DNP can be isolated in human plasma and is elevated in human congestive heart failure [6]. In animals, DNP demonstrates natriuretic and diuretic properties [7]. Thus, these data suggest that this peptide may have an important role in human physiologic and pathophysiologic states. However, the effects this novel natriuretic peptide on the human vasculature are unknown. We also sought to determine if DNP relaxes human arteries and veins in vitro and to compare the effects to other natriuretic peptides. Additionally, we sought to determine the mechanisms of these vasodilating effects. Furthermore, we determined if DNP immunostaining was present in the human vasculature.

2. Methods

2.1. Organ chamber experiments

All study procedures using human tissue were reviewed and approved by the Mayo Foundation Institutional Review Board. Excess internal mammary artery (IMA) and saphenous vein (SV) segments from 79 patients undergoing coronary artery bypass surgery were collected and stored in oxygenated, modified Kreb’s-Ringer solution. No more than two rings from any one vessel were used for any one experiment.

As previously described [8,9] 3–4-mm rings of tissue were dissected and were transferred to organ chambers with 25 ml of Kreb’s-Ringer solution (37°C; pH 7.4) and oxygenated with 94% O₂ and 6% CO₂. The tissue rings were suspended between two stirrups and connected to a strain gauge for continuous recording of isometric tension. After equilibration for 1 h at a resting tension, all vessels were examined for viability by a contractile response to 20 mmol/l KCl at baseline, and at 2, 4 and 6 g, each time after the KCl had been washed out. The vessels were washed with fresh Kreb’s solution, and incubated for an additional 20 min. Then all vessels which had the endothelium mechanically denuded were contracted with 10⁻⁵ mol/l norepinephrine, followed by relaxation with substance P (10⁻⁶ M; Sigma, St. Louis, MO), an endothelium-dependent vasodilator to determine the functional integrity of the vascular endothelium.

After an equilibrium and wash-out period of 30 min, the agents detailed in the specific protocols below were added. All concentrations are expressed as the concentration within the bath solution. After all study drugs were added, 10⁻³ mol/l papaverine was added to obtain maximal vessel relaxation.

2.2. Comparison of the vasodilator effects of DNP with other natriuretic peptides

To determine the arterial and venous vasodilator effects of the natriuretic peptides, both human IMAs and SVs were contracted with 10⁻⁷ mol/l endothelin-1 (Phoenix Pharmaceuticals, Mountain View, CA). After equilibration for 20 min, arteries were relaxed with cumulative concentrations (10⁻¹⁰–10⁻⁷ mol/l) of either α-ANP 1–28, BNP-32, CNP-22, or DNP (Phoenix Pharmaceuticals).

2.3. Potassium and chloride channels

To determine whether potassium channels mediate the vasorelaxing effects to DNP, additional arteries and veins with intact endothelium were exposed to 20 mmol/l KCl for 20 min before exposure to endothelin-1, followed by cumulative concentrations of DNP. To identify the potassium channel altering the vasorelaxing effect to DNP, additional vessel rings were exposed to the following potassium channel inhibitors for 20 min before contraction with endothelin-1 and relaxation with DNP: 10⁻⁶ mol/l glibenclamide (Research Biochemicals International, Natick, MA) (inhibits ATP-sensitive potassium channels), 10⁻⁵ mol/l charybdotoxin (Sigma) (inhibits large-conductance, calcium-activated potassium channels), 10⁻⁷ mol/l apamin (Sigma) (blocks low-conductance, calcium-activated potassium channels), 10⁻⁴ mol/l tetraethyl-ammonium chloride (Sigma) (blocks large conductance calcium-activated potassium channels), and 10⁻³ mol/l barium chloride (Sigma) (blocks inward rectifier potassium channels).

To ensure that the effects of KCl on DNP mediated vasorelaxation were mediated via potassium channels rather than through chloride channels, additional vessel rings were exposed to 10⁻⁷ mol/l 4,4’-disothiocyanato-stilbene-2,2’-disulfonic acid, a non-selective chloride channel blocker, for 20 min before contraction with endothelin-1 and relaxation with cumulative concentrations of DNP.

