C-Type Natriuretic Peptide–Induced Vasodilation Is Dependent On Hyperpolarization in Human Forearm Resistance Vessels

Marina L.H. Honing, Paul Smits, Paul J. Morrison, John C. Burnett, Jr, Ton J. Rabelink

Abstract—Animal studies have demonstrated that CNP causes endothelium-independent vasodilation, which is limited by neutral endopeptidase (NEP) activity. However, the vasodilating mechanism of CNP in humans is still unknown. Therefore, we investigated the vasodilator actions of CNP in human forearm resistance vessels before and after inhibition of nitric oxide (NO) and then prostacyclin production and after inhibition of Ca\(^{2+}\)-dependent potassium channel activation and NEP activity. Three separate studies were performed. In each study, forearm blood flow was recorded by venous occlusion plethysmography in 8 healthy nonsmoking subjects. Brachial artery infusion of CNP (70, 140, 280, and 560 ng per 100 mL forearm volume per minute) caused significant forearm vasodilation in all studies (forearm blood flow from 3.94 to 8.50 mL per 100 mL forearm volume per minute). Inhibition of the endogenous generation of NO by L-NAME-monomethyl arginine (by use of the NO-clamp technique) did not block the maximal vasodilating effects of CNP (forearm blood flow from 3.69 to 6.93). In addition, when the cyclooxygenase system was inhibited by 600 mg of acetylsalicylic acid (aspirin) administered orally 30 minutes before start of measurements, the rise in forearm blood flow remained intact (forearm blood flow from 3.31 to 8.27 mL per 100 mL forearm volume per minute). However, inhibition of Ca\(^{2+}\)-dependent potassium channels with tetraethylammonium chloride (0.1 mg per 100 mL forearm volume per minute) significantly attenuated vasodilation caused by CNP (forearm blood flow from 2.28 to 3.06 mL per 100 mL forearm volume per minute), which suggests that CNP opens vascular potassium channels. Vasodilation to all doses of CNP was significantly increased when activity of NEP was blocked with thiorphan (30 nmol/min), which suggests that NEP activity limits vasodilation of CNP. CNP is a dilator of human resistance vessels that mediates its effects through hyperpolarization of the vessel wall independent of the NO and prostaglandin system. Inhibition of local NEP activity increases CNP bioavailability. This may be of relevance to cardiovascular disease, given that vascular tone is well balanced between NO and an endothelium-derived hyperpolarizing factor, which suggests that in pathological situations, impaired NO activity can be compensated for by enhanced endothelium-derived hyperpolarizing factor release to maintain vascular homeostasis. (Hypertension. 2001;37:1179-1183.)

Key Words: natriuretic peptides ■ hyperpolarizing factor ■ endopeptidase ■ tetraethylammonium chloride ■ nitric oxide

Endothelial cells are known to produce various vasoactive compounds to maintain the balance of vascular tone. Under normal physiological conditions, potentially harmful vasoconstrictive and mitogenic peptides are counteracted by vasodilating and antiproliferative agents such as nitric oxide (NO), prostaglandins, and CNP. Much knowledge has become available about the vasorelaxing properties and bioavailability of NO and prostaglandins in humans. However, the vasodilating mechanism and metabolism of CNP in humans is still under investigation. CNP is an endothelium-derived peptide that acts as a paracrine factor, causing local vasodilation and preventing smooth muscle cell proliferation.\(^1\)\(^-\)\(^3\) In vitro studies have suggested that vasorelaxation induced by CNP is independent of NO.\(^4\) However, in humans, this interaction has not yet been investigated. Therefore, the first part of our present study investigates the role of NO and prostaglandins in CNP-mediated vasodilation in human forearm resistance vessels.

