Pulmonary and cardiac expression of preproendothelin-1 mRNA are increased in heart failure after myocardial infarction in rats. Localization of preproendothelin-1 mRNA and endothelin peptide

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Abstract

Objectives: Recent reports indicate that endothelin (ET) plays an important pathophysiological role in congestive heart failure (CHF). However, existing data on local cardiopulmonary ET production are few. No studies have hitherto examined the specific anatomic localization of cardiopulmonary ET synthesis in CHF. Thus, the aims of the present study were to examine whether cardiopulmonary preproET-1 mRNA synthesis is upregulated in CHF and to determine the anatomic localization of preproET-1 mRNA and the mature peptide. Methods: CHF was induced in rats by occluding the left coronary artery. Only animals with a left ventricular end-diastolic pressure above 15 mmHg after one week were included (n = 28). Sham-operated animals served as controls (n = 24). Hearts and lungs were examined by mRNA slot blot analyses, in situ hybridization (ISH) and immunohistochemistry (IHC). Results: In CHF-rats, slot blot analyses revealed a 3.5 ± 1.1-fold and a 6.4 ± 0.8-fold upregulation of preproET-1 mRNA in the noninfarcted and the infarcted area of the left ventricles, respectively (p < 0.05 for both). ISH revealed that the preproET-1 mRNA was localized predominantly over the granulation tissue in the infarcted region. The ET peptide was predominantly localized to inflammatory cells and remaining cardiomyocytes in the infarcted region as determined by IHC. Lungs from CHF-rats showed a 1.5 ± 0.1-fold upregulation of preproET-1 mRNA (p = 0.01). The most abundant preproET-1 mRNA and ET-1-like immunoreactivity (ET-1-ir) was seen over inflammatory cells and over airway epithelial cells. Some ET-1-ir was also located to bronchial and vascular smooth muscle cells. Conclusion: Increased cardiopulmonary ET synthesis strongly suggest a pathophysiological role for ET in CHF. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Endothelin; Heart failure; Ischemia; Rat

1. Introduction

There is increasing evidence that endothelin (ET) plays a pathophysiological role in congestive heart failure [1–4]. ET receptor blocking agents improve hemodynamic variables both in humans and in rats with heart failure [1,3], and have recently been reported to reduce mortality in a rat model of congestive heart failure [4]. Congestive heart failure is associated both with structural remodelling of the myocardium and pulmonary hypertension and congestion [5]. The structural remodelling of the myocardium implies hypertrophy of the cardiomyocytes and interstitial fibrosis due to the increased synthesis of collagen. Interestingly, ET has been shown to induce hypertrophy of cardiomyocytes [6] and to stimulate collagen synthesis in vitro [7]. However, existing data on local production of ET in the heart are few. Evidence for an increase in local de novo synthesis of preproET-1 mRNA in the failing heart has hitherto been reported by only two research groups [8,9]. However, no studies have reported the anatomic localization of preproET-1 mRNA synthesis in the failing heart, which may provide important clues regarding the pathophysiological role of ET during development of...
congestive heart failure. Accordingly, the first aim of the present study was to examine whether cardiac preproET-1 mRNA is upregulated in congestive heart failure in the rat and to reveal the anatomic localization of preproET-1 mRNA and ET peptide production. We used male Wistar rats in which congestive heart failure was induced by occluding the left coronary artery [8]. To quantify and localize preproET-1 mRNA and the mature ET peptide, we utilized the methods of mRNA slot blot, in situ hybridization and immunohistochemistry.

Pulmonary hypertension and congestion are frequent complications in congestive heart failure [5]. The reported effects of ET make it an interesting candidate as a pathogenic factor in the pulmonary tissue. In addition to primary effects on the heart inducing backward failure resulting in pulmonary congestion, ET might also play a direct pro-congestive role within the pulmonary circulation. ET has been shown to increase permeability of pulmonary capillaries and thus transcapillary fluid filtration, resulting in airway edema [10,11]. Moreover, ET induces postcapillary vasoconstriction in the pulmonary circulation [12] with a subsequent increase in microvascular pressure and transcapillary fluid filtration. Increased production of ET in the lungs in congestive heart failure might therefore contribute to the clinical picture of pulmonary congestion and edema. An increase in pulmonary preproET-1 mRNA would give evidence for a de novo synthesis. Thus, a second aim of the present study was to examine whether preproET-1 mRNA is increased in the lung in rats with heart failure. Furthermore, we aimed at examining the anatomic localization of preproET-1 mRNA and the mature ET peptide within the lungs of rats in heart failure.

