Discrepancy between plasma and aortic wall cyclic guanosine monophosphate in an experimental model of congestive heart failure

Jean-François Arnal, Franz Hofmann, and Jean-Baptiste Michel

Objectives: The state of the vasodilator systems in congestive heart failure is poorly defined. Plasma atrial natriuretic peptide is increased, whereas endothelium derived relaxing factor activity can be decreased. Atrial natriuretic peptide and endothelin derived relaxing factor both cause vascular relaxation by generating cyclic guanosine monophosphate (cGMP), by activating the particulate and the soluble guanylate cyclase, respectively. This study examines the biological effects of atrial natriuretic peptide and endothelium derived relaxing factor in experimental heart failure by assessing the plasma, urinary, and tissue concentrations of their common second messenger cGMP. Methods: Myocardial infarctions (n=31) were induced and sham operations (n=25) were performed on Wistar rats, and the rats were monitored for three months. Aortic and pulmonary cGMP contents were measured, as were the aorta and mainly matrix and smooth muscle cells, and the lung is particularly rich in capillaries, hence in endothelial cells. The concentrations of the other second messenger cyclic adenosine monophosphate (cAMP) was also determined, as were those of cGMP dependent protein kinase in the arteries. Results: 17 of the 31 rats with myocardial infarction had oedema. The total heart weight to body weight ratio and the ratio of the myocardium haemodynamically upstream from the infarcted left ventricle to body weight were increased in proportion to the infarct size. Plasma atrial natriuretic peptide and plasma and urinary cGMP concentrations were increased in proportion to the degree of heart failure (p<0.0001). The pulmonary cGMP concentration was significantly higher in the rats with myocardial infarction than in the control group (p<0.0001). Pulmonary cGMP concentrations were correlated with the plasma concentrations of atrial natriuretic peptide and cGMP (r=0.39 and 0.66 respectively, p<0.0001). The cGMP, cAMP, and cGMP, and cGMP dependent kinase concentrations in the aortic wall of rats with myocardial infarctions were the same as in control rats. Conclusions: The increase in plasma, urinary, and pulmonary cGMP in rats with myocardial infarctions were highly correlated with the increase in circulating atrial natriuretic peptide. By contrast, the aortic cGMP concentration was unchanged in these rats, despite high plasma atrial natriuretic peptide. In congestive heart failure, a discrepancy seems to exist between pulmonary (mainly endothelium) and aortic wall (mainly smooth muscle cells) cGMP.
Methods
Experimental design
The procedures followed in the care and euthanasia of the study animals were in accordance with the European Community standards on the care and use of laboratory animals (Ministère de l’Agriculture, France; authorisation No 00577, 30 April 1989). Normotensive male Wistar rats (Iffa Credo, L’Onve, France) weighing 300-320 g were used. Left ventricular infarction was produced by ligation of the left descending coronary artery under ether anaesthesia as described by Buecher et al. and Pfeffer et al. The left descending coronary artery was ligated proximally to obtain a large infarction leading to animals with uncompensated heart failure. Controls were sham operated by a similar procedure (thoracotomy) without coronary ligation. Systemic blood pressure and body weight were measured weekly by tail cuff method (W+W electronic recorder 8005, Apelab, Bagneux, France) and the animals were examined twice weekly for three months. At the end of the first month, each rat was placed in a metabolic cage and urine was collected for 15 hours. Although the study was designed to examine the disturbances of the peripheral circulation that occur in severe and uncompensated heart failure, the stage of experiment chosen is rapidly lethal, making it difficult to obtain animals with uncompensated heart failure. To avoid losing most of the rats, those showing overt signs of heart failure were killed after the first month if they showed two of the following signs: blood pressure impossible to measure, no right ventricular or weight loss during one day, a large infarction. A control rat was killed on the same day. Because anaesthesia induces changes in circulating hormone concentrations, rats were killed by decapitation to allow hormonal measurements. Blood samples were collected by dissecting 10 ml of blood containing 2.9 mM sodium EDTA, 10 μl phenylmethyl sulphonyl fluoride in ethanol (4.25 mg ml-1), and 25 μl aprotinin (200 000 protease inhibitor units [PIU] ml-1). These samples were centrifuged for 10 minutes at 3000 g, and the plasma was removed and frozen at -70°C. At the same time, arteries and organs were rapidly excised in the order: abdominal aorta plus iliac arteries, thoracic aorta and the lung (within 3 minutes after decapsulation for all organs). The samples were rinsed in cold phosphate buffered saline, frozen in liquid nitrogen, and stored at -70°C. The heart was excised and weighed. The left ventricle was separated from the two atria and the right ventricle was weighed.

