Direct comparison of relaxation and cGMP production in human coronary by-pass grafts in response to stimulation with natriuretic peptides and a nitric oxide donor

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ABSTRACT
In the present study, we investigated the vasodilator properties of A-type, B-type and C-type natriuretic peptides (ANP, BNP and CNP respectively) and the NO (nitric oxide) donor sin-1 (3-morpholino-sydnonimine) in human by-pass grafts. In contrast with previous studies, the same vessel was used to demonstrate a direct link between cGMP production and functional relaxation. Remnants of the IMA (internal mammary artery) and SV (saphenous vein) were obtained from 82 patients undergoing coronary artery by-pass grafting. The responses to cumulative concentrations of ANP, BNP, CNP and sin-1 in vessel rings pre-contracted with a thromboxane A₂ agonist (U46619) were measured in an organ bath. Additionally, intracellular cGMP production after single submaximal dose application of these drugs to vessel rings was determined by a RIA. ANP (P = 0.001) and sin-1 (P < 0.001) caused significant concentration-dependent relaxation of the IMA. In the SV, only sin-1 (P < 0.001) induced marked concentration-dependent relaxation. At a single submaximal concentration, significant relaxation as well as intracellular cGMP production were found in response to ANP, BNP and sin-1 in the IMA. In contrast, in the SV, only sin-1 significantly induced cGMP production and relaxation. There was a moderate, but significant, correlation between intracellular cGMP net production and net relaxation in the IMA. In conclusion, ANP, as the most powerful relaxant of all the natriuretic peptides tested on the IMA, may be a possible alternative vasorelaxant to overcome peri-operative vasospasm in this artery. In contrast with sin-1, ANP and BNP were not effective vasorelaxants of the SV. Net relaxation in response to natriuretic peptides correlated with cGMP net concentrations in the IMA.

INTRODUCTION
The most frequently used coronary artery by-pass grafts are the IMA (internal mammary artery) and SV (saphenous vein). The IMA is considered a better option compared with the SV, showing a superior 10-year patency rate of approx. 90% after CABG (coronary artery by-pass grafting). At this time point, approx. 50% of...
SV grafts are already occluded [1–3]. The SV suffers from different stress factors, such as surgical trauma, hypoxia and hypothermia, during harvesting, which may cause endothelial damage. Additionally, the SV is grafted into the arterial circulation, leading to non-physiological haemodynamic stress with further vascular damage. In the long term, intimal hyperplasia and atherosclerosis may develop [4]. On the other hand, the IMA is more resistant to ischaemic changes due to the high content of elastin with a low metabolic rate; however, the IMA is prone to peri-operative vasospasm, which can lead to graft malfunction and even mortality [5,6].

Therapeutic administration of various NO (nitric oxide) donors, such as nitroglycerine [5,7], to grafts in in vitro experiments to overcome spasm has been shown to be of benefit. However, chronic treatment with nitrates may cause nitrate tolerance and decrease the effectiveness of NO donors [8,9]. Novel NO-donor drugs, such as S-nitrosothiols and S-nitrosated NO amino acids, appear to cause long-lasting (4 h) relaxation without developing vascular tolerance [10]. Also, the metabolite of molsidomine, sin-1 (3-morpholinosydnonimine), is an NO donor that does not show nitrate-tolerance [11].

NPs (natriuretic peptides), which exhibit vasodilating, diuretic and natriuretic properties [12], are potential alternative vasodilators. The biological actions of NPs are mediated by binding to specific transmembrane NP receptors with a subsequent increase in cGMP [12–14]. In previous studies, we [15,16] and others [17,18] have demonstrated that stimulation of human IMAs with NPs caused significant increases in cGMP concentrations. However, studies directly comparing cGMP production with the functional relaxation in the same vessel segment of the IMA or SV in organ baths are still lacking. Therefore the aim of the present study was to investigate both the vasodilator properties and cGMP stimulating capacity of the NPs ANP (A-type NP), BNP (B-type NP) and CNP (C-type natriuretic peptide), and NO in the same vessel ring. For pre-contraction of the frequently used human coronary bypass grafts SV and IMA we used a thromboxane A2 agonist (U46619), because thromboxane A2 is one of the most potent vasoconstrictors released from the endothelium or platelets and elevated plasma levels have been found after CABG.