2. Methods

2.1. Animal preparation

Fifty-two male Wistar rats (250–300 g) were anesthetized with a mixture of 30% oxygen, 70% nitrous oxide and 4% halothane (Zeneca, Macclesfield Cheshire, UK). After induction of anesthesia, the rats were intubated and connected to a rodent ventilator (Model 874092, B. Braun Melsungen, Germany) and ventilated with a mixture of 30% oxygen, 70% nitrous oxide and 1% halothane. The hearts were exposed through a left thoracotomy after removal of the 4th costa and rapidly and gently exteriorized after an incision of the pericardium. In animals undergoing myocardial infarction, the left coronary artery was ligated by a 5-0 silk suture near its origin. In sham-operated animals, the artery was left unligated. Then the heart was replaced into the thorax, and the thoracotomy closed by a string suture during maximum inspiration. The rats were given postoperative analgesia by an s.c. injection of 0.05 ml buphrenorphin (0.3 mg/ml).

Seven days after the first operation, the second operation was performed. Using a 2F micromanometer-tipped catheter (Model SPR-407, Millar Instruments, Houston, TX) inserted through the right carotid artery, we measured left ventricular systolic and end-diastolic pressures (LVEDP). Only the rats with myocardial infarction and an LVEDP above 15 mmHg were considered to be in heart failure and included in the study. The rats were sacrificed by excision of the hearts and lungs. For slot blot analyses, myocardial left ventricles were snap frozen together with pulmonary tissues in liquid nitrogen. Immediately prior to freezing, left ventricles from rats in heart failure were divided into the infarcted area and the remaining non-infarcted area which was easily distinguished macroscopically. To evaluate the degree of pulmonary edema, lungs were weighed immediately after excision from the animals. For in situ hybridization and immunohistochemistry, hearts and lungs were fixated in 4% paraformaldehyde and Bouins solution, respectively.

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 1996).

2.2. Experimental procedure

Rats were randomly allocated to left coronary artery ligation or to sham operation on the day of the first operation. Seven days after the first operation, the second operation was performed and a total of twenty-eight rats were included with congestive heart failure and twenty-four sham-operated animals served as controls. Slot blot analyses were performed on left ventricular tissues from eleven rats in heart failure and ten sham-operated rats and on pulmonary tissues from twelve rats in heart failure and ten sham-operated rats. In situ hybridization and immunohistochemistry were performed on cardiac and pulmonary tissues of five rats in heart failure and five sham-operated rats.

2.3. mRNA slot blot analysis

Poly A+RNA was extracted from homogenized cardiac and pulmonary tissues using oligo dT-conjugated paramagnetic beads according to the manufacturers instructions (Dynal A/S, Oslo, Norway). For slot blot analyses, 0.5, 1.0, and 2.0 μg poly A+RNA from each specimen were loaded onto a nylon membrane by use of a Minifold II (Schleicher and Schuell, Dassel, Germany). The nylon membranes were pre-hybridized at 42°C for 3 h in a solution containing 5×standard saline citrate (SSC), 5× Denhardt’s solution, and 0.1% sodium lauryl sulfate and were then hybridized with 32P-labeled preproET-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes in the same solution at 42°C for 5 and 1 days, respectively. The specificity of the probes has been
verified in a previous publication [8]. The nylon membranes were finally washed twice in SSC/0.1% sodium lauryl sulfate at room temperature for 5×5 min before washing twice with the solution at 60°C for 15 min. Then the nylon membranes were subjected to autoradiography using a phosphorimaging system, and the intensities of the signals subsequently analyzed by densitometric scanning analysis using the ImageQuant Version 3.3 software (Molecular Dynamics Lab., Queensland, Australia). All the slot blot data presented are normalized to the corresponding GAPDH signals.

