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VASODILATOR EFFECT OF ENDOTHELIN IN CUTANEOUS MICROCIRCULATION
OF HEART FAILURE PATIENTS

by

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ABSTRACT

The heart failure syndrome is associated with a reduced vasodilatory capacity in cutaneous microvessels. The aim of this study was to investigate the hypothesis that an altered activity of the endothelin system contributes to this reduction. The skin blood flow was recorded by laser Doppler flowmetry in patients with congestive heart failure and in age- and gender matched controls without clinical signs of heart failure. The vessels were stimulated by iontophoretic administration of endothelin-1 (ET-1). The involvement of the endothelin_A (ET_A) receptor was studied by co-administration of a specific antagonist; the role of the endothelin_B (ET_B) receptor was studied by the administration of the selective agonist sarafotoxin 6c. The plasma levels of ET-1, C-reactive peptide and N-terminal-pro-Brain Natriuretic Peptide were elevated in heart failure patients. Unexpected, ET-1 induced an ET_A mediated vasodilation. In the heart failure group the dilation was reduced to less than half as compared to control. The response to local warming was reduced in parallel indicating that the attenuation of the response in the heart failure group can be explained by the general decline in vascular reactivity. The response to ET_B receptor stimulation did not differ between the groups. The reduction in ET-1 responsiveness is paralleled by a general reduction in microvascular vasodilatory capacity, a phenomenon of increased vascular stiffness in the of heart failure subjects.

INTRODUCTION

Congestive heart failure (CHF) is a clinical syndrome characterized by a recognizable pattern of haemodynamic, renal, hormonal and neural responses (Poole-Wilson 1988). The reduced cardiac output induces compensatory mechanisms that increase the total peripheral vascular resistance (Zelis & Flaim 1982). In addition, heart failure patients have impaired capacity to dilate small and medium sized vessels in response to stimuli like increased metabolic demands and vasorelaxing autacoids (Drexler & Hornig 1999). This combination of an elevated vascular tone and a reduced capacity for vasodilation is likely to be important for the development of subjective symptoms and further deterioration of the condition (Zelis & Flaim 1982; Drexler & Hornig 1999; Andersson *et al.* 2003). Reports differ on whether the reduction in vasodilatory capacity is secondary to an endothelial dysfunction (Drexler & Hornig 1999) or to non-endothelial factors (Negrao *et al.* 2000). In our previous study we observed that elderly heart failure patients had an attenuated response to endothelium independent vasodilators in the cutaneous microcirculation (Andersson *et al.* 2003). The mechanism behind this dysfunction is not fully known. One possibility is that there is a defect within the vascular smooth muscle, a “vascular stiffness” (Zelis & Flaim 1982), but it could also be the result of increased activity in various vasoconstrictor systems acting on the muscle. Some of these mediators are well known, like norepinephrine and angiotensin II, but the activity of those systems cannot fully explain the impairment of the blood flow during exercise (Drexler & Hornig 1999). It has thus been suggested that the endothelin (ET) system is important for the changes in vascular reactivity (Negrao *et al.* 2000).

ET is a 21 amino acid residue peptide produced in various tissues *e.g.* the vascular endothelium, airway epithelium and myocardium (for references see Miyauchi & Goto 1999; Andersson *et al.* 1992). ET-1 is the most abundant of the three known ET isoforms. There are two known forms of ET-receptors, ET_A and ET_B. The latter is mainly expressed on endothelial cells where it causes the release of vasorelaxing factors such as nitric oxide. Both subtypes can be found on vascular smooth muscle cells where they mediate vasoconstriction. This response is mainly ET_A dependent, however (Miyauchi & Goto 1999; Cowburn *et al.* 2005). There are several reports indicating that activation of the ET-system, measured as elevated concentrations of the peptide, is a part of an acute response to severe physical stress and certain forms of inflammation. Elevated plasma levels have been found in patients with critical illness like acute asthma attacks, myocardial infarction, and sepsis (Nomura *et al.* 1989; Haak *et al.* 1994). Similarly, there are transient increases in local concentrations of ET-1 in various inflammatory models (Andersson *et al.* 1992; 1995; 1999). The receptor population is also subject to dynamic changes: *e.g.* in conditions like cerebrovascular disease (Hansen-Schwarz *et al.* 2002) and atherosclerosis (Dagassan *et al.* 1996) the receptor number is reported to be increased. This seems to be associated with the appearance of an ET_B mediated vasoconstriction (Uddman *et al.* 1999, Pernow *et al.* 2000, Stenman *et al.* 2002).