**METHODS**

**Samples and preparation**
IMA and SV samples were obtained intra-operatively from 82 patients undergoing CABG. The study protocol was approved by the Local Ethical Committee, and the investigation conforms with the principles outlined in the Declaration of Helsinki. All patients gave written informed consent. Left IMAs were dissected in a broad pedicle from the left anterior thoracic wall, and the dissected distal segments were used for experimental studies. SV remnants were harvested under standard surgical procedures, but experimentally used segments did not undergo pressure distension. IMA and SV samples were transported to the laboratory in cold (4°C) modified Krebs–Henseleit solution (143.1 mmol/l Na+, 5.9 mmol/l K+, 2.5 mmol/l Ca2+, 1.2 mmol/l Mg2+, 127.8 mmol/l Cl−, 25 mmol/l HCO3−, 1.2 mmol/l SO42−, 1.2 mmol/l H2PO4− and 11.0 mmol/l glucose, pH 7.4) within 1 h. Adherent tissue and excess fat were removed carefully by the aid of a microscope. Each remnant was cut into two or more rings, and thus each vessel served as its own control in the experiments. Rings were only used for one experiment each. Rings of approx. 3 mm were mounted on to two stainless steel hooks in organ baths (Hugo Sachs Electronics), containing 2 ml of modified Krebs–Henseleit solution at 37°C aerated with 95% O2/5% CO2. Vessel rings were allowed to equilibrate for 90 min under progressive stretching to a resting tension of 20 mN, and isometric tension was measured using a transducer (Type 372; Hugo Sachs Electronics). The organ bath solution was changed every 30 min. Indomethacin (10 μmol/l; Sigma) was administered 30 min prior to pre-contraction to inhibit endogenous prostacyclin synthesis.

**Protocol of U46619, NPs and sin-1**
Cumulative DRCs (dose–response curves) were constructed for the thromboxane A2 analogue U46619 (0.1 nmol/l–3 μmol/l; Sigma) to determine a suitable submaximal concentration corresponding to the EC50–80 (effective concentration of drug producing 50–80% of maximum response) for pre-contraction of the vessels. After pre-contraction of new vessel rings with the submaximal concentration of U46619 (30 nmol/l), DRCs were performed to investigate the relaxation responses to ANP [0.1–300 nmol/l; human α-ANP (1–28); Peninsula], BNP (0.1–300 nmol/l; human BNP-32; Peninsula), CNP [0.1–300 nmol/l; human CNP-(32–53); Bachem] or the NO donor sin-1 (1 nmol/l–100 μmol/l; Sigma). If possible, IMA and SV rings of the same patient were stimulated with the same substances (ANP, BNP, CNP or sin-1) in all experiments. The different substances were applied in a random order to the vessel rings of different patients. All absolute vessel tone values were corrected for the baseline vessel tone values after equilibration, which was subtracted from all of the measured values. Relaxation was expressed as the percentage change from vessel tone after pre-contraction. Pre-contracted rings without the administration of relaxing agents served as controls. Because IMA control rings of the long-lasting DRC experiments (approx. 20–25 min) had some spontaneous relaxation, separate experiments for parallel measurement of functional relaxation and cGMP production were done. In these latter experiments, a single submaximal dose
of ANP, BNP or CNP (300 nmol/l for each) or sin-1 (100 µmol/l) was applied to pre-contracted vessel rings not used previously in DRC experiments, and the percentage relaxation was calculated. Vessel rings were shock-frozen in liquid nitrogen and stored at −80 °C for later determination of intracellular cGMP concentrations.