CNP is present in endothelial cells of arteries and veins as an peptide with 53 amino acids.\(^5\) After stimulation of endothelial cells by, for instance, bradykinin, CNP is secreted, after which it can react with its specific guanylate cyclase receptor (natriuretic peptide receptor [NPR]-B) on the vascular smooth muscle cell.\(^6\)\(^-\)\(^7\) NPR-B stimulation increases...
in intracellular cGMP, with subsequent stimulation of potassium efflux and inhibition of calcium influx, resulting in hyperpolarization of the smooth muscle cell membrane. In vitro, we were able to inhibit CNP-mediated relaxation by blocking Ca\(^{2+}\)-dependent potassium (K\(_{Ca}\)) and ATP-dependent potassium channels.\(^7\)\(^,\)\(^8\) The second part of the present study investigates the hypothesis that CNP causes vasodilation through hyperpolarization of the vascular smooth muscle cell in human forearm resistance vessels.

The amount of vasodilation caused by CNP is dependent on the balance of peptide production and breakdown. Organ chamber experiments have demonstrated that the maximal relaxation in response to CNP is attenuated by the presence of endothelium.\(^8\) A possible explanation is the presence of neutral endopeptidase (NEP) on the membrane of the endothelium. Studies have demonstrated that CNP and all other members of the natriuretic peptide family (ANP and BNP) are degraded by NEP.\(^9\)\(^,\)\(^10\) NEP is a plasma membrane–bound zinc metalloproteinase with an integral membrane protein that has its active site facing the extracellular space.\(^11\) In theory, the endothelium may limit the actions of CNP by degrading it directly after it is released from the endothelium. Therefore, the third part of the present study investigates the role of NEP in vasorelaxation induced by CNP, by use of the specific NEP inhibitor thiorphan.

**Methods**

Twenty-four healthy, nonsmoking subjects ranging in age from 18 to 42 years participated in 3 studies. The local ethics committee approved all studies, and written informed consent was obtained from the volunteers before any investigation was started. None of the subjects received vasoactive or nonsteroidal anti-inflammatory drugs from the volunteers before any investigation was started. Alcohol and all caffeine-containing beverages were withheld for 12 hours before study. All studies were performed in a quiet room kept at a constant temperature, between 22°C and 24°C. Each subject was supine, with both forearms resting slightly above heart level.

The brachial artery of the nondominant arm was cannulated with a 20-gauge catheter after local anesthesia of the skin with lidocaine 2% (Astra Pharmaceuticals Ltd). Forearm blood flow was measured simultaneously in both arms by venous occlusion plethysmography with calibrated mercury-in-silastic strain gauges applied to the forearm (Hokanson EC-4).\(^12\) A microcomputer-based R-wave-triggered system for online semicontinuous monitoring was used. During the experiments, upper arm cuffs were intermittently inflated to 40 mm Hg for 4 heartbeats every 15 seconds to prevent venous outflow from the forearm. Wrist cuffs were inflated to 40 mm Hg higher than actual systolic pressure to exclude the hands from the circulation. Forearm blood flow measurements were recorded over a 2-minute period at 5-minute intervals. Intra-arterial blood pressure was continuously monitored. Saline (0.9%, Baxter Healthcare Ltd) was infused for ≥30 minutes, after which forearm blood flow was stabilized before administration of drugs. Drugs and peptides, with the exception of acetylsalicylic acid (aspirin), were dissolved in PSS and infused intra-arterially at locally active doses. The infusion rate was kept constant at 90 mL/h. All solutions were prepared aseptically from sterile stock solutions or ampoules on the day of experiment and stored at 4°C until use. On the day of use, tetraethyiammonium chloride (TEA) was reconstituted from a sterile stock powder, diluted in NaCl 0.9% to a concentration of 1 mg/mL, and passed through a 0.22-μm Millipore filter.