Inflammation is a part of the CHF syndrome and accordingly it seems that the ET system is activated. The plasma levels of ET-1 are elevated and inversely correlated to maximal oxygen consumption. Furthermore, elevation of circulating ET-1 predicts poorer prognosis (see: Duchman *et al.* 2000; Miyauchi & Goto 1999). Part of

the vascular tone in CHF patients appears to be ET_A receptor dependent (Lüscher *et al.* 2002; Cowburn *et al.* 2005. Based on these observations we hypothesized that our previous finding of an attenuated sensitivity to endothelium independent vasodilators might be paralleled by enhanced vascular sensitivity for ET-1. This was investigated by comparing the changes in cutaneous blood flow induced by local administration of either ET-1 (mixed ET_A and ET_B agonist) or a selective ET_B receptor agonist in CHF patients and matched controls. The effects in vascular reactivity were correlated to maximal vasodilatory capacity to local warming, to the plasma levels of ET-1, to inflammatory activity and to the levels of N-terminal pro brain natriuretic peptide (NT-proBNP) as a marker of CHF severity.

MATERIALS AND METHODS

Ethics

The study was conducted in accordance with the Declaration of Helsinki. The Ethics Committee of Lund University approved of the protocol (LU 806-01). Informed consent was obtained from all subjects.

Subjects

Studies were performed on two groups:

1. Fifteen patients (12 men, 3 women) treated at Lund University Hospital for CHF (Table 1). Mean age was 75 years (range 65-81). An echocardiograph was performed and in the cardiologist view the findings were consistent with heart failure. In addition, elevated plasma levels of NT-proBNP were found (Table 2). The criteria for inclusion was NT-proBNP over 700 ng/L. Patients with diabetes mellitus, uremia, ongoing infection, dementia, active smoking habit or the presence of tremor were excluded as well as those on medication with long-acting nitrates or oxygen. Eight patients were judged to be in New York Heart Association functional class IV, one in class IIIb and the remaining in class IIIa. Five patients had atrial fibrillation, one had pacemaker while the remaining were on sinus rhythm. The judged cause of the CHF is given in table 1.
2. Control subjects, matched for age and gender to the CHF patients. These subjects were first seen by a specialist in general medicine, then by a specialist in internal medicine. They were not allowed to have clinical signs of CHF or plasma levels of

NT-proBNP exceeding the cut off level. One male subject was excluded because of elevated NT-proBNP. Basal characteristics for the subjects are given in table 2.

Pharmacological treatment

At the time of the study all of the patients were treated with diuretics. Four were on medication with digitalis, ten on ACE-inhibitors, ten on β -adrenergic antagonists, four on warfarine, three on allopurinol, one on statin, eight on salicylic acid, six on glucocorticosteroids, five on bronchodilators and five on proton pump inhibitors. In the age-matched control group three subjects were taking diuretics, four on β -adrenergic blockers, one on glucocorticosteroids, three on calcium channel blockers, one on a proton pump inhibitor and three on salicylic acid.

The study population has a large variability with regard to origin of the heart failure and the ongoing medication. The aim of the study was to further elucidate the findings in Andersson *et al.* (2003). The populations in these two studies are comparable, with a similar size of the groups, age of participants, underlying diseases, medication and, most important, reduced vasodilatory capacity. The present heart failure group had a more severe disease, however. Despite the heterogeneous consistency of the patient groups we thus find it justified to use the present population for the study of these specific questions study. It can specifically be noted that the abnormal vascular function is present despite ongoing medication with a number of drugs with putatively vasorelaxant effects.

