Small artery tone under control of the endothelium

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Chapter 3

Endothelial dysfunction and infarct size relate to impaired EDHF response in rat experimental chronic heart failure

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Abstract

Background
The rat coronary-ligation model of chronic heart failure (CHF) has been extensively used to investigate its pathophysiology including the role of endothelial dysfunction. Inconsistent results have been obtained concerning the role of endothelial dilative mediators NO and EDHF. Our aim was to investigate involvement of NO and EDHF in aortic endothelial dysfunction in this model and the influence of individual infarct sizes. Furthermore, we investigated whether it is justified to regard rats that failed to develop large infarct sizes as SHAM controls.

Methods
We performed coronary-ligations and SHAM operations and studied acetylcholine (ACh)-induced relaxations and underlying endothelial mediators in isolated aortic rings twelve weeks after infarction. By then, cardiac and hemodynamic parameters were deteriorated in animals with large myocardial infarctions (large-MI, 35±3%), but not those with small myocardial infarctions (small-MI, 5±2%).

Results
Large-MI showed decreased ACh-induced relaxation compared to SHAM due to decreased contribution of EDHF which was inversely correlated with individual infarct size. Interestingly, small-MI showed significantly increased ACh-induced relaxation compared to SHAM due to increased NO contribution.

Conclusions
Our results suggest that impaired aortic endothelial dilatory function in large-MI is mainly due to an impaired EDHF response and strongly depends on individual infarct size. In addition, endothelium-dependent relaxation of small-MI rats differed from SHAM, indicating that both groups may not be pooled to serve as controls. These results emphasize the importance of infarct size and choice of the control group, and may explain inconsistencies in previous studies.
Introduction

Chronic heart failure (CHF) is a complex clinical syndrome characterized not only by cardiac alterations in function and structure, but also by an increase in peripheral vascular resistance, which may further contribute to its progression \(^1\). Apart from neurohumoral activation \(^2-4\), endothelial dysfunction has been implied to importantly contribute to increased peripheral resistance although reports on involvement of local endothelial factors have been less consistent.

Endothelial dysfunction - identified as impaired endothelium-dependent vasodilation (e.g. to acetylcholine) \(^5\) - has been shown clinically in patients with CHF \(^6-8\) as well as in experimental animal models, such as the rat coronary ligation induced CHF model, both for large conductance arteries \(^9,10\) and small arteries \(^11\). Endothelium-derived factors that mediate the dilatory response to ACh are prostacyclin, nitric oxide (NO) and a yet unidentified substance which hyperpolarizes underlying vascular smooth muscle cells (endothelium-derived hyperpolarizing factor, EDHF) \(^12-17\). Some studies have attributed impaired endothelium-dependent relaxation in this rat coronary ligation model to attenuated NO contribution after receptor stimulation \(^18\), and decreased basal NO activity \(^9,19-21\). Other studies, however, have reported normal receptor stimulated and basal NO activity \(^22,23,24\) in this model. Furthermore, alterations in endothelial vasodilator substances in CHF appear not to be restricted to NO but may also apply to EDHF. Indeed, some studies suggest that decreased EDHF may underlie impaired endothelium-dependent relaxation in CHF \(^22\), whereas other studies reported EDHF to be increased in these conditions \(^18\).

Part of these inconsistencies may be explained by differences in vessel-type and -size, or by the post-infarct time studied \(^10\). Nevertheless, even when considering endothelium-dependent relaxation in one vessel type (rat aorta) at the same time-point (10-12 weeks post-MI), data on endothelial function still vary among different studies \(^9,19,22,25\), thus suggesting that additional factors may be involved. In the present study we examined aortic endothelial dysfunction and underlying endothelial dilative mediators in relation to variation in infarct sizes or choice of control groups (SHAM, small-MI). To this end, endothelium-dependent and -independent relaxation and contribution of NO and EDHF pathways were studied in the rat coronary ligation model of CHF 12 weeks after coronary ligation in rats with large myocardial infarctions (35±3%), small myocardial infarctions (5±2%) and SHAM operated rats.