**Study 1: Role of NO and Prostanoids in Vasodilation Caused by CNP**

Venous occlusion plethysmography of the forearm was performed in 8 subjects. CNP 70, 140, 280, and 560 ng per 100 mL forearm volume (FAV) per minute (Clnalfa) and SNP 6, 60, 180, and 600 ng per 100 mL of FAV per minute (Merck) were infused into the brachial artery under 2 conditions: in an intact and in a blocked NO system. The NO system was blocked by infusing L-arginine (L-NMMA) 200 μg per 100 mL of FAV per minute (Institut für Pharmazie, Universität Leipzig), a competitive inhibitor of NO synthase, throughout the experiment. After 10 minutes of L-NMMA infusion, vasocostriction by L-NMMA subsequently was counteracted by concurrent infusion of ascending doses of SNP (30 to 180 ng per 100 mL of FAV per minute) until blood flow had returned to baseline values. L-NMMA and SNP then were coinfused at constant rates for the remainder of the study. In previous studies, we demonstrated that over time, the “NO clamp” was stable and kept baseline forearm blood flow constant during the experiment. To block generation of vasoactive prostaglandins and thromboxanes, aspirin 600 mg (carbasalatum calcium, Dagra Pharma BV) was administered orally 30 minutes before start of the measurements. Previously, 600 mg of aspirin was shown to block cyclooxygenase activity by ≥85%, with recovery occurring during the following 6 hours.\(^13\)

**Study 2: Role of Hyperpolarization in Vasodilation Caused by CNP**

Venous occlusion plethysmography of the forearm was performed in another 8 subjects. The dose-response curves to CNP 70, 140, 280, and 560 ng per 100 mL of FAV per minute were measured alone and after inhibition of large-conductance K\(_{Ca}\) channels with TEA 0.1 mg per 100 mL of FAV per minute (Sigma Chemical Co). Because charbyotoxin and iberiotoxin, the 2 most selective blockers of the calcium-dependent potassium channels are too toxic for human application, we chose TEA to investigate the role of K\(_{Ca}\) channel activation in the vascular effects of CNP. TEA antagonizes different types of potassium channels with varying degrees of potency. However, TEA has been shown to selectively block single K\(_{Ca}\) channels in arterial smooth muscle cells at concentrations <1 mmol/L.\(^14\) We administered TEA intra-arterially at an infusion rate of 0.1 mg per 100 mL of FAV per minute, which correlates with a calculated local plasma concentration of 0.5 mmol/L.\(^15\)

Before CNP infusion, TEA was infused for 30 minutes into the brachial artery to investigate whether inhibition of hyperpolarization with TEA influenced basal forearm blood flow. Cumulative doses of CNP then were coinfused with TEA.

**Study 3: Effects of NEP Inhibition on Vasodilation Caused by CNP**

Venous occlusion plethysmography of the forearm was performed in 8 subjects. Dose-response curves to CNP 70, 140, 280, and 560 ng per 100 mL of FAV per minute and SNP 6, 60, 180, and 600 ng per 100 mL of FAV per minute were measured alone or with coinfusion of thiorphan 30 nmol/min (Clnalfa).\(^16\) Before dose-response curves were obtained, thiorphan was infused for 30 minutes into the brachial artery to achieve maximal inhibition of local neutral endopeptidase in the forearm. After steady-state forearm blood flow was reached, each infusion block of CNP and SNP was repeated during coinfusion of thiorphan.

**Analysis**

Forearm blood flow is expressed as milliliters per 100 mL of FAV per minute. The final 6 blood flow recordings for each infusion step from both measurement and control arm were used to calculate mean forearm blood flow. Recordings made in the first 30 seconds after wrist-cuff inflation were not used for analysis, because initial forearm blood flow values are not representative as a result of redistribution of blood caused by wrist-cuff inflation. Results are expressed as mean±SEM. Statistical analysis was performed by use of 2-way ANOVA for repeated measures, with CNP infusion and the different inhibitors as independent variables. Statistical significance was taken at the 5% level (P<0.05).
CNP plasma concentration levels were calculated using the following formula 17:

\[ C_{\text{plasma}} = \frac{\text{IR}}{(1 - \text{Ht}) \times \text{FBF} \times V}, \]

where \( C_{\text{plasma}} \) is plasma concentration (milligrams per milliliter); IR, infusion rate (milligrams per minute); Ht, hematocrit; FBF, forearm blood flow (milliliters per 100 mL per minute); and V, FAV (milliliters, minus hand volume).