2.4. In situ hybridization

Ten-micrometer cryostat sections from paraformaldehyde-fixed tissues were rehydrated in phosphate-buffered saline (PBS), made permeable with proteinase K, and immersed in 4% paraformaldehyde to stop the reaction. Following three washes in PBS, sections were immersed in a solution of triethanolamine and acetic anhydride for 10 min, dehydrated in increasing concentrations of ethanol and air-dried. Sections were then hybridized with preproET-1 sense and antisense riboprobes labeled with 35S-UTP (1×106 cpm per section) by using a commercial kit (Ambion) as previously described [14]. The sections were incubated for 16 h at 42°C. Unbound probe was removed by incubation in RNase solution for 30 min at 42°C in 2×saline sodium citrate (SSC). This was followed by further washes in graded concentrations of SSC (from 2×SSC to 0.1×SSC) at temperatures ranging from 20°C to 55°C. Sections were then dehydrated in ethanol and ammonium acetate, air-dried, exposed in light-tight boxes for 8 days at 4°C, and developed in D-19 developer (Kodak, Rochester, N.Y.). Negative control experiments involved the use of sense probe and incubation of sections with hybridization mixture in the absence of radiolabeled probe.

2.5. Immunohistochemistry

Immunohistochemistry was performed using a modification of the avidin–biotin–peroxidase complex method as previously described [13,14]. Briefly, after permeabilizing the cryostat sections in 0.3% Triton for 30 min, they were incubated with hydrogen peroxide for 60 min to block endogenous peroxidase activity, followed by further incubation with normal goat serum to reduce non-specific bindings of the antisera. Then, the sections were incubated for 16 h at 4°C with antisera to the C-terminus of ET-1 [15] and von Willebrand factor (factor VIII) used as an endothelial cell marker. After washing in PBS, sections were incubated with biotinylated goat anti-rabbit IgG for 45 min. This was followed by three washes in PBS and incubation with the avidin–biotin–peroxidase complex (Vectastain Elite Kit, Vector Laboratories) for 45 min, and sites of immunoreaction were visualized by use of diaminobenzidine and hydrogen peroxide. Negative controls were prepared with absorption of the first layer antiserum with its respective antigen before incubation with sections.

2.6. Statistical analyses

Values are given as means±SEM. The data followed a normal distribution. Thus, for comparisons between groups we used the Student t-test or one-way ANOVA where appropriate. A p value <0.05 was considered as statistically significant.

3. Results

3.1. Characterization of the animals

Mean body weights, lung weights and hemodynamic variables are shown in Table 1. There was a slight reduction in body weight between the first and second operation in rats in heart failure whereas no significant changes were measured in sham-operated animals. Lung weight was significantly higher in animals in heart failure, indicating pulmonary edema. Systolic blood pressure was lower in rats in heart failure than in sham-operated rats, whereas LVEDP was five times higher.

3.2. Heart

3.2.1. Histology

Sections were examined by light microscopy. Granulation tissue in the infarcted area of the hearts in heart failure

| Weight characteristics and hemodynamic variables of the experimental groups |
|-----------------------------|-----------------------------|-----------------|-----------------------------|
|                            | BWB | BWS | LW  | LVEDP | SBP  |
| Heart failure              | 298.6±4.8 | 281.4±4.2<sup>+</sup> | 1.91±0.13<sup>+</sup> | 21.3±1.0<sup>+</sup> | 96.2±2.7<sup>+</sup> |
| (n=28)                     | (n=28)                     | (n=18)          | (n=27)                     | (n=26)          |
| Sham operated              | 297.7±4.9 | 304.5±4.8 | 1.24±0.04 | 4.7±0.4 | 119.9±1.7 |
| (n=23)                     | (n=23)                     | (n=17)          | (n=21)                     | (n=22)          |

Values are means±SEM. BWB=body weight before primary surgery, BWS=body weight when sacrificed, LW=lung weight, LVEDP=left ventricular end-diastolic pressure, SBP=systolic blood pressure. BWB, BWS and LW are given in grams, whereas LVEDP and SBP are given in mmHg. <sup>+</sup> p<0.001 vs sham operated animals. <sup>†</sup> p<0.01 vs BWB.
was identified morphologically by increased density of capillaries, fibroblasts and inflammatory cells.

3.2.2. Slot blot
Little expression of preproET-1 mRNA was measured in the left ventricles of normal hearts from sham-operated animals \((n=10)\), as depicted in Fig. 1. In contrast, the non-infarcted area of left ventricles from the rats in heart failure \((n=11)\) showed a \(3.5\pm1.1\)-fold upregulation of preproET-1 mRNA compared to sham \((\text{Fig. 1A and B, } p<0.05)\). An even higher upregulation was measured in the infarcted region of the hearts in heart failure \((n=8)\). In this region, a \(6.4\pm0.8\)-fold upregulation was measured compared to sham \((\text{Fig. 1A and B, } p<0.05)\).