Measurement of plasma samples

The ET-1 levels in plasma were quantified by radioimmunoassay as previously described (Hemsén & Lundberg 1991). Using antiserum E1 and iodinated ET-1 (Amersham Pharmacia Biotech) as tracer. The detection limit for the assay was 3.9 pmol/tube and the intra- and interassay variations were 6% and 14%, respectively. The crossreactivity of the E1 antiserum is: ET-1, 100%, ET-2, 27% and ET-3, 8%.

CHF is associated with a low-grade inflammation. In a previous study we also found that CHF was associated with a reduction in LDL-cholesterol and elevated blood glucose levels (Andersson *et al.* 2003). Therefore these parameters, as well as NT-proBNP, CRP and creatinine were measured. Blood samples were analyzed at the dept. of Clinical Chemistry, Lund University Hospital, except for ET-1 determinations, which were analyzed at the Karolinska Institute.

Blood flow measurements

Cutaneous blood flow was measured using the PeriFlux System 5000 (Perimed, Järfälla, Sweden). This method is non-invasive and gives minimal discomfort to the studied subject. Laser-generated light of wavelength 780 nm is directed to the skin using a fiberoptic probe. The light reflected from moving blood cells in the superficial skin micro-vessels undergoes a shift in frequency (Doppler effect) that is proportional to the number and velocity of the moving blood cells. The laser Doppler output is semi quantitative; we have below presented all data as per cent change compared to baseline perfusion value. The temperature of the skin was continuously recorded.

Iontophoresis

Constant current iontophoresis was used to enhance the perfusion of charged molecules into the skin of the dorsal side of the lower arm. This mode of administration was chosen since we wanted to adhere to the former protocol (Andersson *et al.* 2003) and to avoid injections and local irritation in the measurement area. Two series of stimulations were performed, one with human ET-1 and one with S6c.

The Perilont System (Perimed) used in this study contains an applicator with a small recess in the centre and circular temperature probe surrounding the application site. The recess in the centre allows the insertion of a fiberoptic probe to measure the blood flow in the stimulated area. An additional temperature probe containing a fiberoptic probe was placed at suitable distance, avoiding big veins. This was used as a reference during the iontophoresis and was after this used to determine the response to local warming (for references regarding the method see Andersson *et al.* 2003).

Protocol

All studies were performed in a temperature-controlled room 22-24 °C. All subjects were resting in the supine position. Blood pressure and heart rate were measured. The skin of the lower arm was gently cleaned with an alcohol swab. The iontophoretic applicators / fiberoptic probes were applied to the forearm. The basal blood flow was studied for 2 min after which ET-1 was transferred by iontophoresis (40 mA for 60 sec, anodal current). The current alone did not affect the in blood flow (data not

shown). Repeating the iontophoretic stimulation four times at 60-sec intervals produced a stimuli-response curve. Thereafter S6c was administered in the same way using the same current and time intervals. Finally the response to heat was measured following local warming to +44°C for 10 min.

In an additional series the specificity of the ET-1 response was investigated in three subjects. First ET-1 was administered as above. This was then followed about 2-3 minutes later by iontophoresis of ET-1 mixed with the ET_A receptor antagonist FR 139317 given in ten times excess. Administrations were given on the same arm about ten cm apart. In separate series the solvents were studied and found to have no effect on the response (data not shown).

Substances

ET-1 and S6c (both Sigma Chemical Company, St. Louis, Mo, USA) were used in the concentration 10 µM dissolved in MilliQ water with 0.1% bovine serum albumin (BSA) added to avoid adhesion to glass material. FR 139317, (R)2-[(R)-2-[(S)-2[[1-(hexanydro-1H-azepinyl)]carbonyl]amino-4-methyl-pentanoyl]amino-3-[3-(1-methyl-1H-indolyl)]propionyl]]-amino-3-(-2-pyridyl)propionic acid, was a generous gift from Dr. Jo Morii, Fujisawa Pharmaceuticals, Osaka, Japan. It was first dissolved as a 10⁻³ M stock solution in 150 µl ethanol followed by 1850 µl MillQ water. This was further dissolved in MilliQ water with 0,1% BSA. The final solution was calculated to contain no more than 0.08% ethanol.