Results

To allow comparison of data obtained in studies 1 and 2 and to evaluate the effect of cyclooxygenase inhibition, clinical characteristics and laboratory data of the healthy, nonsmoking male volunteers were matched (Table). In all experiments, mean arterial pressure (MAP) and heart rate did not change significantly during intra-arterial infusion of different compounds. Calculated local plasma CNP concentrations were 0.03 mg/mL for CNP 70 ng per 100 mL of FAV per minute, 0.05 mg/mL for CNP 140 ng per 100 mL of FAV per minute, 0.07 mg/mL for CNP 240 ng per 100 mL of FAV per minute), and 0.1 mg/mL for 560 ng per 100 mL of FAV per minute).

Study 1

Effects of Cyclooxygenase Inhibition on Vasodilation Caused by CNP

Vasodilation induced by CNP was independent of the production of vasoactive prostanoids (Figure 1). In an intact cyclooxygenase system (study 2), cumulative doses of CNP increased forearm blood flow from 3.94 (0.72) to 8.50 (1.27) mL per 100 mL of FAV per minute. In an inhibited cyclooxygenase system that used 600 mg of aspirin (study 1), the rise in forearm blood flow was similar; ie, from 3.31 (0.62) to 8.27 (1.18) mL per 100 mL of FAV per minute.

Study 2

Effects of Potassium Channel Inhibition on Vasodilation Caused by CNP

Baseline forearm blood flow was not significantly affected after 30 minutes infusion of TEA (baseline values from 3.59 [0.53] to 3.78 [0.63] mL per 100 mL of FAV per minute). Vasodilation at all dosages of CNP was significantly increased when thiorphan was coinfused (Figure 3; CNP 560 ng, 8.50 (1.27) to 14.48 (1.58) mL per 100 mL of FAV per minute; \( P < 0.05 \)). Endothelium-independent vasodilation was significantly increased when thiorphan was coinflused (Figure 4; CNP 560 ng, 8.50 (1.27) to 14.48 (1.58) mL per 100 mL of FAV per minute; \( P < 0.05 \)). Endothelium-independent vasodilation

Study 3

Effects of NEP Inhibition on Vasodilation Caused by CNP

Baseline forearm blood flow was not significantly affected after 30 minutes’ infusion of thiorphan (baseline values from 3.59 [0.53] to 3.78 [0.63] mL per 100 mL of FAV per minute). Vasodilation at all dosages of CNP was significantly increased when thiorphan was coinflused (Figure 4; CNP 560 ng, 8.50 (1.27) to 14.48 (1.58) mL per 100 mL of FAV per minute; \( P < 0.05 \)). Endothelium-independent vasodilation

Subject Characteristics of Study 1 and 2

<table>
<thead>
<tr>
<th>Variables</th>
<th>Study 1 (n=8)</th>
<th>Study 2 (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>26.7 ± 7</td>
<td>27 ± 6</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22.5 ± 2.6</td>
<td>22.7 ± 3.1</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>85.5 ± 15.5</td>
<td>91.9 ± 14.9</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.6 ± 1.2</td>
<td>4.4 ± 1.6</td>
</tr>
</tbody>
</table>

Figure 1. Forearm blood flow was measured in 8 subjects. Cumulative doses of CNP were infused into the brachial artery before and after blocking generation of vasoactive prostaglandins and thromboxanes with carbasalatum calcium (Ascal) 600 mg. Results demonstrate that vasodilator actions of CNP are independent of an active cyclooxygenase system.

Figure 2. Forearm blood flow was measured in 8 subjects. Cumulative doses of CNP were infused into the brachial artery during coinfusion of saline and inhibition of NO generation (NO clamp). Results demonstrate that vasodilator actions of CNP are independent of an active NO system.