Methods

**Rat coronary ligation myocardial infarction model**

Forty male Wistar rats (250-300 g) were obtained from Harlan (Zeist, The Netherlands) and housed group-wise with free access to food and drinking water. Eleven of these rats were SHAM-operated while the remaining 29 rats were operated for intended myocardial infarction (MI) via chronic coronary occlusion. At the time of operation anaesthesia was induced with isoflurane (2.0-2.5% in oxygen), after which the rats were intubated and mechanically ventilated with this gas-mixture (Amsterdam Infant Ventilator, Hoek/Loos, Schiedam, The Netherlands). Myocardial infarction was induced by direct coronary ligation as described previously \(^26\). Briefly, a left-sided thoracotomy was performed and the anterior descending coronary artery occluded with a 6-0 silk suture 1-2 mm after the bifurcation. Notably, care
was given to obtain a blanching of the myocardium distal to the ligature to confirm MI. Subsequently, the thorax was closed and rats were extubated upon spontaneous respiration. From the total number of 29 rats which were operated for intended myocardial infarction 8 rats died during the operation, one rat died early after the operation (<48h) and three rats died within three weeks after coronary ligation. 17 out of the 29 rats (59%) survived the entire 12 week period and were included for analysis. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Sacrifice
Twelve weeks after infarction, rats were anaesthetized as described above. The right carotid artery was catheterized with a polyethylene catheter filled with 0.9% saline with heparin, 5000 U/L. The carotid catheter was advanced into the thoracic aorta for recording of aortic pressure and heart rate (Statham 23 Db, Gould Instruments, Cleveland, Ohio), followed by administration of heparin (500 IU) via the tail vein. The aorta was removed, rapidly excised hearts were put in cold 0.9% NaCl, and transferred to an organ perfusion set-up. Retrograde perfusion of the aorta (at 38°C), essentially by the Langendorff method, was achieved immediately. All hearts started to beat spontaneously and after equilibration for 10 min the measurements were performed: left ventricular (LV) pressure, systolic and diastolic dP/dt, heart rate, and coronary flow, as described in detail elsewhere. Thereafter, hearts were arrested in diastole in 2 mol/L KCl and weighed. The atria and great vessels were dissected from the ventricles and the right ventricular free wall (RV) was separated from the left ventricle (LV), before ventricular weights were obtained. A transverse slice through the midst of the LV containing the infarcted area was fixed in Bouin’s fluid, embedded in paraffin and 5 µm sections were cut for histological analysis. Infarct size was determined by planimetry and was expressed as the percentage of scar to total LV circumference, as described in detail elsewhere.

Organ bath studies with isolated aortic rings
After removal the thoracic aorta was placed in Krebs solution, cleaned and cut into 2 mm rings which were mounted for isotonic contraction in organ baths filled with 10 ml of Krebs solution (37°C, aerated with 95% O₂ and 5% CO₂) containing 10 µmol/L indomethacin to avoid production and interference of vasoactive prostanoids. Rings were subjected to a preload of 14 mN and allowed to stabilize for 60 min before they were checked for viability by evoking a contraction with 1 µmol/L phenylephrine (PE). After washout and renewed stabilization, rings were precontracted with 1 µmol/L PE to approximately 80% of maximal contraction; statistical analysis showed that preconstriction levels were not significantly different between SHAM (n=11), small-MI (n=7) and large-MI (n=10). Preconstricted rings were subsequently studied for dilatory response to the endothelium-dependent vasodilator acetylcholine (ACh, 10 nmol/L to 100 µmol/L) - in presence and absence of NO inhibition with 100 µmol/L N⁶-mono-methyl-L-arginine (L-NMMA). In pilot experiments we established that this concentration of L-NMMA was sufficient to prevent NO-mediated relaxation as we demonstrated that neither increasing the dosage nor adding another NO inhibitor (L-NAME, 100 µmol/L) induced further inhibition of ACh relaxation. The part of the dilator response sensitive to inhibition with L-NMMA was considered an estimate of NO contribution to total ACh relaxation and was calculated as the difference of the two dose-response curves to ACh with and without L-NMMA. The remaining ACh-evoked relaxation in the presence of L-NMMA and indomethacin was considered an estimate of EDHF
Infarct size relates to EDHF impairment

contribution to total ACh relaxation, in accord with pilot studies demonstrating its complete abrogation in the presence of charybotoxin (100 nmol/L) and apamin (500 nmol/L)\textsuperscript{12,30}. Dilatory responses to the endothelium-independent vasodilator sodium nitrite (SN; 10 µmol/L to 10 mmol/L) were determined in absence of indomethacin and L-NMMA only. Drugs were given in a cumulative fashion and all concentrations represent final bath concentrations.

**Drugs**

The Krebs bicarbonate solution had the following composition (mmol/L): NaCl 120.4, KCl 5.9, CaCl\textsubscript{2} 2.5, MgCl\textsubscript{2} 1.2, NaH\textsubscript{2}PO\textsubscript{4} 1.2, glucose 11.5, NaHCO\textsubscript{3} 25.0 and was freshly prepared daily. These compounds and SN were purchased from Merck, Darmstadt, Germany. All other compounds were purchased from Sigma (St. Louis, MO, USA).

