RESEARCH ARTICLE

Influence of Mechanical Circulatory Support on Endothelin Receptor Expression in Human Left Ventricular Myocardium from Patients with Dilated Cardiomyopathy (DCM)

Florian Gärtner¹, Getu Abraham², Astrid Kassner¹, Daniela Baurichter¹, Hendrik Milting¹*

¹ Erich & Hanna Klessmann Institute for Cardiovascular Research and Development, Clinic for Thoracic and Cardiovascular Surgery, Heart and Diabetescenter NRW, Bad Oeynhausen, Germany, ² Institute of Pharmacology, Pharmacy and Toxicology, University of Leipzig, Leipzig, Germany

* hmitting@hdz-nrw.de

Abstract

Background

In terminal failing hearts ventricular assist devices (VAD) are implanted as a bridge to transplantation. Endothelin receptor (ETR) antagonists are used for treatment of secondary pulmonary hypertension in VAD patients. However, the cardiac ETR regulation in human heart failure and during VAD support is incompletely understood.

Methods

In paired left ventricular samples of 12 dilated cardiomyopathy patients we investigated the density of endothelin A (ET_A) and B (ET_B) receptors before VAD implantation and after device removal. Left ventricular samples of 12 non-failing donor hearts served as control. Receptor quantification was performed by binding of [¹²⁵I]-ET-1 in the presence of nonselective and ET_A selective ETR ligands as competitors. Additionally, the ETR mRNA expression was analyzed using quantitative real-time-PCR.

Results

The mRNA of ET_A but not ET_B receptors was significantly elevated in heart failure, whereas total ETR density analyzed by radioligand binding was significantly reduced due to ET_B receptor down regulation. ET_A and ET_B receptor density showed poor correlation to mRNA data (spearman correlation factor: 0.43 and 0.31, respectively). VAD support had no significant impact on the density of both receptors and on mRNA expression of ET_A whereas ET_B mRNA increased during VAD. A meta-analysis reveals that the ET_A receptor regulation in human heart failure appears to depend on non-failing hearts.

Conclusions

In deteriorating hearts of patients suffering from dilated cardiomyopathy the ET_A receptor density is not changed whereas the ET_B receptor is down regulated. The mRNA and the
proteins of ET\textsubscript{A} and ET\textsubscript{B} show a weak correlation. Non-failing hearts might influence the interpretation of ET\textsubscript{A} receptor regulation. Mechanical unloading of the failing hearts has no impact on the myocardial ETR density.

**Introduction**

Endothelins have been described as potent vasoconstrictor peptides consisting of 21 amino acids secreted by the endothelium [1]. Three isoforms, designated ET-1, ET-2 and ET-3 have been identified [2]. ET-1, the principal isoform is secreted by many cell types beyond endothelial cells including cardiac myocytes [3, 4].

Endothelins mediate their activity mainly in an autocrine/paracrine manner [5, 6] on its receptors of which two have been identified, named endothelin receptor A (ET\textsubscript{A}) and B (ET\textsubscript{B}). Endothelin receptors are G-protein-coupled receptors and mediate their action essentially in a phospholipase C dependent manner. They are expressed in a variety of tissues including the myocardium.

The endothelin system is involved in cardiovascular functions. ET\textsubscript{A} receptor expressed by vascular smooth muscle cells mediates prolonged vasoconstriction whereas the ET\textsubscript{B} receptor which is located on endothelial cells is primarily considered to cause NO-mediated vasodilatation [7].

In the heart, endothelin contributes to contractility [8], chronotropy [9] and fibrosis [10] and induces myocyte hypertrophy [11]. Hypertension, pulmonary hypertension and heart failure are associated with changes of the endothelin system [12, 13]. Bosentan, a dual endothelin receptor antagonist is used for treatment of secondary pulmonary hypertension in patients with mechanical circulatory support (MCS) [14] or awaiting heart transplantation [15].

Ventricular assist devices (VAD) are implanted in terminal failing heart patients as a bridge for heart transplantation or as destination therapy. Cardiac regeneration under mechanical unloading by VAD was repeatedly reported [16, 17]. However, the cellular and molecular changes responsible for these salutary effects as well as the implication of the endothelin system in the cardiac amelioration are incompletely understood. Morawietz et al. found a normalization of increased ET\textsubscript{A} receptor expression levels in 7 of 10 patients with end-stage heart failure after VAD unloading on mRNA level while ET\textsubscript{B} expression remained unchanged [18]. However, data on the protein expression during VAD support are not available so far.

In this study, we investigated the expression of both endothelin receptors on the level of mRNA and protein in parallel in paired myocardial samples of dilated cardiomyopathy (DCM) patients receiving a VAD.