Determination of infarct size
The left ventricle was opened with an incision along the septum from base to apex. Both ventricles were rinsed, blotted dry, and weighed. Myocardial infarct size was measured as described by Chien et al. Incisions were made in the left ventricle so that tissue could be pressed flat. The circumferences of the left ventricle and the region of infarction on both the endocardial and epicardial surfaces were outlined on a clear plastic sheet. Infarct size was calculated and expressed as a percentage of left ventricular surface area, based on the weight of the areas on the sheet. An average of endocardial and epicardial surface areas is reported.

The level of heart failure was further defined by dividing the rats with myocardial infarcts into two subgroups according to one major criterion, oedema, and three minor criteria (heart weight – left ventricle index [LVMI] > 1.5× normal, and the left ventricular end-diastolic dimension > 3× normal). Those rats showing oedema or the three minor criteria were assigned to the severe myocardial infarct subgroup. Rats showing two or fewer minor criteria were assigned to the moderate myocardial infarct subgroup.

Effect of intravenous atrial natriuretic peptide
Normotensive male Wistar rats weighing 300-320 g were used to study the effects of exogenous atrial natriuretic peptide on tissue cGMP. The rats were anaesthetised with inactin (100 mg kg-1) and the right jugular vein was cannulated with an elastomer catheter for injections and blood samples. The experimental protocol was then carried out on unanaesthetised animals. At a control period of one hour, a bolus injection of 3 μg kg-1 of rat atrial natriuretic peptide (1-28, Peninsula) was given to five rats; the control rats (n=5) were given only saline vehicle. Five minutes after the bolus, a 4 ml blood sample was collected into prechilled 5 ml tubes containing 2 mg ml-1 sodium EDTA, 10 μl phenylmethyl sulphonyl fluoride in ethanol (4.25 mg ml-1), and 25 μl aprotinin (200 000 protease inhibitor units [PIU] ml-1). At the same time, the abdominal aorta, thoracic aorta plus iliac arteries and the lung were excised (total time of two to three minutes). They were rinsed in cold phosphate buffered saline, frozen in liquid nitrogen, and stored at -70°C.

Preparation of tissue samples
The thoracic aorta was divided into large proximal (about 30 mg) and small distal (about 20 mg) segments. The abdominal aorta plus iliac arteries were similarly divided into a large segment including the bifurcation and the two iliac arteries (about 25 mg) and the upper part of the abdominal aorta (about 15 mg). The large segments were homogenised in 10 vol buffer (20 mM KPO4, pH 7.0, plus 2 mM benzamidine) with an all glass homogeniser at 4°C for determination of cGMP-dependent concentration. The small segments were homogenised in 10 vol 0.1N HCl with an all glass homogeniser at 4°C for determination of cyclic nucleotides. A cortical segment of lung (about 100 mg) was homogenised in 10 vol 0.1N HCl with an all glass homogeniser at 4°C for determination of cyclic nucleotides. All homogenates were centrifuged at 15 000 g for 30 minutes, and aliquots of the supernatants were stored at -20°C. Protein concentrations of all samples were determined by the Bio-Rad Coomassie brilliant blue G-250 method with bovine serum albumin as standard.

Determination of cGMP and cyclic AMP concentrations
Polyclonal antibody against succinyl cGMP raised in rabbits was provided by Dr B. Buecher (Strasbourg, France). The antigen was prepared by coupling succinyl cGMP to human serum albumin by the method of Steiner et al. Cross-reactivity of the antisemur with cAMP was 0.02%. The radioligand [3H]-cGMP tyrosine methyl ester was prepared by iodinating the succinyl-cGMP tyrosine methyl ester (Sigma) by the method of Greenwood et al with carrier free [3H] (Amersham). The iodination reaction products were separated on Sephadex G-25 fil (Pharmacia LKB). Standard stock solutions of cGMP (40 μM) were prepared in water and the absorbance of the solution was routinely monitored spectrophotometrically (Uvikon 810P, Kontron). Standard dilutions (40-2560 pmol) were made from the stock solution. The HCl extracts containing cGMP were determined radioimmunologically according to Cailla et al as modified by Honna et al to increase sensitivity by succinylating the cGMP before binding to the antibody. The sensitivity of the assay was 2 fmol per tube. The intra-assay reproducibility for duplicate determinations was calculated from 10 measurements of an unknown in the assay. The mean result was 21.8(SD 2.6) fmol. The coefficient of variation was 12%.