**Calculations and statistical analysis**

Vasodilator responses to ACh and SN were expressed as a percentage of precontraction to PE. The concentrations causing half-maximal responses (EC\textsubscript{50} values) are expressed as negative logarithm of the molar concentration (pD\textsubscript{2} values). The part of the total ACh relaxation sensitive to NO inhibition with L-NMMA was considered to represent NO-mediated relaxation (Figure 1B, 3B) and was calculated as the difference of the ACh relaxation curves with and without L-NMMA. The part of the ACh relaxation which persisted in presence of indomethacin and L-NMMA was considered to represent EDHF-mediated relaxation (Figure 1C, 3C). The Area Under each individual Curve (AUC) was determined (Sigma Plot scientific graphing software package, Jandell Scientific) and expressed in arbitrary units to present the individual total response-size to ACh (Figure 2A) and for subsequent analysis of differences in response-sizes with and without L-NMMA to express the NO- and EDHF-mediated relaxation, respectively (Figure 2B and C, Figure 4)\textsuperscript{31}. All data are expressed as mean ± standard error of the mean (SEM). Statistical testing of rat characteristics, cardiac function parameters and curve characteristics (Emax, pD\textsubscript{2}, AUC) was performed using unpaired t-test. Full concentration-response curves were compared using ANOVA for repeated measures. Correlation was tested by linear regression analysis (SPSS). Significance was accepted at P<0.05.

**Results**

**Infarct size, rat characteristics and cardiac function**

In SHAM operated animals infarct-size was 0% in all cases. In the group of intended myocardial infarction (MI) the infarct-size was not normally distributed (Kolmogorov-Smirnov, P=0.007). Consistent with previous studies from our lab\textsuperscript{32} as well as those by others\textsuperscript{33}, the infarct size after intended MI showed a binomial distribution, rendering a group with very small infarct sizes (5±2%, small-MI, n=7) and one with large infarcts (35±3%, large-MI, n=10)\textsuperscript{34}. Rat characteristics and cardiac function are given in Tables 1 and 2, respectively. SHAM and small-MI rats did not significantly differ on characteristics or in vivo and in vitro parameters of haemodynamic and cardiac function. The group of large-MI rats was characterized by decreased systolic blood pressure, increased lung, heart and ventricular weights compared to SHAM controls and small-MI rats (Table 1). Cardiac function measurements of the large-MI group revealed decreased left ventricular pressure, decreased contractility (+dPdt\textsuperscript{-1}) and relaxation (-dPdt\textsuperscript{-1}), and decreased coronary flow (Table 2).
Table 1 Rat characteristics at sacrifice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SHAM (n=11)</th>
<th>Small-MI (5±2%) (n=7)</th>
<th>Large-MI (35±3%) (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infarct size (% LV)</td>
<td>0±0</td>
<td>5±2</td>
<td>35±3*#</td>
</tr>
<tr>
<td>Aortic blood pressure (mmHg)</td>
<td>120±5</td>
<td>110±7</td>
<td>99±4*#</td>
</tr>
<tr>
<td>Heart rate (beats min⁻¹)</td>
<td>313±8</td>
<td>309±5</td>
<td>297±10</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>513±7</td>
<td>502±14</td>
<td>489±20</td>
</tr>
<tr>
<td>Lung weight (g)</td>
<td>1.65±0.05</td>
<td>1.64±0.12</td>
<td>2.37±0.37*#</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>2.03±0.07</td>
<td>2.00±0.09</td>
<td>2.71±0.24*#</td>
</tr>
<tr>
<td>Left ventricular weight (g)</td>
<td>1.23±0.03</td>
<td>1.22±0.05</td>
<td>1.63±0.13*#</td>
</tr>
<tr>
<td>Right ventricular weight (g)</td>
<td>0.26±0.01</td>
<td>0.23±0.01</td>
<td>0.37±0.05*#</td>
</tr>
</tbody>
</table>

Data are mean±SEM, * P<0.05 compared to SHAM. # P<0.05 compared to small-MI.