**Material and Methods**

**Study cohort**

Transmural left ventricular (LV) myocardium of 12 male patients with end stage non-ischemic DCM receiving a continuous flow VAD (DuraHeart, Heartmate II, HeartWare or VentrAssist, respectively) at the age of 46.8 ± 11.1 years was collected from the para-apical region during VAD implantation (VAD-IP) and later from the same patient at heart transplantation (VAD-HTx). Samples without macroscopic signs of fibrosis were snap frozen in liquid nitrogen and stored at -80°C until analysis. The time of VAD-support was 722 ± 436 days (mean ± SD).
patients provided written informed consent for the use of their cardiac tissue samples. For clinical data of the patients, see Table 1. LV myocardial samples were also obtained from non-failing (NF) donor hearts which could not be transplanted for diverse reasons. The donor cohort was sex (12 male) and age (47.0 ± 11.0 years) matched with DCM patients at the time of VAD-implantation. The ethics committee of the Ruhr University Bochum at the Heart and Diabetes-center NRW in Bad Oeynhausen, Germany, approved the study which conforms to the declaration of Helsinki (Vote No. 21/2013).

**mRNA quantification**

RNA was isolated from left ventricles using commercial kits (RNeasy, Qiagen, Hilden, Germany). Reverse transcription of myocardial RNA was done using 250 ng total RNA and 50 units Superscript II (Life Technologies GmbH, Darmstadt, Germany) after random priming with hexamers. mRNA of ET\(_A\) and ET\(_B\) receptors was quantified in duplicates with the ΔΔ\(_C_T\) method in a StepOne Plus (Applied Biosystems, Foster City, US-CA) using TaqMan probes labeled with FAM and TAMRA. Normalization was performed using the geometric mean of the three reference genes RPL13A, TOP2B and TPT1, which were selected from 10 candidate genes and validated using the Visual Basic Application geNorm (M values < 0.5) [19].

Sequences for primers and probes used for real-time experiments were:

ET\(_A\) sense 5'-TG GTTGGACGTGGATCTCC-3'; ET\(_A\) probe 5'-ACCCCTATGACCTGGAGATGTTG-3'; ET\(_A\) antisense 5'-GCAATTCTCAAGCTGCCATTC-3'; ET\(_B\) sense 5'-CGGCCAGGCAACCACT-3'; ET\(_B\) probe 5'-CTCTCCCCGCTGCAAGGACC-3'; ET\(_B\) antisense 5'-TTGATGTA TTGGAAGTCTCCTGATCT-3'; RPL13A sense 5'-TCACGAGGTTGGCTGGATA-3'; RPL13A antisense 5'-CTTGGCT TTCTTTCTCTCTCTC-3'; RPL13A probe 5'-AGGCGCGGTATGCCCG-3'; TPT1 sense 5'-AGTTAATCTGTCTTCCGTTACC TT-3'; TOP2B sense 5'-GCTAAAGGAAAAACCGCTTCC-3'; TOP2B antisense 5'-AGTTTACAGCTCTACTACTCTTT

**Table 1. Clinical data at the time of MCS-implantation and medication during VAD support.**

| patient No. | gender | disease  | age [y] | LVEDD | LVESD | EF   | CI   | PCW  | CVP  | PAP    | Medication during VAD support |
|-------------|--------|----------|---------|-------|-------|------|------|------|------|--------|--------------------------------|
| #1          | M      | DCM      | 41      | n.a.  | n.a.  | 20   | 2.7  | 20   | 18   | n.a.   | βB; MA; AI                      |
| #2          | M      | DCM      | 51      | 72    | 65    | 20   | 1.4  | 33   | 23   | 38     | βB, MA; AI; EB                  |
| #3          | M      | DCM      | 59      | 61    | 56    | 20   | 1.3  | 27   | 10   | 32     | n.a.                            |
| #4          | M      | DCM      | 30      | n.a.  | n.a.  | 15   | n.a. | n.a. | n.a. | 14     | βB, MA; AI                      |
| #5          | M      | DCM      | 54      | 78    | 72    | 25   | 2.2  | 12   | 19   | 45     | βB, MA; AI                      |
| #6          | M      | DCM      | 64      | n.a.  | n.a.  | 20   | 1.5  | 29   | 18   | n.a.   | βB, MA; AI                      |
| #7          | M      | DCM      | 32      | 59    | 57    | 10   | 1.8  | 15   | n.a. | n.a.   | MA; AI                          |
| #8          | M      | DCM      | 45      | 104   | 94    | 20   | 2.0  | 38   | 19   | 48     | βB, MA; Al                       |
| #9          | M      | DCM      | 37      | 88    | 82    | 20   | 2.9  | 17   | 16   | 46     | βB, MA; AI                      |
| #10         | M      | DCM      | 55      | 75    | 67    | 27   | 2.0  | 19   | 1.4  | 33     | βB, MA; Al                      |
| #11         | M      | DCM      | 58      | 70    | 62    | 30   | 1.8  | 19   | 1.4  | 33     | βB, MA; Al                      |
| #12         | M      | DCM      | 35      | 80    | 72    | 20   | 1.8  | 19   | 1.4  | 33     | βB, MA; Al                      |
| means (SD)  | 47 (11)| 76 (13)  | 70 (11)| 21 (5)| 2.0 (0.5)| 23 (8)| 15 (5)| 36 (11)|