The plasma was deproteinized by addition of 0.1 volume of 10 μl of 15% trichloroacetic acid to increase sensitivity by succinylating the cGMP before binding to the antibody. The sensitivity of the assay was 2 fmol per tube. The intra-assay reproducibility for duplicate determinations was calculated from 10 measurements of an unknown in the assay. The mean result was 21.8(SD 2.6) fmol. The coefficient of variation was 12%.

The plasma was deproteinized by addition of 0.1 volume of 15% trichloroacetic acid to increase sensitivity by succinylating the cGMP before binding to the antibody. The sensitivity of the assay was 2 fmol per tube. The intra-assay reproducibility for duplicate determinations was calculated from 10 measurements of an unknown in the assay. The mean result was 21.8(SD 2.6) fmol. The coefficient of variation was 12%.

Determination of plasma atrial natriuretic peptide
Plasma atrial natriuretic peptide was extracted on a C18 cartridge and determined by non-equilibrium radioimmunoassay with a double antibody to separate the free and bound fractions. Peptide tracer was labelled with [125I] by a modified chloramine T method.

Determination of cGMP dependent kinase concentration
The tissue concentration of cGMP dependent kinase was determined by an enzyme linked immunosorbent assay (ELISA) with affinity purified anti cGMP kinase antibodies. Microsomal plasma membrane homogenates were incubated with pure cGMP kinase for 16 hours at 4°C. The coating solution was removed by aspiration, and the residual binding sites were saturated by incubation with buffer A (20 mM KPO4, pH 7.5, 150 mM NaCl, 0.01% NaN3) containing 2% bovine serum albumin (BSA) pH 7.0, at room temperature for one hour. The plates were washed twice with buffer A containing 0.1% BSA. The affinity purified rabbit antibodies were preincubated with three dilutions of tissue extracts or pure cGMP dependent kinase for 16 hours at 4°C. These probes were transferred to the plates and incubated for two hours at 4°C. The plates were then washed twice. A goat anti rabbit IgG-peroxidase conjugate was added and the plates incubated for one hour at room temperature. They were washed three times and substrate solution (0.4 mg/ml 1,2 phenylene diamine plus 0.01% H2O2 in a phosphate citrate buffer, pH 5.0) was added. The reaction was stopped with 2N HCl after 15 minutes and the reaction product was measured at 492 nm. The cGMP-dependent kinase concentration was calculated as described previously.

Statistical methods
Results are expressed as means(SEM). Differences in blood pressure and body weight were evaluated by ANOVA with repeated measures (comparison of the three groups). A non-paired test was used to compare the differences of survival between subgroups of myocardial infarction. One way analysis of variance was followed by the Scheffe F test to compare the effect of the pathophysiological condition on the different variables. Linear regression curves and correlation coefficients were obtained by the least squares method.

Results
Mortality, blood pressure, and body weight
The mortality was 28 of 59 rats with myocardial infarction and 1 of 26 sham operated rats. Seven rats with myocardial infarction (and seven controls) were killed early after the end
of the first month because of overt signs of heart failure. A total of 31 rats with myocardial infarction and 25 sham operated rats survived to the end of the experiment. Induction of the myocardial infarction caused a significant fall in systolic blood pressure that persisted throughout the study (F=14.70, p<0.0002).

The total heart weight to body weight ratio was significantly greater in the myocardial infarction subgroup than in the sham operated rats (F 73.46, p<0.0001). Both the absolute weights of the myocardium haemodynamically upstream from the infarcted left ventricle (the left atrium, right ventricle, and right atrium) and the ratios of their weights to body weight were significantly increased (F 85.05, p<0.0001). The infarction involved 29(1)% of the left ventricle in the rats with moderate myocardial infarction and 37(1)% in the rats with severe myocardial infarction (t=3.85, p<0.05) (table 1).