Homogenization
IMA and SV rings were weighed whilst still frozen, and were immediately homogenized with a Micro-Dismembrator® (Braun Biotech International). In brief, a frozen vessel ring was crushed with a 5 mm tungsten ball in a pre-cooled 5 ml teflon container for 30 s at 2500 rev./min. Subsequently, 1 ml of 5% trichloric acetate was added to destroy cellular membranes, and the tissue was crushed for another 30 s. The homogenate was allowed to thaw and was then centrifuged at 1000 × g for 15 min at 4 °C. The supernatant was extracted with 3 ml of diethyl ether. This procedure was repeated three times, and residual diethyl ether was allowed to evaporate by shaking the samples for a further 1 h, before extracted cGMP was frozen at −80 °C until measured.

RIA
Concentrations of cGMP in homogenized vessel rings were determined using an RIA (Amersham), according to the manufacturer’s protocol and as reported previously [15]. The production of cGMP concentration was normalized by the wet weight of the tissue fragments and expressed as pmol/g of tissue.

Statistical analysis
Data analysis of the DRC for U46619 and the mathematical fitting of functions to data using a least-squares method were performed by the program Origin (version 4.1; MicroCal). The data were fitted to the equation: $y = (A_1 - A_2)/(1 + (x/x_0)^p) + A_2$, where $A_1$ is the initial absolute mN value (equilibration value), $A_2$ is the final absolute mN value (maximum contraction value), $p$ is the slope, and $x_0$ is the EC_{50} value.

Statistical comparison of the DRCs for NPs or sin-1 with controls was done using ANOVA (SPSS® for Windows 11.0). To evaluate effects of single dose application of drugs, the Mann–Whitney $U$ test was performed, and Spearman rank correlation coefficients were calculated. For the correlations, net cGMP production and net relaxation were calculated by subtracting the control values from the values obtained after stimulation of the vessel rings with the different substances. Data are means ± S.E.M., if not specified otherwise. A $P$ value of < 0.05 was considered to indicate statistical significance.

### RESULTS

Patient characteristics are listed in Table 1.

#### DRCs for U46619
The maximum contractile responses to U46619 were significantly higher in the SV than in the IMA (86 ± 7 mN and 54 ± 5 mN respectively; $P = 0.008$). The EC_{50} value for the IMA was $12.4 ± 3.1$ nmol/l ($n = 14$ IMA rings) and for the SV was $12.2 ± 1.9$ nmol/l ($n = 14$). As determined from these experiments, pre-contraction of vessel rings was optimal at a concentration of 30 nmol/l U46619 (ED_{77} for IMA and ED_{72} for SV). At this concentration, U46619 caused similar contractile responses in the IMA and SV. However, the IMA had some spontaneous relaxation after approx. 15 min, which did not reach statistical significance, whereas the SV was stable for at least 20 min.

#### DRCs to NPs and sin-1
In the presence of indomethacin (10 µmol/l) and after pre-contraction with 30 nmol/l U46619, ANP, BNP, CNP and sin-1 were applied cumulatively to the IMA and SV rings (Figures 1A and 2). ANP (77 ± 9%, $P = 0.001$) and sin-1 (84 ± 5%, $P < 0.001$) were the most powerful relaxants of the IMAs, and significantly relaxed IMA.