**Abbreviations:** AI = ACE-inhibitor (enalapril or ramipril); βB = β-adrenergic receptor blocker (carvedilol or bisoprolol); CI = cardiac index [l min\(^{-1}\) m\(^{-2}\)]; CVP = central venous pressure [mm Hg]; DCM = dilative cardiomyopathy; EB = endothelin receptor blocker (Bosentan); EF = ejection fraction (echo) [%]; LVEDD = left ventricular end diastolic diameter [mm Hg]; LVESD = left ventricular end systolic diameter [mm Hg]; MA = mineralocorticoid-receptor antagonist (spironolactone or eplerenone); PAP = pulmonary artery pressure [mm Hg]; PCW = pulmonary capillary wedge pressure [mm Hg].

doi:10.1371/journal.pone.0169896.t001

VAD and Cardiac ETR Expression in DCM Hearts

PLOS ONE | DOI:10.1371/journal.pone.0169896 January 17, 2017 3 / 12
CTTCTGTGTT-3'. PCR conditions were: 95°C, 10'; 95°C, 15’; 60°C, 1’; 40 cycles; 300 nM primer; 150 nM probe.

Membrane preparation

150 mg of LV myocardium was minced with scissors and homogenized in 1 mL ice-cold homogenization buffer (50 mM Tris-HCl buffer, pH 7.4, with 1 mM EDTA and 2% BSA) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, US-MO, #P2714) using a Ribo-lyser (Peqlab Erlangen, Germany) for 20 seconds 3 times in 3-minute intervals. Samples were diluted to 20 mL with homogenization buffer and centrifuged at 700 g for 15 minutes. The supernatant was passed through four layers of cheesecloth and centrifuged at 50,000 g for 25 minutes. The pellet was washed once by resuspension in homogenization buffer without BSA and centrifugation and finally resuspended in resuspension buffer (39.8 mM HEPES, pH 7.4, 135 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl₂, 0.3 mM Na₂HPO₄, 1.2 mM CaCl₂, 0.4 mM MgSO₄, 4 mM NaHCO₃) containing protease inhibitor cocktail. Protein content was determined using bicinchoninic acid and BSA as a standard. Membranes were diluted in incubation buffer (0.2% BSA in resuspension buffer) containing protease inhibitor to yield a protein concentration of 15 μg/40 μL and stored at -80°C.

Radioligand binding studies

For determination of the total amount of endothelin receptors, competitive binding experiments were performed in a total of 1 mL incubation buffer. A constant amount of ~10,000 cpm of [¹²⁵I]-ET-1 in 100 μL was mixed with 6 different concentrations of unlabeled ET-1 (500 μL) as a competitor ranging from 2 to 486 pM in the final solution. Membranes were diluted 1:10 and 400 μL of the membrane suspension (15 μg of protein) was added to the ligands and incubated for 60 minutes at 37°C in siliconized (Sigmacote, Sigma-Aldrich) glass tubes in a shaking water bath. Binding was stopped by the addition of 14 mL incubation buffer. Bound ligand was separated by vacuum filtration through glass fiber filters (Millipore, Billerica, US-MA) presoaked with incubation buffer containing 4% BSA and a washing step with another 14 mL of incubation buffer. The activity of the filters was determined in a gamma counter (Multi Crystal LB2111, Berthold Technologies, Bad Wildbad, Germany). The total binding without competitor which yields the top plateau of the competition curve was determined without ET-1 in quadruplicates. Residual activity not displaced by 1 μM Bosentan was defined as nonspecific binding (bottom plateau of the competition curve) and determined in duplicates. For every sample the displacement experiment was repeated once. Analyses were performed using the software GraphPad Prism 6 (GraphPad Software, La Jolla, US-CA) and a nonlinear regression (One site–Fit log IC₅₀). The receptor density (Bₘₐₓ) was calculated as (Bₒ x IC₅₀) / [L] [20]. Bₒ was obtained by subtracting the nonspecific binding from the total binding; IC₅₀ was regarded as constant and set to 49.3 pM which was the mean IC₅₀ value of all competition curves.

To calculate the ratio of ETₐ and ETₜ, displacement was performed with 8 different concentrations of the ETₐ selective antagonist BQ-123 ranging from 50 μM to 640 pM. Total binding and nonspecific binding was determined in duplicates each. The displacement experiment with BQ-123 was repeated once for every sample. The ratio of ETₐ and ETₜ receptors were calculated from a nonlinear regression (Two sites–Fit log IC₅₀) using the software GraphPad Prism 6.

