Naloxone prevents increased atrial natriuretic peptide release during regional myocardial ischaemia and stunning in awake dogs

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Background. Atrial natriuretic peptide (ANP) release is increased in patients with ischaemic left ventricular dysfunction. A beneficial effect of naloxone on recovery from myocardial stunning was shown previously. The aim of this study was to investigate the effects of naloxone on ANP release during regional myocardial ischaemia and stunning in awake dogs.

Methods. Ten dogs were chronically instrumented for measurement of heart rate, left atrial, aortic, and left ventricular pressure (LVP), LV dP/dtmax/min, and myocardial wall-thickening fraction. An occluder around the left anterior descending artery (LAD) allowed induction of reversible ischaemia in the LAD-perfused myocardium. Each dog underwent two ischaemic episodes (randomized crossover fashion; separate days): 10 min of LAD occlusion (1) after application of naloxone (63 µg kg⁻¹), and (2) without naloxone. ANP levels were measured at baseline (BL) and at predetermined time points until complete recovery of myocardial stunning occurred.

Results. LAD ischaemia-induced release of ANP (peak level: 182 (30) vs 27 (7) pg ml⁻¹ BL) only in the control group without naloxone. Between 1 and 180 min of reperfusion, ANP levels were significantly higher only in the control group (P<0.05).

Conclusion. Pre-ischaemic application of naloxone prevents this ischaemia-induced ANP-release in conscious dogs.

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The natriuretic peptide family consists of a group of structurally similar but genetically distinct peptides. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), both of myocardial origin, are reactive hormones whose elevated blood levels correlate with myocardial dysfunction, in the presence of cardiac failure. They are similarly upregulated in heart failure and counteract neurohormones that induce vasoconstriction and fluid retention.¹ However, responses of the natriuretic peptides during regional myocardial ischaemia and stunning remain uncertain. Myocardial stunning is a general term for the mechanical dysfunction that persists after reperfusion even in the absence of irreversible damage and despite the return of normal, near-normal, or supranormal perfusion.² In a previous investigation, we demonstrated that naloxone attenuates myocardial stunning in conscious dogs through its action on the central nervous system.³ This effect of naloxone might be explained by antagonizing endogenous opioid peptides (EOPs) which are released upon myocardial ischaemia and have negative inotropic properties.⁴

The present investigation in chronically instrumented conscious dogs tests the hypothesis that (1) ANP and BNP
are released in response to myocardial regional ischaemia and stunning and (2) that naloxone prevents this ischaemia-induced release.

Methods
This investigation is performed in accordance with the Guide for the Care and Use of Laboratory Animals\(^5\) and was approved by the District Government of Münster. After overnight fasting, 10 mongrel dogs (either sex, weight 23–32 kg) received i.m. pre-medication with piritramide 1 mg kg\(^{-1}\) and ketamine 5 mg kg\(^{-1}\). The animals were anaesthetized i.v. with propofol 5 mg kg\(^{-1}\). After tracheal intubation, anaesthesia was maintained with isoflurane in a mixture of oxygen (35%) in air and supplemented by fentanyl (5 μg kg\(^{-1}\)). Perioperative antibiotic prophylaxis was achieved with cefamandole 30 mg kg\(^{-1}\). Details of the instrumentation methods have been published previously\(^6\) and are summarized briefly below. A left thoracotomy was performed at the fifth intercostal space under aseptic conditions. Size 18 F gauge catheters were inserted into the descending aorta and the left atrium for pressure measurement, injection of microspheres, administration of naloxone, and withdrawal of blood. A pressure microtransducer (Janssen Pharmaceutica, Beerse, Belgium) was inserted into the left ventricle through an apical stab wound to measure left ventricular pressure (LVP). Pulsed Doppler blood flow velocity probes (20 MHz; Baylor College of Medicine, Houston, TX, USA) were fitted around the left anterior descending coronary artery (LAD). To measure the regional myocardial wall-thickening fraction (WTF), 10 MHz pulsed Doppler crystals were sutured to the myocardium in the LAD-perfused area. Proximal to the Doppler flow probe, a pneumatic occluder was positioned around the LAD (proximal to the first main diagonal branch) to induce reversible brief ischaemic episodes in the LAD-perfused myocardium. After closure of the thorax, all leads were tunnelled subcutaneously and exteriorized between the scapulae. After instrumentation, the animals were trained daily to accustom them to the experimental environment and to ensure that they could quietly lie in the cage when connected to the data acquisition system. Aortic and left atrial pressures were measured using disposable pressure transducers. Pressure, flow velocity, and wall-thickening signals were processed using a six-channel pulsed Doppler system (Baylor College of Medicine). The left ventricular micromanometer was calibrated to the pressures measured in the aorta and left atrium. The LVP signal was electronically differentiated (Gould Inc., Cleveland, OH, USA) to obtain LV dP/dt\(_{\text{max}}\)\(^{-1}\) and LV dP/dt\(_{\text{min}}\)\(^{-1}\). All signals were digitally recorded. Experiments were only conducted after the animals had recovered completely from the instrumentation, and measurements of blood gas values and haemodynamic variables showed normal values, which took between 10 and 12 days after surgery.