2.4. Determination of the role of the endothelium and nitric oxide in the vasorelaxation effects to DNP

To determine the role of the endothelium in the vasodilator effects to DNP, the endothelium was mechanically removed before precontraction with endothelin-1 and relaxation with DNP.

To determine the effect of the nitric oxide pathway, 10⁻⁴ mol/l of L-NAME monomethyl-arginine (L-NMMA) (Sigma), a nitric oxide synthase inhibitor was added 20 min before the addition of endothelin-1 to vessel rings, followed by cumulative concentrations of DNP.

2.5. Cyclic 3’,5’-guanosine monophosphate (cGMP)

To determine the role of cGMP on DNP mediated vasorelaxation, additional vessels with the endothelium intact were exposed to 10⁻³ mol/l 1H-[1,2,4]oxadiazolo(4,3-α) quinoxaline-1-one (ODQ; Biomol Research Laboratories, Plymouth Meeting, PA), an inhibitor of soluble guanylate cyclase, 20 min before contraction with
endothelin-1, with subsequent relaxation using DNP. Additionally, DNP mediated stimulation of cGMP production was measured by radioimmunoassay.

2.6. Radioimmunoassay for cyclic 3',5'-guanosine monophosphate (cGMP)

As previously described [10], vascular rings were isolated and transferred to minimal essential medium with 1% fetal calf serum. The tissue was incubated for 1–2 h at 37 °C (5% CO₂). Then, indomethacin (1.1×10⁻⁴ mol/l, Sigma) and 3-isobutyl-1-methylxanthine (1.3×10⁻³ mol/l, Sigma) were added to the medium and the tissue was incubated for an additional 30 min. Each study drug (1.4×10⁻⁶ mol/l DNP and 1.4×10⁻⁶ mol/l ANP) was added to the tissue for 8 min and then the tissue was removed from the medium and frozen in liquid nitrogen. The tissue was homogenized and a cGMP radioimmunoassay kit (Amersham) was used to determine the cGMP level [11].

2.7. Prostaglandins

In additional experiments, the role of prostaglandins in mediating the DNP vasodilatation was elucidated by the exposure of vessels to 10⁻⁵ mol/l indomethacin for 20 min prior to precontraction with endothelin-1 and vasorelaxation to cumulative concentrations of DNP.

2.8. DNP immunostaining

Paraffin sections of human IMA and SV were cut in 5-μm sections and mounted on positively charged slides from five patients. The slides were deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked by placing the slides in 1.5% hydrogen peroxide and 50% absolute methanol and then rinsing. To enhance the immunostaining signal, the slides were incubated in 0.125% trypsin (37°C). To block non-specific binding sites, the tissue was incubated with 10% horse serum (Vector, Burlingame, CA)/phosphate buffered saline/Tween 20 for 10 min. Polyclonal rabbit antibody to IMA and SVs, with a greater effect to DNP on the IMA and SV (10 mol/l, endothelial cells) to determine the specific cell types which had DNP immunostaining [12].

Serial paraffin sections of each vessel were stained using monoclonal antibodies for alpha-actin (a marker for myointimal cells) and von Willebrand factor (a marker for endothelial cells) to determine the specific cell types which had DNP immunostaining [12].

2.9. Data analysis

Data are expressed as mean±S.E.M. The contraction obtained with endothelin-1 for each vessel was considered as baseline (0% relaxation) [8,9]. Subsequent measurements of IMA relaxation are expressed as a percent reduction in contraction, with the maximal relaxation attained with papaverine being 100% relaxation. For statistical analysis, the comparison of relaxation curves between experiments was achieved with a two-way ANOVA. An unpaired t-test (two-tailed) was used for comparison between groups for different concentration points of the relaxation curves when the ANOVA demonstrated a difference between the relaxation curves and for cGMP values. EC₅₀ values were calculated using Prism statistical software. Statistical significance was accepted with a P-value<0.05.