Data, Statistical analysis

Data are given as mean \pm standard error of the mean. Statistical analysis was performed by the Mann-Whitney U test. This non-parametric test was chosen since we have no reliable data that our material has a normal distribution in all groups. For analysis of the stimuli-response curves an estimate for the area under the curve (AUC) was performed for each patient by adding the values for the respective stimulations. The resulting numbers was then used for determination of statistical significance. Calculations were performed using StatView 5.0, Berkeley, CA.

RESULTS

Blood flow responses

Base line blood flow did not differ significantly between the two groups for any of the modes of stimulation. For ET-1 the basal flow was 6.9 ± 1.3 flow units in the CHF and 7.9 ± 1.1 in the control group. Corresponding values for S6c were 8.8 ± 1.5 and 8.6 ± 1.8 . Before warming the flows were 11.3 ± 1.5 and 8.4 ± 1.0 .

Iontophoresis of ET-1 induced a step-wise increase in local blood flow. This response was attenuated in the CHF group. The value for AUC was reduced in parallel with the reduced response to heat. Also S6c induced hyperemia. This response was smaller in magnitude in the controls than the response to ET-1. There was no difference in S6c response between the groups (Table 3).

In order to elucidate the mechanism behind the ET-1 induced vasodilation additional control experiments were performed on three healthy volunteers. In these, ET-1 was administered together with the ET_A receptor antagonist FR 139317 given in ten times excess. This abolished the vasodilatory response almost entirely (Fig. 1).

Plasma samples

The mean NT-proBNP level was more than thirty-fold higher in the heart failure group compared to the controls (Table 2). There was also a significant elevation of the ET-1 concentration. The elevation of CRP indicates an ongoing inflammatory reaction. The renal function seemed to be reduced in the heart failure group as indicated by the higher creatinine level. The LDL-cholesterol level was significantly lower in the CHF-

group but there was no significant difference between the groups in HDL-cholesterol or blood glucose levels.

Correlations

The sensitivity to ET-1 measured as the AUC for the relative increase in blood flow correlated negatively to the NYHA level ($r=-0.4$; $p<0.05$). There were positive correlations to LDL-cholesterol ($r=0.4$; $p<0.05$), to the hyperemic response to heat and to age (both: $r=0.4$; $p<0.05$).

The response to warming correlated negatively to the severity of heart failure, measured as plasma NT-proBNP ($r=-0.4$; $p<0.05$), NYHA functional class ($r=-0.6$; $p<0.001$) and sCRP concentration ($r=-0.4$; $p<0.05$). There was a positive correlation to the LDL-cholesterol level ($r=0.4$; $p<0.05$).

The response to S6c expressed as AUC relative to the response to heat correlated to BMI ($r=0.5$; $p<0.05$) and creatinine ($r=0.4$; $p<0.05$). There was a negative correlation to LDL-cholesterol ($r=-0.4$; $p<0.05$).

The NT-proBNP level was correlated to the NYHA class ($r=0.6$; $p<0.001$) and to plasma creatinine ($r=0.5$; $p<0.001$). There was also a negative correlation between NT-proBNP and LDL-cholesterol ($r = -0.4$, $p<0.05$).

The plasma ET-1 level correlated negatively to the LDL-cholesterol level ($r=-0.4$; $p<0.05$) and to the MAP ($r=-0.4$; $p<0.05$).

DISCUSSION

This study was performed to test the hypothesis that the part of the increased vascular tone in patients with CHF is due to an altered sensitivity for ET-1.

Unexpected, we found that ET-1 increased the blood flow. To our knowledge, there are no reports of a direct ET_A mediated vasodilation, which leaves the possibilities that the effect was either mediated by ET_B receptors, unspecific with regard to ET receptors, or an indirect ET_A mediated response.