Heart variables and oedema (table 1)
The total heart weight to body weight ratio was significantly greater in the myocardial infarction subgroups than in the sham operated rats (F 73.46, p<0.0001). Both the absolute weights of the myocardium haemodynamically upstream from the infarcted left ventricle (the left atrium, right ventricle, and right atrium) and the ratios of their weights to body weight were significantly increased (F 85.05, p<0.0001). The infarction involved 29(1)% of the left ventricle in the rats with moderate myocardial infarction and 37(1)% in the rats with severe myocardial infarction (t=3.85, p<0.0001). The infarct size was correlated with the total heart weight ratio (r2=0.67, p<0.0001, n=56), the ratio of the myocardium weight haemodynamically upstream from the left ventricle to body weight (r2=0.65, p<0.0001, n=56), and with the infarction size (r=28.9x-89.6, r2=0.334, p<0.001, n=56). The plasma concentrations of cGMP were significantly increased in both groups of rats with heart failure (F 52.63, p<0.0001). There was a close correlation between plasma cGMP and plasma atrial natriuretic peptide (r2=0.72, p<0.0001, n=56). Plasma cAMP did not change in either group. Urinary cGMP was significantly raised in the model of heart failure (F 34.36, p<0.0001) and was closely correlated with plasma concentrations of atrial natriuretic peptide (r2=0.49, p<0.0001, n=56).

Tissue variables
The pulmonary cGMP concentration was significantly higher in the rats with myocardial infarcts than in the control group (F 11.13, p<0.0001) (table 2). There was a close correlation between pulmonary cGMP and plasma atrial natriuretic peptide (r2=0.59, p<0.0001, n=56) (fig 2) and plasma cGMP (r2=0.66, p<0.0001, n=56) (fig 3). The cGMP contents of both the thoracic and abdominal aorta were not significantly different in the myocardial infarction group (2218(155) and 1933(145) fmol.mg-' protein, respectively) and in the control group (2782(273) and 2358(217) fmol.mg-' protein, respectively). Table 2 shows the data from cGMP in the two subgroups as previously defined.

The cAMP concentrations of the arterial wall and the lung remained unchanged in the rats with myocardial infarcts (table 3). The cGMP dependent kinase concentrations in segments of thoracic aorta and abdominal aorta plus iliac arteries from sham operated rats (n=8) and rats with severe myocardial infarction (n=8) were similar (table 3).

Effect of exogenous atrial natriuretic peptide (table 4)
Plasma atrial natriuretic peptide in anaesthetised rats given only saline vehicle was 332(36) pg/ml. Plasma atrial natriuretic peptide in rats given a bolus injection of 3 μg ANP/kg was 2452(771) pg/ml. Exogenous atrial natriuretic peptide increased plasma and pulmonary cGMP content by about fivefold and 9.5-fold respectively. Bolus injection of 3 μg atrial natriuretic peptide·kg-' did not significantly influence thoracic or abdominal aortic cGMP content, however.

Table 1 Body weight, heart weight, infarction size, and oedema at the end of the experimental period.

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Table II  Plasma atrial natriuretic peptide and cGMP concentrations.

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<th>Severe MI (n=19)</th>
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<tr>
<td>Plasma ANP (pg·ml⁻¹)</td>
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<td>694(83)*</td>
<td>1011(61)*</td>
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<td>Plasma cGMP (pmol·m⁻¹)</td>
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<td>16(1)*</td>
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<td>Urinary cGMP (pmol·100 g⁻1·15 h⁻¹)</td>
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<td>9.3(1.0)*</td>
<td>20.1(1.8)*</td>
<td>&lt;0.001</td>
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<td>Pulmonary cGMP (fmol·mg⁻¹)</td>
<td>191(31)</td>
<td>560(238)</td>
<td>1075(189)*</td>
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<td>Thoracic Ao cGMP (fmol·mg⁻¹)</td>
<td>2782(273)</td>
<td>2174(267)</td>
<td>2243(194)</td>
<td>NS(0.123)</td>
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<td>Abdominal Ao cGMP (fmol·mg⁻¹)</td>
<td>2358(217)</td>
<td>2056(229)</td>
<td>1862(190)</td>
<td>NS(0.227)</td>
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Data are means(SEM). Sham operated=sham operated rats; MI=myocardial infarction rats; ANP=atrial natriuretic peptide; nmoles.100 g⁻¹.5 h⁻¹=amount of cGMP in nmoles.100 g⁻¹ of body weight.urinary volume excreted in 15 hours⁻¹; Ao=aorta.