Effects of NO Bioavailability on Vasodilation Caused by CNP

Inhibition of NO synthase with L-NMMA for 10 minutes caused a significant vasoconstriction, which reduced blood flow from 3.93 (0.53) to 2.09 (0.30) mL per 100 mL of FAV per minute (\( P < 0.05 \)), which was counteracted with incremental dosages of SNP until baseline forearm blood flow was restored (without NO clamp, 3.93 [0.53], and with NO clamp, 3.69 [0.46] mL per 100 mL of FAV per minute). Baseline forearm blood flow was kept constant for \( \geq 20 \) minutes, until infusion of CNP was started. Inhibition of NO synthase did not influence vasodilation induced by CNP (Figure 2); ie, forearm blood flow increased from 3.69 (0.46) to 6.93 (0.93) mL per 100 mL of FAV per minute.

Study 3

Effects of NEP Inhibition on Vasodilation Caused by CNP

Baseline forearm blood flow was not significantly affected after 30 minutes' infusion of thiorphan (baseline values from 3.59 [0.53] to 3.78 [0.63] mL per 100 mL of FAV per minute). Vasodilation at all dosages of CNP was significantly increased when thiorphan was coinfused (Figure 3; CNP 560 ng, 8.50 (1.27) to 14.48 (1.58) mL per 100 mL of FAV per minute; \( P < 0.05 \)). Endothelium-independent vasodilation was significantly increased when thiorphan was coinflused (Figure 4; CNP 560 ng, 8.50 (1.27) to 14.48 (1.58) mL per 100 mL of FAV per minute; \( P < 0.05 \)). Endothelium-independent vasodilation

Figure 2. Forearm blood flow was measured in 8 subjects. Cumulative doses of CNP were infused into the brachial artery during coinfusion of saline and inhibition of NO generation (NO clamp). Results demonstrate that vasodilator actions of CNP are independent of an active NO system.

Effects of NO Bioavailability on Vasodilation Caused by CNP

Inhibition of NO synthase with L-NMMA for 10 minutes caused a significant vasoconstriction, which reduced blood flow from 3.93 (0.53) to 2.09 (0.30) mL per 100 mL of FAV per minute (\( P < 0.05 \)), which was counteracted with incremental dosages of SNP until baseline forearm blood flow was restored (without NO clamp, 3.93 [0.53], and with NO clamp, 3.69 [0.46] mL per 100 mL of FAV per minute). Baseline forearm blood flow was kept constant for \( \geq 20 \) minutes, until infusion of CNP was started. Inhibition of NO synthase did not influence vasodilation induced by CNP (Figure 2); ie, forearm blood flow increased from 3.69 (0.46) to 6.93 (0.93) mL per 100 mL of FAV per minute.

Study 2

Effects of Potassium Channel Inhibition on Vasodilation Caused by CNP

Baseline forearm blood flow was not significantly affected after 30 minutes infusion of TEA (baseline values, 2.58 (0.32) to 2.29 (0.21) mL per 100 mL of FAV per minute). Vasodilation caused by infusion of CNP was significantly attenuated when the dose-response curve was repeated in the presence of TEA (Figure 3; CNP 560 ng from 2.40 (0.22) to 5.88 (0.67) mL per 100 mL of FAV per minute and CNP 560 ng in the presence of TEA from 2.28 (0.24) to 3.06 (0.33) mL per 100 mL of FAV per minute), which suggests that the mechanism of action of CNP is mediated by opening of vascular potassium channels. Vasodilation induced by SNP was not inhibited by TEA (P.S., unpublished data, 1998), which indicates that TEA has no inhibitory effect on endothelium-independent vasodilation.
measured by infusion of SNP was not affected by NEP inhibition. Infusion of SNP alone increased forearm blood flow from 3.55 (0.65) to 15.70 (2.19) mL per 100 mL of FAV per minute, and coinfusion of SNP with thiorphan increased forearm blood flow from 4.03 (0.61) to 16.14 (1.66) mL per 100 mL of FAV per minute.