The ET_B receptors could mediate both vasodilation and constriction. It has been shown in a recent study that the predominating ET_B effect in CHF is to induce vasorelaxation and to remove ET-1 from the circulation (Cowburn *et al.* 2005). In accordance, we found that the specific ET_B agonist S6c induced vasodilation, a likely ET_B mediated effect. There is thus a possibility that these receptors are involved in the observed ET-1 response. However, since ET_A blockade almost entirely abolished the response to ET-1 and there was no co-variation between the responses to ET-1 and S6c it seems unlikely that this was the only mechanism behind the hyperemia.

Iontophoresis of vehicle alone did not affect the local blood flow, which together with the results from receptor blockade makes an unspecific response less plausible. Furthermore, the receptor antagonist FR139317 has been evaluated before and found to be fairly selective for ET_A receptors (Adner *et al.* 1994)

The most likely explanation for the ET-1 mediated vasorelaxation is thus an indirect ET_A receptor dependent mechanism. Such responses have been described previously: When ET-1 is injected into the skin it induces an intense

vasoconstriction at the injection site with a surrounding flare. Results suggest that the flare component is partially histamine-dependent and the result of an axon reflex (Crossman *et al.* 1991). A likely interpretation of our data is thus that S6c induced an ET_B mediated vasodilation but that the ET-1 response was mainly via an indirect ET_A mediated effect.

The response to heat was markedly reduced in the heart failure group indicating a general vasomotor dysfunction. In our previous study (Andersson *et al.* 2003) this parameter did not reach statistical significance. The patients in the present study had a more severe CHF disease. It could thus be that the vasodilatory capacity is reduced in parallel with the severity of the disease. This is supported by the correlation between the response to heat and degree of CHF, measured either as NT-proBNP or NYHA functional class. The response to local warming correlated negatively to the CRP concentration, which is in accordance with the hypothesis that the reduction in vascular function is secondary to the inflammatory response. It should be noted that the vascular dysfunction was present despite ongoing medication for heart failure with drugs that in many cases have vasodilatory effects. This impairment is likely to contribute to the patient's subjective symptoms like intolerance for high temperatures and reduced exercise capacity. It can also lead to an increased cardiac workload and thus further deterioration of the condition.

The vasodilatory responses to ET-1 and to local warming were correlated and it can be concluded that the sensitivity to ET-1 follows the general decline in vascular reactivity. It can be concluded that it is not possible to find any evidence for an ET_A mediated increase in vascular tone using the present method. Since it has

been shown that a part of the tone is ET-1 dependent (Lüscher *et al.* 2002; Cowburn *et al.* 2005) and since the counteracting vasodilatory ability is severely impaired it is still a likely possibility that the relative importance of ET-1 is raised. The response to S6c relative to the heat response was higher in the heart failure group, but the difference did not reach statistical significance. There is thus a possibility of some up-regulation of vasodilatory ET_B receptors in skin vessels but additional studies are needed to confirm this.

In accordance with several previous reports we found that the mean plasma level of ET-1 is elevated in CHF patients. There was no correlation between this parameter and the vasodilatory responses, inflammatory activity or severity of the disease, however. In CHF most of the circulating ET-1 is produced in the lung (Lepailleur-Enouf *et al.* 2001) and it seems plausible that the plasma concentration to a large extent is determined by the degree of pulmonary hypertension. ET-1 has a short half-life in the circulation and is believed mainly to act in a paracrine way. The plasma concentration is thus less likely to be a good measure of the activity of the ET-system in the peripheral vasculature. Still, there was an inverse relation between the plasma concentration and MAP, however, suggesting some involvement in blood pressure regulation. Also from this parameter an increased production of ET-1 could be more important in a situation with vasodilatory dysfunction.

The response to S6c correlated to BMI which makes a connection between obesity and the ET-system possible. There are some other reports linking these; so does leptin up-regulate ET-1 production in endothelial cells (Quehenberger *et al.* 2002).