*p<0.05 v sham operated value; fp<0.05 v moderate MI value.

Discussion

The state and the role of two major vasoconstrictor systems, the sympathetic adrenergic system and the renin angiotensin system, in the pathogenesis of the disturbances of the peripheral circulation have been extensively studied in heart failure.6,7 Because of their more recent characterisation,7-11 the state of the endogenous vasodilatory systems have been less thoroughly investigated. The increase in the plasma concentration of atrial natriuretic peptide in relation to the severity of heart failure was confirmed in our present study.5,6 Fifty five per cent of the rats with myocardial infarction had oedema and thus provide a suitable model of decompensated heart failure. The sodium and water retention, which characteristically occurs in heart failure, illustrates the greater influence of the antinatriuretic (over the cardiac natriuretic) system, despite the large increase in the plasma concentrations of atrial natriuretic peptide. Indeed, the renal effects of exogenous atrial natriuretic peptide seem to be dramatically blunted in both human18,19 and experimental23-24 heart failure. The effects of 8-bromo-cGMP on renal excretory function are also abolished or greatly attenuated in experimental heart failure.32 This pathophysiological state is also characterised by increased vasoconstriction, which suggests an imbalance between vasoconstrictor and vasodilator systems. The first seems to be activated and the second to be depressed. The hypertensive effect of atrial natriuretic peptide is blunted in rats with myocardial infarction.32 Diminished endothelium derived relaxing factor activity has been reported in experimental35-36 and human37-38 heart failure. The results for plasma endothelium derived relaxing factor-NO concentrations (indirectly measured by its stable metabolite, NO⁻) in patients with severe heart failure are conflicting.38-39 The impairment of both the endothelium dependent, and possibly the endothelium independent cGMP mediated vasodilation in patients with heart failure raises the question of the biological efficacy of the vasodilating factors in heart failure.27-30 These data raise the possibility that an intracellular defect beyond cGMP production is involved in the tolerance to atrial natriuretic peptide in heart failure. cGMP dependent kinase seems to mediate the effects of cGMP, and possibly also of cAMP, in smooth muscle cells.41 An increased cGMP dependent kinase activity has been reported in hypertensive rats.42 Our results show no change, however, in the arterial concentration of cGMP dependent kinase in rats with heart failure.

The concentration of cGMP, which is the second messenger of both NO and atrial natriuretic peptide, is raised in extracellular fluids, including plasma2 and urine,41 in heart failure. The presence of cGMP in extracellular fluids is believed to result from its egress through the cellular membrane, which is impermeable to its re-entry unless the molecules are modified to make them lipophilic.

Figure 2  Correlation between plasma atrial natriuretic peptide concentration and pulmonary cGMP (y=0.001x+2.029, r²=0.59, p<0.0001). Semilogarithmic transformation was employed because of the scatter of the cGMP values (90-3114 fmol·mg⁻¹·protein).  ○, sham operated rats; □, moderate myocardial infarction rats; ▲, severe myocardial infarction rats.

Figure 3  Correlation between plasma cGMP concentration and pulmonary cGMP (y=0.066x+1.826, r²=0.56, p<0.0001). Semilogarithmic transformation was employed because of the scatter of the cGMP values (90-3114 fmol·mg⁻¹·protein).  ○, sham operated rats; □, moderate myocardial infarction rats; ▲, severe myocardial infarction rats.
Pulmonary, and aortic cGMP content. Thoracic Pulmonary cAMP (pmol/mg-I) Abdominal Ao cAMP (pmol/mg-I) Thoracic Ao G kinase (pmol/mg-I) Abdominal Ao G kinase (pmol/mg-I)

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Data are means(SEM). Sham-operated=sham operated rats; MI=myocardial infarction rats; Ao=aorta; Abdominal Ao cAMP (pmol/mg-I) Data are means(SEM). ANP=atrial natriuretic peptide; Ao=aorta, cGMP dependent kinase concentrations.