### Table 1 Characteristics of patients undergoing CABG who donated IMA and SV tissue for the present study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DRCs</th>
<th>Single dose application</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>51*</td>
<td>49*</td>
</tr>
<tr>
<td>Female (n)</td>
<td>16 (31.4)</td>
<td>9 (18.4)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>71 (38–85)</td>
<td>70.5 (44–85)</td>
</tr>
<tr>
<td>Risk factors (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>6 (11.8)</td>
<td>8 (16.0)</td>
</tr>
<tr>
<td>Smoking</td>
<td>12 (22.6)</td>
<td>10 (20.4)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>37 (72.5)</td>
<td>32 (65.3)</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>37 (72.5)</td>
<td>25 (51.0)</td>
</tr>
<tr>
<td>Hypertriglyceridaemia</td>
<td>12 (23.5)</td>
<td>11 (22.4)</td>
</tr>
<tr>
<td>Drugs (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrates</td>
<td>23 (45.1)</td>
<td>16 (32.7)</td>
</tr>
<tr>
<td>Acetyl salicylic acid</td>
<td>39 (76.5)</td>
<td>38 (77.6)</td>
</tr>
<tr>
<td>β-blocker</td>
<td>29 (56.9)</td>
<td>32 (65.3)</td>
</tr>
<tr>
<td>Diuretics</td>
<td>29 (56.9)</td>
<td>32 (65.3)</td>
</tr>
<tr>
<td>Calcium antagonists</td>
<td>6 (11.8)</td>
<td>9 (18.4)</td>
</tr>
<tr>
<td>ACE-inhibitors</td>
<td>19 (37.3)</td>
<td>15 (30.6)</td>
</tr>
</tbody>
</table>
Isolated rings were pre-contracted with U46619 and then cumulative concentrations of NPs were added (0.1–300 nmol/l). The vasorelaxing effects of the NP are expressed as percentages of the initial U46619-induced pre-contraction corrected for the initial resting tension. Values are means ± S.E.M. P values between the different DRCs were calculated using ANOVA.

rings when compared with control rings. BNP (32 ± 8%) and CNP (43 ± 7%) did not cause significantly different relaxation compared with control rings, as control IMA rings had some spontaneous relaxation (29 ± 4%; Figure 1A). In the SVs, only sin-1 (Figure 2) caused significant (P < 0.001) and concentration-dependent relaxation (56 ± 8%). NPs were poor and non-significant relaxants of SVs (Figure 1B). Control SV rings did not show significant spontaneous relaxation during the whole experiment.

cGMP production in the vessel rings
Applications of single submaximal relaxing concentrations of ANP, BNP or CNP (300 nmol/l each) or sin-1 (100 µmol/l) were used for the determination of intracellular cGMP concentration in the same vessel ring. Relaxation in response to these concentrations (as median values) was 93% for ANP (P < 0.001), 70% for BNP (P < 0.003), 47% for CNP (P < 0.05) and 94% for sin-1 (P < 0.001) in pre-constricted IMA rings (Figure 3). Intracellular cGMP concentrations from homogenized vessels were determined at the time point of maximum relaxation response after drug application. These time points were comparable between the different drugs. Intracellular cGMP production in IMAs was increased significantly after ANP, BNP and sin-1 stimulation (Figure 4). Sin-1 caused the most pronounced increase in intracellular cGMP (7.4-fold; P < 0.001), followed by ANP (5.6-fold; P < 0.001) and BNP (4-fold; P < 0.001), compared with control cGMP concentrations [median (minimum–maximum), 21.10 (8.98–75.09) pmol/g]. A moderate, but significant, correlation was observed between intracellular net production of cGMP and net relaxation in IMAs (r = 0.473, P = 0.003).

In SVs, only application of 100 µmol/l sin-1 induced a significant and powerful relaxation (median, 44.8%; P < 0.001; n = 27; Figure 3). The NPs tested neither caused significant relaxation nor significant increase in cGMP levels. Only sin-1 stimulation in SVs significantly increased intracellular cGMP (14.4-fold; P < 0.001; Figure 4) compared with control rings.
Intracellular cGMP concentrations following stimulation with a single submaximal dose of NPs (300 nmol/l) or sin-1 (100 µmol/l) in IMAs and SVs

Comparison between IMA and SV

We additionally compared the relaxation responses of SVs with IMAs. Although IMAs had some spontaneous and significantly greater relaxation in control rings compared with control SV rings (Figure 3), intracellular cGMP concentrations were not significantly different between the two vessel types. NO and NPs, except for CNP, were significantly more powerful relaxants of the IMA than of the SV (P values ranging from 0.001–0.002; Figure 3). After ANP and BNP stimulation, intracellular cGMP concentrations were significantly higher in IMAs than in SVs (P values ranging from <0.0001–0.009; Figure 4). In contrast with NPs, sin-1 application caused a significantly higher increase in intracellular cGMP concentration in SVs than in IMAs (P = 0.03; Figure 4), despite the fact that relaxation was significantly greater (P = 0.002) in IMAs than in SVs.