3. Results

3.1. Vasorelaxation effects to DNP on human internal mammary artery and saphenous vein compared with other natriuretic peptides

DNP (10⁻¹⁰–10⁻⁷ mol/l) significantly relaxed human IMAs and SVs, with a greater effect to DNP on the IMAs (P<0.001) (Table 1 and Fig. 1A). In the IMAs, DNP caused equal vasorelaxation compared with ANP (P=0.35) and BNP (P=0.22) (Table 1). However, DNP caused significantly greater vasorelaxation compared with CNP

<table>
<thead>
<tr>
<th>Natriuretic peptide (10⁻¹⁰ mol/l)</th>
<th>% Arterial relaxation</th>
<th>Artery log EC₅₀</th>
<th>% Venous relaxation</th>
<th>Vein log EC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrial natriuretic peptide (ANP)</td>
<td>69.5±5.6*</td>
<td>−7.9±0.4*</td>
<td>19.3±2.6*</td>
<td>−7.1±1.0</td>
</tr>
<tr>
<td>Brain natriuretic peptide (BNP)</td>
<td>65.6±7.6</td>
<td>−9.8±1.4*</td>
<td>22.8±2.9*</td>
<td>−7.3±1.0</td>
</tr>
<tr>
<td>C-Type natriuretic peptide (CNP)</td>
<td>48.9±6.8*</td>
<td>−8.5±1.3</td>
<td>15.2±3.4*</td>
<td>−5.7±1.1</td>
</tr>
<tr>
<td>Dendroaspis natriuretic peptide (DNP)</td>
<td>80.6±4.1</td>
<td>−6.1±0.6</td>
<td>33.4±4.1</td>
<td>−5.7±1.1</td>
</tr>
</tbody>
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*P<0.05 compared with DNP.
(P=0.02). In the SVs, DNP caused the greatest vasorelaxation of all of the natriuretic peptides (Table 1 and Fig. 1B).

3.2. Role of potassium and chloride channels in DNP mediated vasorelaxation

Potassium chloride significantly attenuated the dose-response to DNP mediated vasorelaxation in the IMAs (no KCl added (n=13 rings), relaxation from $10^{-7}$ mol/l DNP: 80.6±4.1%; 20 mmol/l KCl and relaxation from $10^{-7}$ mol/l DNP (n=6 rings): 23.4±7.0%, P<0.001) and SVs (no KCl added (n=14 rings), relaxation with $10^{-7}$ mol/l DNP: 33.4±4.1%; 20 mmol/l KCl and relaxation from $10^{-7}$ mol/l DNP (n=4 rings): 4.4±5.4%, P=0.004).

DNP mediated relaxation of the IMAs was not attenuated by glibenclamide (n=9 rings, relaxation from
10⁻⁷ mol/l DNP: 77.9±3.8%), charybdotoxin (n=9 rings, relaxation from 10⁻⁷ mol/l DNP: 64.8±7.6%), apamin (n=8 rings, relaxation from 10⁻⁷ mol/l DNP: 68.0±8.2%), or 10⁻⁴ mol/l tetraethyl-ammonium chloride (n=7 rings, relaxation from 10⁻⁷ mol/l DNP: 74.4±4.3%). Similarly in the SVs, DNP mediated relaxation was not attenuated by glibenclamide (n=8 rings, relaxation with 10⁻⁷ mol/l DNP: 26.7±6.8%), charybdotoxin (n=10 rings, relaxation with 10⁻⁷ mol/l DNP: 32.1±8.1%), apamin (n=9 rings, relaxation with 10⁻⁷ mol/l DNP: 32.6±8.0%) or 10⁻² mol/l tetraethyl-ammonium chloride (n=8 rings, relaxation with 10⁻⁷ mol/l DNP: 27.7±2.8%). However, relaxation of the IMA segments was significantly attenuated by the inward rectifier potassium channels inhibitor, barium chloride (n=11 rings, relaxation from 10⁻⁷ mol/l DNP: 40.1±4.9%, P<0.001) as were the SV segments (n=10 rings, relaxation from 10⁻⁷ mol/l DNP: 5.9±3.5%, P<0.001) (Fig. 2).

4,4'-Diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS) did not inhibit DNP mediated IMA vasorelaxation (DIDS: n=8 rings, relaxation from 10⁻⁷ mol/l DNP: 77.3±7.9%) or SV vasorelaxation (DIDS: n=8 rings, relaxation from 10⁻⁷ mol/l DNP: 26.2±8.2%). Thus, inhibition of DNP mediated vasorelaxation by potassium chloride was not mediated via chloride channels.