Determination of collagen content using 4-hydroxyproline measurements

The collagen content in tissue samples was determined by aminoacid analysis of 4-hydroxyproline using the chloramin-T method [21]. Two independent tissue samples were weighted
and hydrolyzed in 6 M HCl. Analysis was done in duplicate for each sample as published before [22].

**Determination of plasma Big-ET1**

Plasma Big-ET1 before and 28 ± 11 (mean ± SD) days after VAD-implantation was measured using a commercially available ELISA according to manufacturer’s conditions (Biomedica, Vienna, Austria).

**Results**

We quantified endothelin receptors by quantitative real-time-PCR in paired (pre- vs. post-VAD) myocardial samples of 12 VAD patients with DCM. In parallel the receptor density was quantified by radio ligand binding with $^{[125]}$I-ET-1. The total receptor number was obtained according to DeBlasi *et al.* by competition with unlabeled ET-1 [20] (see S1 Fig for representative competition curves). For the assessment of the relative amount of the two endothelin receptor subtypes ET$_A$ and ET$_B$ the binding of the radioligand was inhibited by BQ-123. This resulted in biphasic competition curves (see S2 Fig for representative curves). We compared our findings to 12 rejected donor hearts which were matched according to gender and age.

The LV mRNA of the ET$_A$ receptor was significantly higher ($p < 0.001$, one way ANOVA and Bonferroni’s multiple comparisons test) in the failing myocardium of patients compared to NF hearts (0.61 ± 0.22 vs. 0.34 ± 0.09 ([arbitrary units] means ± SD); Fig 1A). After VAD-support the elevated mRNA of ET$_A$ (0.69 ± 0.25) was not changed in comparison to the time of device implantation and remained on a significantly ($p < 0.01$) elevated level compared to control myocardium. In contrast, we did not find different concentrations of ET$_B$ mRNA in failing myocardium compared to rejected donor hearts (NF, pre-VAD: 0.91 ± 0.45, 0.61 ± 0.22; Fig 1B). However, the mRNA of ET$_B$ was significantly upregulated after VAD (0.99 ± 0.29; $p < 0.05$).

Thus, the ET$_A$ receptor mRNA was increased in terminal failing hearts and not regulated by...
mechanical unloading, whereas the ET\textsubscript{B} receptor mRNA was only regulated during VAD support but not different between NF and samples from failing hearts. Radioligand binding was done for total endothelin receptor binding (Fig 2A) and for receptor subtype analysis (Fig 2B). The mean $K_D$ of $[^{125}\text{I}]-\text{ET}-1$ was replaced by varying amounts of unlabeled ET-1. B) Receptor subtype analysis. For the assessment of the relative amount of receptor subtypes, $[^{125}\text{I}]-\text{ET}-1$ was replaced by varying amounts of the selective ET\textsubscript{A} receptor antagonist BQ-123. C-D) Density of the ET\textsubscript{A} receptor (C) and the ET\textsubscript{B} receptor (D) as calculated from both replacement experiments. All experiments were performed in duplicates. Shown are the mean values. During VAD-support, patients were treated (open circles) or not (closed circles) with Bosentan. * $p < 0.05$ and *** $p < 0.001$ compared to NF-samples using One Way ANOVA and Bonferroni’s multiple comparisons test.

doi:10.1371/journal.pone.0169896.g002

Fig 2. Expression of endothelin receptors determined by radioligand binding studies. A) Total amount of endothelin receptors in 12 paired myocardial samples from VAD-receivers (pre-VAD and post-VAD) and 12 non-failing (NF) donor hearts. Binding of $[^{125}\text{I}]-\text{ET}-1$ was replaced by varying amounts of unlabeled ET-1. B) Receptor subtype analysis. For the assessment of the relative amount of receptor subtypes, $[^{125}\text{I}]-\text{ET}-1$ was replaced by varying amounts of the selective ET\textsubscript{A} receptor antagonist BQ-123. C-D) Density of the ET\textsubscript{A} receptor (C) and the ET\textsubscript{B} receptor (D) as calculated from both replacement experiments. All experiments were performed in duplicates. Shown are the mean values. During VAD-support, patients were treated (open circles) or not (closed circles) with Bosentan. * $p < 0.05$ and *** $p < 0.001$ compared to NF-samples using One Way ANOVA and Bonferroni’s multiple comparisons test.