DISCUSSION

In contrast with most previous reports, in the present study we have directly measured cGMP production after NP and NO stimulation in human by-pass graft segments, in which relaxation was measured in the organ bath, and found significantly increased cGMP concentrations in vessels with significant relaxation responses. Furthermore, there was a moderate, but significant, correlation between net relaxation and cGMP net production in IMAs. Sin-1 was a more powerful vasorelaxing substance in IMAs than in SVs, and the NPs only induced significant relaxation in IMAs. ANP caused the most pronounced relaxation of all of the NPs tested in IMAs. BNP and CNP induced only weak relaxation in the DRC evaluation. In SVs, only sin-1 caused significant relaxation, whereas all NPs were ineffective. These results are in accordance with two previous studies using endothelin-1 and phenylephrine [19] or noradrenaline [20] for pre-constriction. However, in our present study, dose-dependent relaxation induced by BNP did not reach statistical significance in IMAs. These discrepancies may be partly due to the different pre-contracting substances used. We chose U46619 as a pre-contraction substance, because U46619 is a thromboxane A2 agonist and thromboxane A2 is released during CABG. U46619 has been shown to be the most potent constrictor of IMAs [5] and, therefore, relaxation activities to NPs may be different. In our DRC experiments, the control rings had some spontaneous, but non-significant, relaxation as well. This could additionally and partially mask any effective relaxation activities in response to different substances. Recently, a spontaneous time-dependent relaxation of IMA control rings was observed as well [21]. In agreement with our findings, concentration-dependent relaxation by ANP in several human arteries pre-constricted with noradrenaline or prostaglandin F2α was reported by Hughes et al. [22], whereas ANP, as in our present study, was without effect on SVs. The different distribution of NP receptors in IMAs and SVs may explain the different relaxation responses to ANP which we observed in our present study. In human arteries (gastroepiploic arteries and IMAs), the gene transcript for NP receptor A, whose preferential ligand is ANP, followed by BNP, was found to be four times more intense compared with the gene transcript in SVs [18].

The most potent vasorelaxing substance in our present study was sin-1, and it significantly, but not fully, reversed vasoconstriction in IMAs (84 % relaxation) and SVs (56 % relaxation). Previous studies have found complete or nearly complete relaxation after incubation with the NO donor nitroglycerine (10 µmol/l) in IMAs and SVs pre-contracted with U46619 [7] or phenylephrine [10]. The new S-nitrosothiol RIG200, SNP (sodium nitroprusside) and S-nitrosoglutathione were also powerful relaxants and had sustained vasorelaxation responses in IMAs and SVs [10]. We used a different NO donor (sin-1) than these two latter studies, and did not achieve complete relaxation in our experiments using higher concentrations of 100 µmol/l. A possible influence of relaxation due to nitrate tolerance in our patients with chronic treatment with nitrates can be excluded, because sin-1 has been reported to be unaffected by this phenomenon [11]. It is more likely that lower relaxation responses are due to the effect of different pre-contraction substances used. NO donors, such as nitroglycerine or SNP, had lower relaxation responses in IMAs when pre-contracted with endothelin-1, one of the most potent vasoconstrictors, instead of angiotensin II or phenylephrine [23,24]. However, the powerful relaxation after sin-1 stimulation in IMAs...
observed in the present study concurs very well with previous results showing high relaxation capacities of various NO donors [5,23,25].