3.2.3. In situ hybridization
In situ hybridization was performed to display the localization of preproET-1 mRNA in myocardial tissues. The most striking difference between normal hearts and hearts in heart failure was the strong hybridization signals in the region subjected to myocardial infarction in the latter group. Especially the border zone between granulation tissue and remaining cardiomyocytes in the ischemic area displayed strong hybridization signals \((\text{Fig. 2A})\). Both in the non-infarcted areas of hearts in heart failure \((\text{Fig. 2B})\) and in normal hearts \((\text{Fig. 2C})\), scattered silver grains were seen over cardiomyocytes. Weak signals were seen over endothelial cells and over vascular smooth muscle cells of large and small intramyocardial vessels in both groups.

3.2.4. Immunohistochemistry
To reveal the subset of cells containing the mature ET peptide, we performed immunohistochemistry. Little ET-1-
like-immunoreactivity (ET-1-ir) was seen located to the fibrotic tissue of the infarcted area (Fig. 3A). However, remaining cardiomyocytes adjacent to the granulation tissue showed strong ET-1-ir (Fig. 3A). The most striking difference in ET-1-ir between the normal hearts and those in heart failure was the strong immunostaining of the inflammatory cells of the granulation tissue of the failing hearts as seen in Fig. 3C. ET-1-ir was seen over cardiomyocytes both in the non-infarcted area of hearts in heart failure (Fig. 3D) and in normal hearts (Fig. 3B). Occasionally, there was ET-1-ir located to endothelial cells and the underlying smooth muscle cells of both large and small intramyocardial vessels, with no apparent difference between failing and normal hearts.

3.3. Lung

3.3.1. Slot blot

Fig. 4 shows a representative slot blot of normal lungs from sham-operated animals and lungs obtained from rats in heart failure one week after induction of myocardial infarction. Normal lungs (n=10) showed little expression of preproET-1 mRNA (Fig. 4A and B). In lungs from rats in heart failure (n=12) there was a mean 1.5±0.1-fold upregulation of preproET-1 mRNA (p=0.01).

3.3.2. In situ hybridization

In rats in heart failure, in situ hybridization revealed scattered silver grains over bronchial epithelial cells as seen in Fig. 5A. Moreover, preproET-1 mRNA was also observed over inflammatory cells within the pulmonary tissues (Fig. 5A, arrows). Some preproET-1 mRNA could also be observed along the alveolar linings and located to intrapulmonary vessels (Fig. 5C).

3.3.3. Immunohistochemistry

The strongest ET-1-ir was located to the Clara cells of the epithelial lining of bronchioles (Fig. 6A) and to inflammatory cells (Fig. 6B) in lungs from rats in heart failure. Moreover, ET-1-ir was seen located to vascular...
Fig. 3. Localization of endothelin-1-like immunoreactivity (ET-1-ir) in left ventricular tissue. Panel A shows ET-1-ir in the infarcted region. The strongest staining is seen in the subendocardial area (arrows) and over the remaining cardiomyocytes (arrowheads). Panel B shows ET-1-ir over the left ventricular free wall of a sham-operated rat. Panel C shows immunostaining of inflammatory cells in the infarcted region. Panel D represents a section from the non-infarcted left ventricular tissue from a rat in heart failure. Strong immunostaining is seen over cardiomyocytes, whereas little or no ET-1-ir is seen in the intramural vessel (arrowhead). Panel E represents a section from the infarcted region stained after absorption of the first layer antiserum with ET-1 before incubation with the section. Panels A, B and E original magnification ×100, panels C and D original magnification ×400.

(Fig. 6C) and bronchial (Fig. 6D) smooth muscle cells and to bronchial epithelial cells (Fig. 6D). Small variations were seen in the intensities of immunostaining between different pulmonary areas. However, we did not find any correlation between the degree of immunostaining and calibre of the airways and vessels.
Fig. 4. Panel A shows a representative slot blot analysis of preproendothelin-1 (ppET-1) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA of pulmonary tissues from rats in ischemic heart failure (HF) and sham-operated rats (SHAM). Each horizontal line represents mRNA from one animal. Panel B shows bar graphs representing quantitation of the slot blots normalized according to the GAPDH signals. * p<0.05 vs SHAM.