doi:10.1371/journal.pone.0169896.g002

mechanical unloading, whereas the ET\textsubscript{B} receptor mRNA was only regulated during VAD support but not different between NF and samples from failing hearts. Radioligand binding was done for total endothelin receptor binding (Fig 2A) and for receptor subtype analysis (Fig 2B). The mean $K_D$ of $[^{125}\text{I}]-\text{ET}-1$ was 49.3-pM ± 15.7 (SD). The $K_D$ of NF, pre-VAD and post-VAD samples were not significantly different. Furthermore, there was no significant difference in the $K_D$-values of patients receiving Bosentan or not. The ET\textsubscript{A} receptor in pre-VAD samples (88.4 ± 28.0 fmole/mg protein) was not different from donor hearts (81.0 ± 18.4 fmole/mg protein; Fig 2C). We also did not observe an effect of mechanical unloading on
the ET$_A$ receptor density (post-VAD: 90.2 ± 25.2 fmole/mg protein). In contrast, we observed a significant lower expression of ET$_B$ receptors in terminally failing hearts (p < 0.001 vs. NF; NF 142.6 ± 30.4 and pre-VAD: 87.4 ± 26.6 fmole/mg protein; Fig 2D). ET$_B$ receptor expression was also not influenced by mechanical unloading (post-VAD: 87.6 ± 22.3 fmole/mg protein). Three patients received Bosentan during VAD support (see Table 1) which was far too low to draw any conclusion on an influence of this adjuvant therapy on the ET$_A$ or ET$_B$ receptor expression during mechanical unloading, respectively.

The data from mRNA and protein analysis for both endothelin receptors measured in the same specimen were plotted for correlation tests. We found only a weak correlation with a R$^2$ in the linear regression model of 0.20 and 0.12 for ET$_A$ or ET$_B$ receptors. We also compared the data by the non-parametric Spearman correlation tests which revealed coefficients of 0.43 and 0.31 for the ET$_A$ or ET$_B$ receptors, respectively (Fig 3).

We compared ET$_A$ and ET$_B$ receptor expression with the myocardial collagen content but did not find any evidence for a correlation (see S3 Fig).

Plasma Big-ET1 is significantly lower (p < 0.05, t-Test) after 28 ± 11 days of VAD-support compared to the time of VAD-implantation (see S4 Fig). There is no evidence for a correlation of myocardial ET$_A$ or ET$_B$ receptor density and plasma Big-ET1 at VAD-implantation or after VAD-support, respectively (see S5 Fig).

**Discussion**

In our study, we investigated the ET$_A$ and ET$_B$ receptor mRNA expression as well as the density of endothelin receptors in LV samples of DCM patients. Furthermore, for the first time the effect of VAD unloading on cardiac endothelin receptor expression on mRNA as well as on protein level was analyzed in parallel.

As obtained by real-time-PCR, the mRNA of the ET$_A$ receptor was up regulated in left ventricular pre-VAD samples, whereas the ET$_B$ mRNA expression was unaltered compared to NF samples (Fig 1). In numerous previous studies the myocardial regulation of endothelin receptor
mRNA in human heart failure was analyzed. The results, however, were not always concordant. ET<sub>A</sub> receptor mRNA in the LV of DCM patients has been reported to be increased [18, 23] or not regulated [24–26], respectively. In the same manner, ET<sub>B</sub> receptor mRNA was down regulated [25] or not altered [18, 23, 26] in LV DCM samples.

On the protein level, we found no alteration in the density of the ET<sub>A</sub> receptor in LV DCM samples (pre-VAD) compared to the NF samples. However, the ET<sub>B</sub> receptor density was significantly decreased in the ventricles of DCM patients explaining the reduced total endothelin receptor binding sites in this study (Fig 2). As it is the case for receptor mRNA, examination of literature concerning the density of ET<sub>A</sub> and ET<sub>B</sub> receptors in LV samples of DCM patients, yield inconsistent data [23, 25–30]. Some authors found no alteration in the density of ET<sub>A</sub> receptors in heart failure [26, 27, 29] whereas others reported increased levels of myocardial ET<sub>A</sub> binding sites [23, 25, 28, 30]. It appears that the study outcome is highly dependent on the control hearts which were used in the given study (see S6 Fig). NF hearts represent not well characterized tissue samples by their nature. In previous reports the control myocardium contained between 14 and 204 fmole/mg ET<sub>A</sub> receptor. Of note, in studies where the control samples contained low densities of this receptor (between 14 and 42 fmole/mg) [23, 25, 28, 30] the DCM samples showed significantly increased numbers of endothelin receptors. Whereas in studies where the NF control hearts had values above 70 fmole/mg there was no significant change of ET<sub>A</sub> receptor density in DCM hearts [26, 27, 29]. In our study, we found 81 fmole/mg ET<sub>A</sub> receptors in NF control hearts and could not find significant changes of the ET<sub>A</sub> receptor densities in patients with need for VAD support. Thus, our data on ET<sub>A</sub> expression agree with previously reported receptor regulation in the human heart and do not suggest ET<sub>A</sub> receptor regulation in DCM.

In contrast data from the ET<sub>B</sub> receptor in the literature appear to be not dependent on the control hearts which were used although in the literature a remarkable scatter of data on the ET<sub>B</sub> receptor density in NF myocardium was reported. We and others [23, 25, 29] found a significant down regulation of the ET<sub>B</sub> receptor in the myocardium of patients with DCM whereas others [26–28, 30] did not find a significant regulation of the ET<sub>B</sub> receptor in human heart failure. This discrepancy might be due to the comparably small number of control hearts used in some studies (n = 4 [30], n = 5 [27, 28], n = 6 [29] or n = 7 [26], respectively).