Discussion

We demonstrated that the endothelium-derived relaxing factor CNP caused a significant vasodilation in resistance vessels of the human forearm. Organ-chamber experiments that used arteries and veins of different tissues and species had already demonstrated that CNP is an arterial and venous dilator.2,4,8 In addition, Clavell et al18 demonstrated that systemic infusion of CNP in anesthetized dogs decreased total peripheral resistance and cardiac output, which indicates that peripheral arterial and venous dilation could account for the hemodynamic changes. However, these results could not be confirmed by others. Charles et al19 performed the same experiments in conscious sheep and found only decreased cardiac output with increased natriuresis but no influence on blood pressure. In addition, Barletta et al20 systemically infused low-dose CNP in humans and found no cardiovascular or renal effects.

CNP is produced in endothelial cells and exerts its effects in a paracrine fashion.1,2 In addition, activity of CNP may be limited because of rapid degradation by NEP present on the membranes of endothelial and vascular smooth muscle cells.10 We were able to demonstrate that in human forearm, actions of CNP are limited by direct degradation by NEP. Therefore, actions of NEP could be a rate-limiting factor for vasodilator actions of CNP in human circulation. Thus, because of low abluminal concentrations or rapid degradation by NEP, cardiovascular effects of low-dose CNP infusion may be limited.21 In contrast, we infused high-dose CNP into the brachial artery, which created a condition with high luminal and abluminal CNP concentrations. This condition may explain the more-pronounced hemodynamic effects in the present study. However, one should consider that infusion of high-dose CNP could increase local plasma concentrations of ANP through competitive displacement of ANP from natriuretic peptide clearance receptors on the vascular smooth muscle cells. Therefore, in theory, vasodilator effects of CNP in part could be due to increased local ANP plasma levels. The real physiological relevance of CNP probably can be determined only by use of physiological stimuli. Preliminary data from our laboratory suggest that in certain vascular beds, bradykinin could be such a stimulator.6

The present study also demonstrates for the first time that in human resistance vessels, vasorelaxation caused by CNP is independent of NO and prostaglandins. These results are in agreement with in vitro investigations that used arterial rings for organ-chamber experiments.4,8 Those studies demonstrate that CNP causes vasodilation through hyperpolarization of vascular smooth muscle cells. The evoked hyperpolarization could be inhibited with large-conductance KCa channel inhibitors and ATP-dependent potassium channel inhibitors. In agreement with these in vitro studies, we also show that vasodilation caused by CNP could be inhibited by coinfusion of TEA, an inhibitor of large-conductance KCa channels, which indicates that in the human forearm, CNP acts as a hyperpolarizing agent.

Thus, in human resistance vessels, CNP is a dilator that mediates its effects through hyperpolarization of the vessel wall independent of the NO and prostaglandin system. Given that we demonstrated that CNP is an important hyperpolarizing agent through its activation of KCa channels and that infusion of TEA as well as thiorphan had no effect on baseline flow, CNP is unlikely to be an important regulator of basal tone in healthy humans. However, 2 in vitro studies demonstrated that physiological vasodilator stimuli such as shear stress stimulate release of CNP from endothelial cells.22,23 At this time, the clinical relevance of these findings is under investigation. In contrast, CNP may well play a role in disease conditions associated with impaired NO activity, given that enhanced endothelium-derived hyperpolarizing factor release has been demonstrated in these situations.24 In addition, Nir Amiram et al25 demonstrated that endothelin stimulates the release of CNP, which indicates that in disease conditions with elevated endothelin levels, CNP could act as a feedback mechanism to counterbalance the constrictor actions of endothelin. Also, we recently demonstrated that bradykinin is an important agonist of CNP release,6 which
indicates that some of the beneficial effects of ACE inhibitors therefore also could be attributable to increased CNP production. Because our present study demonstrated that CNP is an endothelium-derived hyperpolarizing factor in humans, further research should be directed toward the role of CNP in vascular function in patients with cardiovascular disease.

References
C-Type Natriuretic Peptide–Induced Vasodilation Is Dependent On Hyperpolarization in Human Forearm Resistance Vessels
Marina L. H. Honing, Paul Smits, Paul J. Morrison, John C. Burnett, Jr and Ton J. Rabelink

Hypertension. 2001;37:1179-1183
doi: 10.1161/01.HYP.37.4.1179

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/37/4/1179

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/