Arterial blood samples for measurement of ANP and BNP were drawn from the aortic catheter, collected into chilled syringes and transferred to polypropylene tubes containing EDTA (1 mg ml\(^{-1}\) of blood) and aprotinin (500 kIU ml\(^{-1}\) of blood). Thereafter, the samples were centrifuged and the plasma stored at –70°C until analysis. ANP and BNP plasma concentrations were analysed by radioimmunoassays using a polyclonal rabbit IgG-antisera raised to the following peptides (Peninsula Laboratories, Belmont, CA, USA): α-ANP 1–28 (human) and BNP-32 (human). Peptides were extracted from 3 ml plasma (Sep-Pak C\(_{18}\), Waters Associates, Milford, MA, USA) and eluted with 3 ml of a mixture of 60% acetonitrile, 0.1% trifluoroacetic acid, and 39% distilled water (by volume). All samples were assayed in triplicate. Standard curves were constructed with standard human ANP and BNP in radioimmunoassay buffer. The mean recovery of added natriuretic peptides from plasma was 60–80%, and the lower detection limits as defined by 95% of the upper plateau of the standard curve were 0.1 nmol per tube for ANP and 0.5 nmol per tube for BNP. Cross reactivity between natriuretic peptides was less than 0.1%. The intra-assay and inter-assay coefficients of variation were 3.8 and 9.6% for ANP and 6.1 and 7.9% for BNP, respectively.

The experimental design was as follows: All 10 dogs were used in two ischaemic experiments. Each dog had one ischaemic episode without pre-treatment and the other ischaemic episode after pre-treatment with 63 μg kg\(^{-1}\) naloxone (Curamed Pharma, GmbH, Ch.-B.: 0060697) i.v. 5 min before ischaemia.\(^3\) Five animals received their first coronary artery occlusion without pre-treatment. The other five received naloxone before their first ischaemia.

The following observations were made.

1. Measurement of baseline (BL) values in the awake state and induction of 10 min of LAD ischaemia, with follow-up of WTF, ANP, and BNP levels until complete recovery occurred.

2. Measurement of BL values in the awake state, application of 63 μg kg\(^{-1}\) naloxone, induction of 10 min of LAD ischaemia, with follow-up of WTF, ANP, and BNP levels until complete recovery occurred.

A second ischaemic episode was only induced when there was complete recovery of regional myocardial function in the LAD-perfused area; the minimum time interval between the two experiments was 4 days.

Regional myocardial blood flow was measured using coloured microspheres (Triton Technology, San Diego, CA, USA). For each measurement, a total of \(9 \times 10^6\) microspheres suspended in a volume of 3 ml 0.9% NaCl was injected into the left atrium. The reference blood sample was withdrawn from the aortic catheter at a rate of 10 ml min\(^{-1}\). Animals were killed by injection of potassium chloride into the LA catheter during general anaesthesia when regional myocardial function had completely recovered after the last ischaemic episode. The heart was dissected and three tissue samples were
obtained from the LAD-perfused left ventricle in each dog. LAD samples were taken from the immediate vicinity of the wall-thickening probes. Only samples from animals with severe ischaemic dysfunction, as determined by the wall-thickening probe, were included (no animal was excluded due to insufficient dysfunction). Samples were further dissected into the subendocardial, subepicardial, and mid-myocardial layers. Measurement of microspheres in the tissue samples was performed as described previously. Measurement of regional myocardial blood flow to the regions described was carried out four times during the experiment, as follows: (1) without naloxone and without ischaemia (control), (2) without naloxone during ischaemia, (3) after naloxone and without ischaemia, and (4) after naloxone and during ischaemia.

Experiments were conducted in chronically instrumented conscious dogs to avoid the effects of acute surgical trauma, anaesthesia, volume and ionic imbalances, and temperature on recovery from stunning. As multiple stun manoeuvres may induce extensive development of coronary collaterals thus precluding the induction of post-ischaemic dysfunction, the number of ischaemic episodes was restricted to two in each animal.