Vascular risk factors, CHF and blood flow

In our previous study (Andersson *et al.* 2003) we observed that several vascular risk factors were correlated to the changes in vascular function in CHF patients. One of these, the LDL-cholesterol, was however, found to be reduced and negatively correlated to the vascular function. This unexpected finding was confirmed in the present study. The LDL-cholesterol was the one parameter with most correlations to other measures: It correlated negatively to disease severity (NT-proBNP and NYHA functional class), to the plasma ET-1 concentration and to blood vessel function (responses to heat and ET-1). There was a positive correlation to blood pressure. It could thus be suggested that LDL is associated with important mechanisms in the CHF syndrome. This is in line with reports that a low cholesterol concentration is associated to poor clinical outcome in the CHF syndrome (Rauchhaus *et al.* 2000). The details of this connection and its importance are not fully known. LDL-cholesterol is known to be a negative acute phase reactant (Carpentier & Scrueel 2002) and could thus be reduced by the inflammatory reaction. In our previous study we found that the LDL concentration correlated to the plasma level of interleukin-6. As in the present study there was no significant co-variation with the CRP level, however.

Taken together the present study shows that ET-1, administered by iontophoresis, is a vasorelaxant in cutaneous microvessels. This response is attenuated in CHF patients, in parallel with a general decline in vasodilatory capacity. No specific alteration in sensitivity to ET-1 could be detected but the peptide could as

a vasoconstrictive agent have an increased influence due to the impairment of the vasorelaxant ability.

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FIGURE and TABLE LEGENDS.

Figure 1.

The vasodilatory response (increase in AUC flow units) following iontophoretic application of ET-1 (10 μ M) followed by ET-1 combined with the ET_A receptor antagonist FR 139317 (100 μ M) to three healthy volunteers.

Table 1.

Underlying disease judged as cause for the heart failure

Table 2.

Basal characteristics and laboratory values for the subjects at the day of study.

Table 3.

Changes in blood flow following stimulation with local warming or by ET-1 and S6c administered by iontophoresis. Values are given as increase in AUC for ET-1 and s6c and as the maximal increase for heat. Statistical significance calculated using Mann-Whitney U-test.

Table 1

Ischemic heart disease:	5 patients
Dilated cardiomyopathy:	2
Toxic cardiomyopathy:	1
Hypertension	2
Valvular disease	1
Myocardial sarcoidosis	1
Unknown	3

Table 2

	CHF	Control	Significance
NYHA	3.6 \pm 0,1	0 \pm 0	p<0.0001
BMI (kg/m ²)	27.2 \pm 1.5	24.4 \pm 0.9	p=0.25
MAP (mm Hg)	92.9 \pm 5.0	95.1 \pm 2.1	P=0.69
Body temp. (C)	37.0 \pm 0.06	37.0 \pm 0.02	p=0.9
Pro-BNP (ng/l)	6082 \pm 1403	183 \pm 44	p<0.0001
LDL-cholesterol (mM)	2.5 \pm 0.21	3.56 \pm 0.20	p=0.0024
HDL-cholesterol (mM)	1.5 \pm 0.16	1.7 \pm 0.12	p=0.21
Creatinine (μ M)	96.4 \pm 7.0	76.5 \pm 3.4	p=0.067
B-glucose (mM)	6.8 \pm 0.6	5.6 \pm 0.2	p=0.14
sCRP (mg/l)	25.0 \pm 6.4	2.9 \pm 0.7	p=0.0018
Heart rate (bpm)	76.7 \pm 4.2	74.5 + 2.5	p=0.8
ET-1 (pM)	7.2 \pm 0.5	5.8 \pm 0.2	p=0.026

Table 3

Stimuli	CHF	Control	Significance
ET-1	483 \pm 103 %	1334 \pm 378 %	p=0.029
Heat	776 \pm 99 %	2014 \pm 304%	p=0.0003
S6c	463 \pm 133 %	546 \pm 87 %	p=0.26
ET-1 / Heat	72.4 \pm 15.2 %	85.7 \pm 23.7 %	p=0.95
S6c / Heat	57.1 \pm 19.6 %	35.6 \pm 7.8 %	p=0.66