We did not find an effect of mechanical circulatory support on the regulation of endothelin receptor mRNA or protein. This is in accordance to mRNA data obtained by a global gene expression analysis of 30 paired samples from VAD-supported DCM hearts using Affymetrix HG-U133 Plus 2.0 arrays [31]. However, in a previous study Morawietz et al. found down regulation of increased mRNA during mechanical circulatory support [18]. Unfortunately, the authors investigated a mixed heart failure cohort containing DCM and ischemic cardiomyopathy (ICM) samples. Serneri et al. reported different regulation of endothelin receptors in patients with DCM or ICM, respectively [26]. In addition, patients in that study were supported by first generation pulsatile VADs, which might unload the ventricle stronger compared to continuous flow systems.

Endothelin receptor mRNA and protein showed only a weak correlation which might reflect that transcription and translation of these receptors are regulated on different levels (Fig 3). Endothelin receptor mRNA and protein and might have different half-lifes. An independent regulation of mRNA and protein was observed previously for the myocardial beta 1 adrenergic receptor [32] and other proteins [33]. It appears that the analysis of mRNA does not contribute significantly to the understanding of endothelin receptor regulation on the protein level.

It was discussed that endothelin receptor activation might be associated with fibrosis regulation [10]. Therefore, we quantified the total collagen of the hearts used for endothelin
receptor determination. We did not find any correlation between the total collagen content and the ET\textsubscript{A} or ET\textsubscript{B} receptor density, respectively. Thus, our data do not support a correlation of endothelin receptor regulation and cardiac fibrosis in human heart failure. However, fibrosis regulation might be more complex and a comparison between endothelin receptor regulation and myocardial collagen content is not sufficient to rule out an impact of endothelins for fibrosis development in human heart failure [34]. However, the role of endothelin receptor activation for myocardial fibrosis development was beyond the goals of this study.

In summary, conflicting data of endothelin receptor expression in DCM hearts exist which might be due to the control hearts used in a given study. In our patient population, we could not detect any influence of VAD-unloading on ET\textsubscript{A} or ET\textsubscript{B} receptor density, respectively.

Supporting Information

S1 Fig. Representative competition of ET-1 on 10,000 cpm \textsuperscript{125}I-ET-1. All experiments were performed in duplicates. The dashed lines represent the total binding (TB) and the non-specific binding (NSB).

(TIF)

S2 Fig. Representative biphasic competition of BQ-123 on 10,000 cpm \textsuperscript{125}I-ET-1. All experiments were performed in duplicates. The dashed lines indicate the portion of each receptor subtype or represent the total binding (TB) and the non-specific binding (NSB), respectively.

(TIF)

S3 Fig. Correlation of receptor expression with the collagen quantity. Myocardial tissue was analyzed for ET\textsubscript{A} (A) and ET\textsubscript{B} (B) receptor density and hydroxyproline content. The spearman correlation coefficient is indicated. The graph contains NF, pre-VAD and post-VAD samples.

(TIF)

S4 Fig. Influence of VAD implantation on Plasma Big-ET1 level. Plasma Big-ET1 was measured using a commercially available ELISA according to manufacturer’s conditions (Biomedica). Plasma Big-ET1 in patients supported by LV assist devices. Plasma Big-ET1 is significantly lower after 28 ± 11 days (mean ± SD) VAD-support (p < 0.05).

(TIF)

S5 Fig. Correlation of endothelin receptor density and Plasma Big-ET1 level at the time of (A) VAD implantation and (B) after VAD support. There is no evidence for a correlation of myocardial ET\textsubscript{A} or ET\textsubscript{B} receptor density and plasma Big-ET1 at VAD implantation or after VAD support, respectively. Of note, post VAD plasma samples were measured 28 ± 11 days (mean ± SD) after VAD implantation whereas the receptor density was measured at the time of heart transplantation.

(TIF)

S6 Fig. Literature evaluation of ETR binding data. Ratios of the means for relative endothelin receptor expression in human DCM hearts plotted against the endothelin receptor density in NF control hearts derived from the literature (see [22, 24–29] in the main manuscript). (A) The published ET\textsubscript{A} receptor density regulation appears to be dependent on the receptor density measured in rejected donor hearts (nonlinear regression; one phase decay; r\textsuperscript{2} = 0.8). Significant regulation of ET\textsubscript{A} was only detected in studies using control hearts with receptor densities below 42 fmoles/mg. (B) In contrast the regulation of ET\textsubscript{B} receptor density is not dependent on the receptor densities in control myocardium. (●) ratios of the means reflecting significant changes compared to controls; (○) ratios of the means reflecting no significant changes compared to
controls. Of note, results of this study were given as large symbols.