3.3. Role of the endothelium and nitric oxide in DNP mediated vasorelaxation

DNP mediated vasorelaxation was mildly but significantly attenuated with the removal of the endothelium in both IMAs (P<0.001 by ANOVA) (relaxation from 10⁻⁷ mol/l DNP with endothelium: 80.6±4.1%; without the endothelium: 75.0±5.3%, P=0.17) and in SVs (relaxation from 10⁻⁷ mol/l DNP with endothelium: 33.4±4.1%; without the endothelium: 23.9±6.3%, P<0.001) (Fig. 3).

L-NMMA, an inhibitor of nitric oxide synthase, did not attenuate DNP mediated relaxation in IMAs (n=16 rings) (relaxation from 10⁻⁷ mol/l DNP without L-NMMA: 78.1±4.4% vs. without L-NMMA: 80.6±4.1%, P=0.72). Similarly in SVs, L-NMMA did not attenuate DNP mediated relaxation (n=13 rings, relaxation from 10⁻⁷ mol/l DNP with L-NMMA: 25.3±4.7%, P=0.31).

3.4. Role of cGMP in DNP mediated vasorelaxation

Addition of DNP to rings from SVs increased cGMP ~7-fold over control vessels without the addition of any cGMP stimulating agents (control: 1.7±0.3 pmol/mg protein; DNP: 13.0±2.0 pmol/mg protein, P<0.001) (Fig. 4). ANP also significantly increased cGMP (5.6±0.8 pmol/mg protein, P<0.001), but this increase was only 3-fold over control vessels, which was significantly less than the increase with DNP (P=0.003). In the rings from the IMAs, DNP significantly increased cGMP compared with control rings (P<0.004), but there was no difference in the response to DNP compared with ANP (P=0.5) (control: 2.5±0.4 pmol/mg protein; DNP: 47.1±4.0 pmol/mg protein; ANP: 47.3±16.4 pmol/mg protein).

ODQ, a specific inhibitor of soluble guanylate cyclase did not significantly attenuate the relaxation of the IMAs (n=10 rings) to DNP (relaxation from 10⁻⁷ mol/l with ODQ: 76.0±3.9% vs. relaxation without ODQ: 80.6±4.1%, P=0.7). ODQ also did not attenuate relaxation to DNP in the SVs (n=8 rings, relaxation with 10⁻⁷ mol/l DNP: 34.9±6.2, P=0.67).

Fig. 2. The role of potassium channels in DNP mediated vasorelaxation in IMAs. Potassium chloride significantly attenuated DNP mediated vasorelaxation in the IMAs. Additionally, barium chloride inhibited DNP mediated vasorelaxation, thus demonstrating that the inward rectifying potassium channels mediate this relaxation.
Fig. 3. The role of the endothelium in DNP mediated vasorelaxation in human internal mammary arteries and saphenous veins.

3.5. Role of prostaglandins in DNP mediated vasorelaxation

Indomethacin mildly, but significantly inhibited vasorelaxation to DNP in IMAs (n=12 rings) (relaxation from $10^{-7}$ mol/l DNP with indomethacin: 59.6±8.8% vs. without indomethacin: 80.6±4.1%, $P<0.001$) and in SVs (n=13 rings) (relaxation from $10^{-7}$ mol/l DNP with indomethacin: 12.0±6.9% vs. without indomethacin: 33.4±4.1%, $P=0.02$) (Fig. 5).

Fig. 4. Tissue cGMP levels in human saphenous veins after stimulation with ANP or DNP.
3.6. Immunostaining to DNP in human internal mammary arteries and saphenous veins

DNP immunoreactivity was present primarily in the endothelium and subendothelial region of both the human IMAs and SVs (Fig. 6). In serial sections, these cells stained for both von Willebrand factor and for alpha-actin.

4. Discussion

The results of the present studies demonstrate that DNP relaxes human vessels in vitro. The mechanism for DNP mediated vasorelaxation involves the delayed rectifying potassium channels and prostaglandins, and utilizes cGMP as a second messenger. Additionally, DNP immunoreactivity is present in both human IMAs and SVs. Thus, this study supports a potential for DNP to have a physiologic role as a vasorelaxing peptide in human vessels.