Statistical analysis
Data were analysed using repeated-measures two-way ANOVA followed by Bonferroni-corrected Student’s t-test as appropriate; P<0.05 was considered significant. Data are presented here as mean plus or minus standard error of the mean (SD).

Results
None of the animals was excluded from the analysis due to insufficient dysfunction. The maximum degree of regional ischaemic dysfunction was similar during the first and the second ischaemic episodes in each dog.

ANP and BNP levels
Regional myocardial ischaemia in the LAD-perfused area caused a significant release of ANP (Fig. 1A) only in the control without naloxone (peak levels: 182 (SD 30) vs 29 (7) pg ml⁻¹ at BL). Between 1 and 180 min of reperfusion, ANP levels were significantly higher in the control (P<0.05) as compared with the experiment with naloxone pre-treatment. After naloxone pre-treatment, ANP levels remained unchanged during and after regional ischaemia. Myocardial BNP (Fig. 1B) release during regional ischaemia and reperfusion was not different between experiments with or without naloxone.

Arterial blood pressure, left atrial pressure, heart rate, LV dP/dtₘₐₓ⁻¹, and LV dP/dtₘᵢₙ⁻¹
There were no significant changes in arterial pressure in either experimental group during or after ischaemia (Table 1). Left atrial pressure during LAD ischaemia increased significantly only in the control. Induction of regional ischaemia did not induce a significant alteration of LV dP/dtₘₐₓ⁻¹ (Table 1) in the control. After naloxone pre-treatment, LV dP/dtₘₐₓ⁻¹ was significantly elevated during ischaemia and remained so until 30 min of reperfusion. After naloxone, LV dP/dtₘᵢₙ⁻¹ was significantly lower during ischaemia and remained so until 720 min of reperfusion, as compared with the control experiments (Table 1). Heart rate during LAD ischaemia increased significantly in both experiments, but there was no significant difference between the two experiments during or after ischaemia.

Blood flow velocity in the LAD artery
During LAD occlusion, LAD flow velocity decreased to zero and increased to significantly higher values compared with the BL for the first 15 min during reperfusion (Table 1). There were no statistically significant differences between the experimental groups in blood flow velocity in the LAD-perfused area.

Regional myocardial wall thickening
In all animals, severe regional myocardial dysfunction occurred during LAD occlusion. Induction of regional ischaemia led to a significant reduction in WTF to negative values (‘wall thinning’) in both experimental conditions (Fig. 2). During ischaemia, WTF in the LAD-perfused area expressed as a percentage of the BL value was reduced to −56 (13)% in the experiment with naloxone and to −58 (14)% in the experiment without naloxone. During reperfusion, WTF in relation to pre-ischaemic BL recovered significantly faster with naloxone as compared without naloxone at time points between 1 and 30 min of reperfusion (94 (10) vs 23 (39)% at 1 min; 99 (7) vs 33 (48)% at 5 min; 98 (6) vs 40 (50)% at 15 min; 94 (12) vs 47 (36)% at 20 min; and 92 (9) vs 49 (29)% at 30 min). After 30 min of reperfusion no significant difference in WTF occurred. BL WTF values were reached after 48 h of reperfusion with and without naloxone.

Regional myocardial blood flow
During LAD occlusion, sub-endocardial blood flow decreased significantly from 0.74 (0.24) to 0.08 (0.01) ml g⁻¹ min⁻¹ without (P<0.05 vs BL), and from 1.1 (0.19) to 0.1 (0.05) ml g⁻¹ min⁻¹ with naloxone (P<0.05 vs BL). Administration of naloxone in the absence of ischaemia led to a significant increase in sub-endocardial blood flow to the LAD-perfused areas (0.74 (0.24) vs 1.1 (0.19) ml g⁻¹
min^-1). Without ischaemia, naloxone caused a significant increase in the blood flow ratio between the sub-endocardial and the sub-epicardial regions (endocardial/epicardial ratio) in the LAD-perfused area (1.2 (0.74) vs 2.21 (1.04)). Blood flow ratio to the sub-endocardial layers between the occluded (LAD-perfused area) and the normal, non-ischaemic zone (occluded/normal ratio) during ischaemia was not different with and without naloxone.

**Discussion**

In this chronically instrumented dog model ANP levels increased significantly during regional LAD ischaemia and remained elevated during myocardial stunning. Pre-ischaemic administration of naloxone completely prevented this increased ANP release. Myocardial BNP release during regional ischaemia and reperfusion was not different with or without naloxone.