Because performing DRCs is time-consuming, which resulted in spontaneous relaxation of control IMA rings, we used single drug dose stimulation of the vessel rings for comparison of relaxation with cGMP production. This eliminated the spontaneous relaxation effect in the control rings, which may have partially masked the relaxation responses in the DRCs. When a single submaximal dose of NPs or sin-1 was administered to pre-contracted IMA rings, the relaxation responses were markedly greater than in the DRC experiments. Thus, in these experiments, BNP and CNP also exhibited a considerable relaxation capacity in IMAs.

In the present study, we demonstrated that vasorelaxation to NPs was related to a significant increase in cGMP in the same vessel segment. These results extend the results of our previous study [16] and earlier studies [17,18] in which vessel segments were incubated with NPs for a certain time period without functional investigations. NPs, with the exception of CNP, were more effective in IMAs than in SVs with regard to vasorelaxation and cGMP production. The greater effect of NPs on IMAs than on SVs seen in the present study has also been observed by Best et al. [20], who demonstrated increased intracellular cGMP concentrations which were higher in IMAs than in SVs after ANP stimulation lasting for 8 min. However, in contrast with our present study, cGMP concentrations were measured in separate vessel segments not used for relaxation studies. Only one study on newborn lamb vessels directly compared vasorelaxation and cGMP concentrations in the same vessel segments [26]. ANP induced greater relaxation and cGMP concentrations in pulmonary arteries pre-constructed with endothelin-1 than in the veins. CNP was more potent in the veins, but caused only moderate cGMP production, and a cGMP-independent mechanism of CNP-induced relaxation was speculated [21]. On the other hand, dose-dependent increases in intracellular cGMP concentrations were shown after NP (ANP, BNP or CNP) or SNP stimulation in membrane preparations of rat thoracic aorta [27]. Potent vasorelaxation capacities of various NO donors in arteries of different species [27-29] were associated with a significant increase in intracellular cGMP levels tested in separate vessel segments. The discrepancies in relaxation responses and cGMP production between NPs and NO stimulation seen in the present study were also observed previously in mouse ventricular myocytes [30]. Although CNP and the NO donor SNAP (S-nitroso-N-acetyl-dl-penicillamine) induced similar changes in the contractile responses, intracellular cGMP concentrations were more pronounced after SNAP than CNP application. This was attributed to the different subcellular localization of the distinct GCs (guanylate cyclases). The particulate GCs stimulated by NPs are found in subsarcolemmal areas and may be close to its effectors. The soluble GC is distributed in the cytosol which contains cytoskeletal structures that could influence cGMP diffusion to the effectors. Furthermore, the different responses of IMAs and SVs to sin-1 stimulation may be explained by the different morphologies of these two vessels. The IMA is like the aorta, an elastic artery, and has a distinct layering of the media with a large number of elastic laminae and few smooth muscle fibres, which are orientated in an oblique angle. This structure allows the IMA a further extension of the wall. On the other hand, the SV has a highly variable structure with longitudinal muscle bundles in the media adjacent to the intima as well as in the adventitia. In the media, collagen predominates with some circumferential muscle fibres. Therefore relaxation in SVs may require a more pronounced production of the second messenger cGMP than in IMAs.

In summary, we have demonstrated potent vasorelaxation and significant intracellular cGMP increases in the same vessel rings after NP stimulation. Additionally, we found a moderate, but significant, correlation between intracellular cGMP net production and net relaxation, supporting a direct link between the production of the second messenger cGMP and relaxation. Among all of the NPs tested, the most powerful relaxation was found in the IMA after ANP stimulation, and sin-1 was an effective relaxant of both IMAs and SVs. ANP was capable of achieving a similar relaxation (93 %) in IMAs as the NO donor sin-1 (94 %) and could serve as an alternative relaxing mechanism to overcome peri-operative vasospasm in the IMA.

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