Acknowledgments

We thank Désirée Gerdes and Birte Bohms for excellent technical assistance and Dr. Cenk Oezpeker and Dr. Antoon J van den Bogaerdt for revising of the manuscript. Finally, we are grateful to all the patients participating in this study.

Author Contributions

Conceptualization: FG HM GA.
Data curation: FG HM.
Formal analysis: FG HM.
Funding acquisition: HM.
Investigation: FG AK DB.
Methodology: FG.
Project administration: HM.
Resources: HM.
Supervision: GA HM.
Validation: FG HM.
Visualization: FG.
Writing – original draft: FG HM.
Writing – review & editing: FG HM.

References

1. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 1988; 332(6163):411–5. doi: 10.1038/332411a0 PMID: 2451132
2. Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K et al. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc. Natl. Acad. Sci. U.S.A. 1989; 86(8):2863–7. PMID: 2649896
3. Nunez DJ, Brown MJ, Davenport AP, Neylon CB, Schofield JP, Wyse RK. Endothelin-1 mRNA is widely expressed in porcine and human tissues. J. Clin. Invest. 1990; 85(5):1537–41. doi: 10.1172/JCI114601 PMID: 1692036
4. Resink TJ, Hahn AW, Scott-Burden T, Powell J, Weber E, Bühler FR. Inducible endothelin mRNA expression and peptide secretion in cultured human vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 1990; 168(3):1303–10. PMID: 2161221
5. MacCumber MW, Ross CA, Glaser BM, Snyder SH. Endothelin: visualization of mRNAs by in situ hybridization provides evidence for local action. Proc. Natl. Acad. Sci. U.S.A. 1989; 86(18):7285–9. PMID: 2674952
6. Wagner OF, Christ G, Wojta J, Vierhapper H, Parzer S, Nowotny PJ et al. Polar secretion of endothelin-1 by cultured endothelial cells. J. Biol. Chem. 1992; 267(23):16066–8. PMID: 1644793
7. Seo B, Oemar BS, Siebenmann R, Segesser L von, Lüscher TF. Both ETA and ETB receptors mediate contraction to endothelin-1 in human blood vessels. Circulation 1994; 89(3):1203–8. PMID: 8124808
8. Ishikawa T, Yanagisawa M, Kimura S, Goto K, Masaki T. Positive inotropic action of novel vasoconstrictor peptide endothelin on guinea pig atria. Am. J. Physiol. 1998; 255(4 Pt 2):H970–3.
9. Ishikawa T, Yanagisawa M, Kimura S, Goto K, Masaki T. Positive chronotropic effects of endothelin, a novel endothelium-derived vasoconstrictor peptide. Pflugers Arch. 1988; 413(1):108–10. PMID: 2464160

10. Hafizi S, Wharton J, Chester AH, Yacoub MH. Profibrotic effects of endothelin-1 via the ETA receptor in cultured human cardiac fibroblasts. Cell. Physiol. Biochem. 2004; 14(4–6):285–92. doi: 10.1159/000080338 PMID: 15319352

11. Sakai S, Miyauchi T, Kobayashi M, Yamaguchi I, Goto K, Sugishita Y. Inhibition of myocardial endothelin pathway improves long-term survival in heart failure. Nature 1996; 384(6607):353–5. doi: 10.1038/384353a0 PMID: 8934519

12. Krum H, Denver R, Tzanidis A, Martin P. Diagnostic and therapeutic potential of the endothelin system in patients with chronic heart failure. Heart Fail Rev 2001; 6(4):341–52. PMID: 11447309

13. Touyz RM, Schiffren EL. Role of endothelin in human hypertension. Can. J. Physiol. Pharmacol. 2003; 81(6):533–41. doi: 10.1139/y03-009 PMID: 12839265

14. LaRue SJ, Garcia-Cortes R, Nassif ME, Vader JM, Ray S, Ravichandran A et al. Treatment of secondary pulmonary hypertension with bosentan after left ventricular assist device implantation. Cardiovasc Ther 2015[Epub ahead of print].

15. Hefke T, Zittermann A, Fuchs U, Schulte-Eistrup S, Gummer JF, Schulz U. Bosentan effects on hemodynamics and clinical outcome in heart failure patients with pulmonary hypertension awaiting cardiac transplantation. Thorac Cardiovasc Surg 2012; 60(1):26–34. doi: 10.1055/s-0030-1250726 PMID: 21432755

16. Dipla K, Mattielo JA, Jeevanandam V, Houser SR, Margulies KB. Myocyte recovery after mechanical circulatory support in humans with end-stage heart failure. Circulation 1998; 97(23):2316–22. PMID: 9639375

17. Maybaum S, Mancini D, Xydias S, Starling RC, Aaronson K, Pagani FD et al. Cardiac improvement during mechanical circulatory support: a prospective multicenter study of the LVAD Working Group. Circulation 2007; 115(19):2497–505. doi: 10.1161/CIRCULATIONAHA.106.633180 PMID: 17485581

18. Morewitz H, Szibor M, Goettsch W, Bartling B, Barton M, Shaw S et al. Detaching the hf ventricle by ventricular assist device normalizes increased expression of endothelin ET(A) receptors but not endothelin-converting enzyme-1 in patients with end-stage heart failure. Circulation 2000; 102(19 Suppl 3):III188–93.