Table 2 Cardiac function of the isolated Langendorff heart

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SHAM (n=11)</th>
<th>Small-MI (5±2%) (n=7)</th>
<th>Large-MI (35±3%) (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV pressure (mmHg)</td>
<td>104±1</td>
<td>104±3</td>
<td>71±6*#</td>
</tr>
<tr>
<td>Contractility (mmHg sec⁻¹)</td>
<td>4178±137</td>
<td>4104±119</td>
<td>3187±4228*#</td>
</tr>
<tr>
<td>Relaxation (mmHg sec⁻¹)</td>
<td>3706±137</td>
<td>3514±163</td>
<td>1859±149*#</td>
</tr>
<tr>
<td>Heart rate (beats min⁻¹)</td>
<td>267±6</td>
<td>295±21</td>
<td>266±9</td>
</tr>
<tr>
<td>Coronary flow (ml min⁻¹ g⁻¹)</td>
<td>13.8±0.7</td>
<td>14.4±1.1</td>
<td>11.7±0.7*#</td>
</tr>
</tbody>
</table>

Data are mean±SEM, * P<0.05 compared to SHAM. # P<0.05 compared to small-MI.
Infarct size relates to EDHF impairment

**Endothelial function**

In the present study potential interference of vasoactive prostanoids in the dilatory responses was avoided with indomethacin to block cyclooxygenase in all experiments, hereafter referred to as total aortic relaxation to ACh.

**Large-MI rats**

Total aortic relaxation to ACh was significantly decreased in large-MI rats (Emax=44±6%, pD2=5.8±0.2, AUC=98±21) compared to SHAM controls (Emax=60±6%, pD2=6.7±0.1, AUC=157±18, P<0.05 for all three) *(Figure 1A)*. NO inhibition similarly decreased total ACh relaxation in SHAM and in large-MI. Hence, the NO-mediated relaxation was not significantly different in large-MI compared to SHAM *(Figure 1B, P=NS)*. In contrast, EDHF-mediated relaxation was significantly decreased in large-MI rats (Emax=15±2%, AUC=50±9) compared to SHAM (Emax=29±4%, AUC=80±11, P<0.05 for both) *(Figure 1C)*. The individual response-size for total ACh-induced relaxation *(Figure 2A)* as well as for EDHF-mediated relaxation *(Figure 2C)* were inversely correlated to individual infarct size (r=-0.71, P=0.02 and r=-0.64, P=0.04, respectively), whereas the relation of NO-mediated relaxation to infarct-size *(Figure 2B)* did not reach significance (r=-0.48, P=0.16). Finally, endothelium-independent relaxation to sodium nitrite was similar in both groups (Emax, pD2, AUC for SHAM and large-MI were 86±5%, 4.7±0.1, 145±16 and 85±4%, 4.5±0.2, 121±11, respectively, P=NS for all three) and was not related to infarct size.

**Small-MI rats**

Total relaxation to ACh in small-MI rats (Emax=71±4%, pD2=7.1±0.1, AUC=206±17) was significantly increased compared to SHAM controls (Emax=60±6%, pD2=6.7±0.1, AUC=157±18, P<0.05 for all three) *(Figure 3A)*. Furthermore, NO-mediated relaxation *(Figure 3B)* was significantly higher in small-MI compared to SHAM controls (P<0.05), whereas EDHF contribution was not significantly different *(Figure 3C)*. Endothelium-independent relaxation to sodium-nitrite was similar in both groups (Emax, pD2, AUC for SHAM and small-MI were 86±5%, 4.7±0.1, 145±16 and 91±4%, 4.7±0.1, 155±11, respectively, P=NS for all three).

**Discussion**

The rat coronary ligation model of CHF has been extensively used as an experimental model to investigate the pathophysiology of heart failure, including the development of endothelial dysfunction. The results concerning endothelial function and contribution of mediators remain controversial. Therefore, we studied the contribution of the endothelial mediators NO and EDHF in aortic endothelial dysfunction in this experimental model of CHF 12 weeks after coronary ligation. The main finding of this study is that the decreased aortic endothelium-dependent relaxation observed in rats with large infarct sizes may be due to an impaired EDHF pathway and relates to individual infarct size.

Rats with large-MI had increased lung and ventricular weights, displayed decreased aortic pressure and impaired *in vitro* cardiac function, demonstrating the presence of chronic cardiac failure. Consistent with the development of chronic cardiac failure rats showed endothelial dysfunction. The present study suggests an important role for impairment of the EDHF-
Figure 1 Total acetylcholine (ACh)-induced relaxation (A), NO-mediated relaxation (B) and EDHF-mediated relaxation (C) in rats with large myocardial infarctions (large-MI, n=10) compared to SHAM operated rats (n=11). * P<0.05
Figure 2 Scatterplot of individual infarct size versus total acetylcholine (ACh)-induced relaxation (A), NO-mediated relaxation (B), and EDHF-mediated relaxation (C) in rats with large myocardial infarctions.
**Figure 3** Total acetylcholine (ACh)-induced relaxation (A), NO-mediated relaxation (B) and EDHF-mediated relaxation (C) in rats with small myocardial infarctions (small-MI, n=7) compared to SHAM operated rats (n=11). * P<0.05
mediated dilative response in endothelial dysfunction in the rat coronary ligation model. This may be evidenced by the significant decrease in EDHF-mediated relaxation in large-MI rats compared to SHAM (which was similar in size to the total decrease in ACh relaxation) and by the significant inverse relation between EDHF-mediated relaxation and individual infarct size. However, NO-mediated relaxation was mainly preserved in large-MI rats and the relation between NO-mediated relaxation and infarct size did not reach significance. Until now, the involvement of the EDHF pathway in endothelial dysfunction in CHF has been controversial.