The natriuretic peptide family plays a role in regulating vascular tone and growth. These peptides are particularly important in pathophysiological states such as congestive heart failure, hypertension and atherosclerosis. In these conditions, the natriuretic peptides may alter vascular and cardiac remodeling and modulate vascular tone [13,14]. DNP is the newest member of the natriuretic peptide family and has vasorelaxing effects in isolated rat and canine arterial vessels [4,5]. The current study extends these studies to demonstrate that DNP relaxes isolated human arteries and veins.

In human IMAs, DNP caused vasorelaxation similar to ANP and BNP, but greater relaxation than CNP. In human SVs, the greatest vasorelaxation of all of the natriuretic peptides was seen with DNP. The lowest concentration of DNP utilized in these studies is similar to concentrations seen in the human circulation and similar to the concentration of the other natriuretic peptides in normal and pathophysiologic states [6,15,16]. The higher concentrations of DNP used in the current studies may represent concentrations seen at the cellular level when DNP is functioning in a paracrine fashion. These studies suggest DNP may have a physiologic role in arterial and venous vasodilatation in humans.

Our current study demonstrates that DNP-immunostaining is present in both human arteries and veins. This extends the previous study that demonstrates the presence of DNP in human plasma by showing that DNP is endogenous to the human vasculature [6]. DNP immunostaining localized to both the endothelial cells and smooth muscle cells in both human arteries and veins. Therefore, DNP has the potential to act as vascular-derived autocrine and paracrine peptide in humans.

Although a prior study suggested that DNP acts via the NPR-A receptor and increases cGMP in bovine aortic endothelial cells, little is known regarding how DNP mediates vasorelaxation [4]. The current study extends this previous observation and demonstrates that DNP increases cGMP in human vessels. Furthermore, we showed that this increase in cGMP is mediated through particulate and not soluble guanylate cyclase, as ODQ, a specific inhibitor of
Fig. 6. Immunostaining of DNP in human internal mammary arteries and saphenous veins (100×). DNP immunoreactivity was present primarily in the endothelium and subendothelial cell region of both the human internal mammary arteries (in panel A) and saphenous veins (in panel D) in all vessels stained. These cells stained for both alpha-actin (in panel B in the internal mammary artery and panel E for the saphenous vein) and for von Willebrand factor (in panel C in the internal mammary artery and panel F for the saphenous vein).

soluble guanylate cyclase failed to attenuate DNP mediated vasorelaxation. This is similar to ANP and BNP, which also modulate their activity via the NPRA, and thereby activates particulate guanylate cyclase [1]. Additionally, DNP was a more potent simulator of cGMP production in human veins than ANP.

Cyclic GMP can activate potassium channels by cGMP-dependent dephosphorylation [17]. ANP, BNP and CNP all mediate some of their effects through potassium channel activation [18–24]. The current study demonstrates for the first time that DNP also mediates vascular relaxation through the potassium channels and DNP uniquely affects inward rectifying potassium channels. In the vasculature, these channels are important in the maintenance of resting potential of the cells. Inward rectifying potassium channels are also altered by shear stress and regulate vascular reactivity in blood vessels due to passive wall tension [25,26]. Thus, DNP may function as a novel hyperpolarizing factor, via potassium channel activation.

We demonstrated that DNP mediated vasorelaxation was
attenuated by indomethacin, which supports the role of prostaglandins in DNP mediated vasorelaxation. Prostaglandins such as prostacyclin are produced in the vascular endothelium and are important in the regulation of vascular tone. These vasoactive substances not only exert their own effect on vascular tone, but also act synergistically with other vasoactive substances such as ANP [27]. In addition to the interactions of ANP and prostaglandins, CNP-induced vasodilatation is mediated in part through the prostaglandin pathway, as demonstrated by the inhibitory effects of indomethacin on CNP-mediated vasodilatation [28]. Moreover, our current study supports the role of the prostaglandin pathway in modulating DNP mediated vasorelaxation of human vessels.

In summary, the current study demonstrates that DNP, a new member of the natriuretic peptide family mediates vasorelaxation in human vessels. The vasodilatation is mediated via the inward rectifying potassium channels and prostaglandin production and involves the generation of cGMP. Moreover, DNP immunoreactivity is present in the vascular wall of human vessels. This study supports the role for DNP as an endogenous peptide in humans, which may have a physiologic role in vascular tone.

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