Plasma concentrations of the cardiac natriuretic peptides ANP and BNP may be of prognostic value for risk stratification after myocardial infarction. ANP is released from granules in atrial myocytes during atrial stretch, whereas BNP is released from the ventricle. All effects mediated by natriuretic peptides, for example inhibition of sympathetic stimulation of the heart, serve to reduce arterial and venous pressures, and blood volume. They are similarly upregulated in heart failure, whereas BNP seems to be superior in detecting myocardial cell damage. In our
Table 1. Arterial blood pressure (ABP), left atrial pressure (LAP), rate of increase in left ventricular pressure (LV dP/dtmax), rate of decrease in left ventricular pressure (LV dP/dtmin), heart rate (HR), and blood flow velocity in the left anterior descending coronary artery (BFV LAD) for the control group and naloxone groups at BL, during ischaemia, and at predetermined time points during reperfusion. Data are presented as mean values (SD). *Significant as compared with BL. Significantly as compared with control group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Experiment</th>
<th>BL occlusion</th>
<th>Ischaemia (5 min after occlusion)</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 min</td>
<td>5 min</td>
</tr>
<tr>
<td>ABP (mm Hg)</td>
<td>Control</td>
<td>125 (34)</td>
<td>121 (22)</td>
<td>124 (25)</td>
</tr>
<tr>
<td></td>
<td>Naloxone</td>
<td>126 (33)</td>
<td>123 (28)</td>
<td>127 (47)</td>
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<td>LAP (mm Hg)</td>
<td>Control</td>
<td>2.7 (5)</td>
<td>8.6 (10)*</td>
<td>3.4 (6)</td>
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<td>Naloxone</td>
<td>2.3 (7)</td>
<td>5.2 (8)</td>
<td>2.7 (3)</td>
</tr>
<tr>
<td>LV dP/dtmax (mm Hg s⁻¹)</td>
<td>Control</td>
<td>123 (98)</td>
<td>1162 (595)</td>
<td>1189 (688)</td>
</tr>
<tr>
<td></td>
<td>Naloxone</td>
<td>-631 (47)</td>
<td>-313 (353)</td>
<td>-539 (411)</td>
</tr>
<tr>
<td>HR (beats min⁻¹)</td>
<td>Control</td>
<td>87 (15)</td>
<td>110 (21)*</td>
<td>117 (11)*</td>
</tr>
<tr>
<td></td>
<td>Naloxone</td>
<td>90 (13)</td>
<td>112 (13)*</td>
<td>114 (27)*</td>
</tr>
<tr>
<td>BFV LAD (kHz)</td>
<td>Control</td>
<td>5.9 (4.2)</td>
<td>0*</td>
<td>6.26 (0.3)*</td>
</tr>
<tr>
<td></td>
<td>Naloxone</td>
<td>5.6 (3.6)</td>
<td>0*</td>
<td>6.26 (0.3)*</td>
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</tbody>
</table>
not use ischaemic preconditioning before the ischaemic episode. Therefore, the different results may only reflect the different experimental settings. Acute instrumentation induces myocardial damage due to massive sympathetic stimulation and opioid agonism mediates cardioprotective effects during acute surgical stress. This is not inevitably at variance to our results. Nevertheless, selective opioid receptor block might provide a starting-point for effectively treating the negative inotropic effects of myocardial stunning. However, further investigations in this area will be needed.

Naloxone not only improved systolic function, but also left ventricular diastolic function during regional ischaemia and myocardial stunning. Naloxone also prevented a significant left atrial pressure increase and improved endocardial blood flow during ischaemia. These improvements explain, at least in part, the absence of an increased ANP release during ischaemia and myocardial stunning with naloxone pre-treatment. The absent ANP release, therefore, reflects the positive effects of naloxone on ischaemic and post-ischaemic myocardial dysfunction. No cardiovascular effects of naloxone alone, or a direct inhibitory effect on ANP secretion were found under BL conditions.

This study has some limitations. First, the results obtained are restricted to dogs; there may be relevant species differences in severity and duration of the ischaemic response and its modulation by naloxone. Secondly, the study design does not allow any conclusion of possible mechanisms of the observed effect. Thirdly, a dose–response relationship regarding the protective effect of naloxone was not established.

In conclusion, ANP release, in contrast to BNP release, is enhanced during regional myocardial ischaemia and stunning in conscious dogs. Naloxone completely prevents this increased ANP release. This study is the first description showing that ANP is part of the neurohumoral profile that occurs during myocardial stunning.

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