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<td>(ng·ml⁻¹)</td>
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<td>Pulmonary cGMP (pmol·mg⁻¹)</td>
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<td>Thoracic Ao cGMP</td>
<td>2216(325)</td>
<td>2355(628)</td>
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<td>Abdominal Ao cGMP</td>
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<td>2048(362)</td>
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Data are means(SEM). ANP=atrial natriuretic peptide; Ao=aorta.

As recently shown, aortic wall cGMP seems to be mainly dependent on NO synthase: soluble guanylate cyclase activity. Indeed, endothelium derived relaxing factor seems to be an ideal modulator of local vasomotor tone, because stimulation of its release integrates local (shear stress), neural (acetylcholine mediated), and hormonal (bradykinin, ADP, etc) signals. Several reasons could account for the absence of change of aortic cGMP in response to increased circulating atrial natriuretic peptide. Firstly, atrial natriuretic peptide may be rapidly degraded by the clearance receptors and neutral endopeptidase on both endothelial and smooth muscle cells. Secondly, atrial natriuretic peptide could depress endothelium derived relaxing factor-N0 activity, as suggested by in vitro studies, leading to decreased soluble guanylate cyclase activity. Thirdly, at least two phosphodiesterases catalyse cGMP in vascular smooth muscle, and thus influence its concentration.

In summary, it is commonly assumed that the sympathetic adrenergic system and the renin angiotensin system are responsible for the increased peripheral vascular resistance in heart failure. The state of the vasodilatory systems in heart failure are, however, poorly defined. In this experimental model aortic and pulmonary cGMP concentrations were used as in vivo bioassays of circulating atrial natriuretic peptide and basal endothelium derived relaxing factor-N0 activity. The pulmonary cGMP concentration was significantly higher in the rats with myocardial infarcts than in the control group. The increase in plasma, urinary, and pulmonary cGMP in rats with myocardial infarction was highly correlated with the increase in circulating atrial natriuretic peptide. By contrast, the aortic cGMP concentration was unchanged in degradation of cGMP. This might explain the large increase in pulmonary cGMP concentration in response to increased circulating atrial natriuretic peptide, and cGMP egression might predominantly contribute to the regulation of intracellular cGMP concentration in the endothelium. Similarly, the close correlation between plasma atrial natriuretic peptide and urine cGMP is in agreement with the work of Wong et al., showing that increased urinary cGMP reflects the effect of atrial natriuretic peptide on the tubular cells. Lastly, cultured endothelial cells contain an active cAMP hydrolysing system, which might account for the unchanging cAMP concentrations in lung and in plasma.

Cyclic GMP plays an important part in the relaxation of smooth muscle cells, and thus in vasorelaxation. The cGMP in the thoracic and abdominal aorta in this model of heart failure was not different from sham operated rats, despite increased concentrations of atrial natriuretic peptide. Furthermore, intravenous atrial natriuretic peptide leading to comparable plasma atrial natriuretic peptide concentrations, failed to increase aortic cGMP content. Thus atrial natriuretic peptide within a pathophysiological range failed to influence aortic wall cGMP. As recently shown, aortic wall cGMP seems to be mainly dependent on NO synthase: soluble guanylate cyclase activity. Indeed, endothelium derived relaxing factor seems to be an ideal modulator of local vasomotor tone, because stimulation of its release integrates local (shear stress), neural (acetylcholine mediated), and hormonal (bradykinin, ADP, etc) signals. Several reasons could account for the absence of change of aortic cGMP in response to increased circulating atrial natriuretic peptide. Firstly, atrial natriuretic peptide may be rapidly degraded by the clearance receptors and neutral endopeptidase on both endothelial and smooth muscle cells. Secondly, atrial natriuretic peptide could depress endothelium derived relaxing factor-N0 activity, as suggested by in vitro studies, leading to decreased soluble guanylate cyclase activity. Thirdly, at least two phosphodiesterases catalyse cGMP in vascular smooth muscle, and thus influence its concentration.
these rats, despite high plasma atrial natriuretic peptide. In
congestive heart failure a discrepancy seems to exist between
pulmonary (mainly endothelium) and aortic wall (mainly
smooth muscle cells) cGMP.

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