19. Vandesompele J, Preter K De, Pattyn F, Poppe B, Roy N Van, Paepe A De et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 2002; 3(7):research0034.1–0034.11.

20. DeBlasi A, O’Reilly K, Motulsky HJ. Calculating receptor number from binding experiments using same compound as radioligand and competitor. Trends Pharmacol. Sci. 1989; 10(6):227–9. PMID: 2773043

21. Stegemann H. Mikrobestimmung von Hydroxprolin mit Chloramin-T und p-Dimethylaminobenzaldehyd. Hoppe-Seyler’s Z. Physiol. Chem. 1958; 311(1–3):41–5. PMID: 13548908

22. Schäfer S, Ellinghaus P, Janssen W, Kramer F, Lustig K, Milting H et al. Chronic inhibition of phosphodiesterase 5 does not prevent pressure-overload-induced right-ventricular remodelling. Cardiovasc. Res. 2009; 82(1):30–9. doi: 10.1093/cvr/cvp002 PMID: 19131365

23. Asano K, Bohlmeier TJ, Westcott JY, Zisman L, Kinugawa K, Good M et al. Altered expression of endothelin receptors in failing human left ventricles. J. Mol. Cell. Cardiol. 2002; 34(7):833–46. PMID: 12099722

24. Hasegawa K, Fujiwara H, Koshiji M, Inada T, Doyama K et al. Endothelin-1 and its receptor in hypertrophic cardiomyopathy. Hypertension 1996; 27(2):259–64. PMID: 8567049

25. Zolk O, Quattek J, Sitzler G, Schrader T, Nickenig G, Schnabel P et al. Expression of endothelin-1, endothelin-converting enzyme, and endothelin receptors in chronic heart failure. Circulation 1999; 99 (16):2118–23. PMID: 10217651

26. Serneri GG, Cecioni I, Vanni S, Paniccia R, Bandinelli B, Vetere A et al. Selective upregulation of cardiac endothelin system in patients with ischemic but not idiopathic dilated cardiomyopathy: endothelin-1 system in the human failing heart. Circ. Res. 2000; 86(4):377–85. PMID: 10700441

27. Pöncke K, Vogelsang M, Heinroth M, Becker K, Zolk O, Böhm M et al. Endothelin receptors in the failing and nonfailing human heart. Circulation 1998; 97(8):744–51. PMID: 9498537

28. Pieske B, Beyermann B, Breu V, Löffler BM, Schlotthauer K, Maier LS et al. Functional effects of endothelin and regulation of endothelin receptors in isolated human nonfailing and failing myocardium. Circulation 1999; 99(14):1802–9. PMID: 10199875

29. Walker CA, Ergul A, Grubbs A, Zile MR, Zeilner JL, Crumby AJ et al. beta-Adrenergic and endothelin receptor interaction in dilated human cardiomyopathic myocardium. J. Card. Fail. 2001; 7(2):129–37. doi: 10.1054/jcaf.2001.24125 PMID: 11420764
30. Del Ry S, Andreassi MG, Clerico A, Biagini A, Giannessi D. Endothelin-1, endothelin-1 receptors and cardiac natriuretic peptides in failing human heart. Life Sci. 2001; 68(24):2715–30. PMID: 11400914
31. Schwientek P, Ellinghaus P, Steppan S, D’Urso D, Seewald M, Kassner A et al. Global gene expression analysis in nonfailing and failing myocardium pre- and postpulsatile and nonpulsatile ventricular assist device support. Physiol. Genomics 2010; 42(3):397–405. doi: 10.1152/physiolgenomics.00030.2010 PMID: 20460602
32. Milting H, Scholz C, Arusoglu L, Freitag M, Cebulla R, Jaquet K et al. Selective upregulation of beta1-adrenergic receptors and dephosphorylation of troponin I in end-stage heart failure patients supported by ventricular assist devices. J. Mol. Cell. Cardiol. 2006; 41(3):441–50. doi: 10.1016/j.yjmcc.2006.04.010 PMID: 16765375
33. Maier T, Güell M, Serrano L. Correlation of mRNA and protein in complex biological samples. FEBS Lett. 2009; 583(24):3966–73. doi: 10.1016/j.febslet.2009.10.036 PMID: 19850042
34. Milting H, Ellinghaus P, Seewald M, Cakar H, Bohms B, Kassner A et al. Plasma biomarkers of myocardial fibrosis and remodeling in terminal heart failure patients supported by mechanical circulatory support devices. J. Heart Lung Transplant. 2008; 27(6):589–96. doi: 10.1016/j.healun.2008.02.018 PMID: 18503956