The relationship between impairment of EDHF and infarct size in animals with overt cardiac failure in our study suggests that the EDHF pathway may respond according to the severity of cardiac failure. The exact underlying mechanism of this relation can not be determined from the present study. However, CHF is known to be associated with profound hemodynamic alterations, among others with decreased blood pulsatility, blood flow and shear stress. These mechanical stimuli are believed to modulate the activity of endothelial mediators, such as NO and EDHF. Recently, it has been shown that EDHF synthesis is stimulated by pulsatile stretch. It may be hypothesized therefore, that hemodynamic alterations in CHF associated with decreased blood pulsatility may have contributed to decreased EDHF-mediated relaxation seen in the present study.

In the present study, coronary ligation was performed under control of blanching of the myocardium to induce large-MI. Notwithstanding the intended efforts for large-MI, infarct size was quite variable. It appeared to either cluster at 5% (small-MI) or at 35% (large-MI), as found in previous studies. As many studies employ the rats with small infarct sizes as controls, or pool them together with SHAM operated animals, we explicitly studied their endothelial function. Rats with small-MI showed normal haemodynamics and intact cardiac function. Consistently, they were devoid of any signs of heart failure. Interestingly, small-MI rats showed significantly increased endothelium-dependent relaxation due to increased NO-mediated relaxation as compared to the SHAM group. This phenomenon emphasizes the importance of the choice of the control group for the interpretation of endothelial function data in this model and may have contributed to some of the inconsistencies regarding endothelial dysfunction in CHF. As cardiac and hemodynamic function was identical in small-MI and SHAM rats, they do not explain the difference in endothelial function and NO-mediated relaxation between both groups. On the other hand, hearts of rats in the small-MI group - but not the SHAM group - experienced partial ischemia, at least temporarily. This may have provoked certain compensatory mechanisms in rats of the small-MI group, such as up-regulation of eNOS. An important argument against this explanation would be that these processes are likely to occur early after coronary ligation. Consequently, one could question whether they would still be of relevance 12 weeks after coronary ligation. An alternative explanation may be that an increased NO-mediated relaxation was inherent to individual rats prior to the infarction. As the level of vascular NO activity is rather variable among individuals in a normal population (cf. SHAM animals, Figure 4), the small-MI rats may represent a selected group of individuals characterized by increased NO activity that was already present before induction of MI. Indeed, the distribution of NO-mediated relaxation in the SHAM group overlaps those of the combined large- and small-MI group together (Figure 4). Such view would be compatible with previous speculations on the relation between genetically determined NOS activity and the occurrence of end-organ disease and also with findings of Baker et al. who reported that some rat strains revealed significantly smaller infarct sizes after induction of myocardial ischemia compared to other rat strains suggesting a genetic component of cardioprotection. In this respect it may be interesting to
perform further studies with a prospective study design to test if individual NO-mediated relaxation measured before induction of coronary ligation may determine development of individual infarct-size.

In summary, we showed the decreased endothelium-dependent relaxation in experimental CHF mainly due to impairment of the EDHF-mediated response. Endothelium-dependent relaxation as well as EDHF contribution were inversely related to individual infarct size. Moreover, care should be taken to use rats with very small infarcts as controls as their endothelial dilatory function was significantly increased compared to SHAM rats due to increased NO-mediated relaxation, despite similar cardiac and hemodynamic function. These results emphasize the important role of EDHF in endothelial dysfunction in CHF and underline the importance of the choice of control groups and included infarct sizes for interpretation of results on endothelial dysfunction.

Figure 4 Individual variation in NO-mediated relaxation in SHAM operated rats (n=11), in the total group of rats with myocardial infarctions (MI, n=17), and in rats which developed small myocardial infarctions (small-MI, n=7) and large myocardial infarctions (large-MI, n=10).
References