4. Discussion

The present study shows that preproET-1 mRNA is upregulated 1.5-fold in the lungs of rats in heart failure. Moreover, we demonstrate that the preproET-1 mRNA and the mature ET peptide are located predominantly to inflammatory cells and bronchial and bronchiolar epithelial cells, but also to bronchial and vascular smooth muscle cells within the pulmonary tissue of animals in heart failure. Furthermore, we report that there is a 3.5-fold and 6.4-fold upregulation of preproET-1 mRNA in the non-infarcted and infarcted area of left ventricles of hearts in heart failure, respectively. The most abundant expression of preproET-1 mRNA in the hearts in heart failure was located to the granulation tissue in the infarcted area in regions adjacent to remaining cardiomyocytes. The most abundant immunostaining for the mature ET peptide was seen located to inflammatory cells in the infarcted region and to the adjacent remaining cardiomyocytes, extending the observations of a previous in vitro study reporting that macrophages and monocytes are potential sources of ET-1 production [16]. We [8] and Sakai et al. [9] have previously reported increased expression of preproET-1 mRNA in the hearts of rats in heart failure. In our previous study [8], we reported a transient upregulation of preproET-1 mRNA, with the highest expression measured one week after induction of myocardial infarction. The present data therefore also extend these previous observations to the cellular level.

The finding of increased ET in inflammatory cells and cardiomyocytes adjacent to the infarcted region suggests a role for ET in heart failure after myocardial infarction, possibly related to tissue repair and tissue differentiation. We have previously reported increased myocardial synthesis of ET-1 in the acute phase of myocardial infarction in the pig [14]. Myocardial infarction is associated with
tissue inflammation and formation of granulation tissue. Basic findings of granulation tissue are neovascularisation and angiogenesis which might be stimulated by increased myocardial ET-1 [12]. Secondly, ET has been demonstrated to exert mitogenic effects [17] and to facilitate collagen synthesis [7] both of which are important in wound healing following an acute myocardial infarction. A local production of ET by granulation tissue including inflammatory cells in the infarcted region as demonstrated in the present study, is therefore compatible with a role for ET in the tissue repair process. The increased ET-1 synthesis in the viable left ventricular tissues in rats in heart failure might also facilitate the remodelling process in this region. Furthermore, this finding indicates that also processes other than tissue necrosis per se might induce increased ET-1 synthesis.

In the present study we provide evidence for an increased de novo synthesis of ET-1 in the lungs of rats in congestive heart failure as seen by a 1.5-fold upregulation of preproET-1 mRNA. The most abundant preproET-1 mRNA and ET-1-ir in the lungs of rats in heart failure were found in inflammatory cells and in the bronchial and bronchiolar epithelium, whereas vascular endothelial cells showed less expression. This is in contrast to a recent study by Sakai and coworkers [18]. In that study they observed increased ET-1-ir in the vascular endothelium of lungs from rats in congestive heart failure, whereas staining of airway epithelium was not reported. The discrepancies between their study and the present might be explained by the longer time span between induction of heart failure and examination of the lungs in their study. Sakai and coworkers sacrificed the animals two weeks after induction of myocardial infarction. Since in situ hybridization was not performed in that study, it is not known whether the increased immunostaining of the pulmonary vascular endothelium might be a result of increased extraction of circulating plasma ET which was also increased in their study, or a result of de novo synthesis. Another possible explanation might be that expression of preproET-1 mRNA and ET-1-ir in airway

Fig. 5. Panel A shows expression of preproendothelin-1 (ppET-1) mRNA in pulmonary tissues from rats in heart failure as demonstrated by in situ hybridization. Scattered silver grains are seen over bronchial epithelial cells (large arrowheads), over inflammatory cells (arrows) and along the alveolar linings (small arrowheads). Panel B shows a matching region from a control animal. Panel C shows an intrapulmonary vessel (arrowheads) from a rat in heart failure. Panel D represents a section from lung from a rat in heart failure hybridized with the ppET-1 sense probe. All panels original magnification ×400.
epithelial cells and inflammatory cells are linked to the degree of pulmonary edema. Sakai and coworkers do not report whether their animals had significant pulmonary edema when sacrificed. In the present study we measured an increase in lung weight by more than 50% in rats in heart failure, indicating severe pulmonary congestion and edema.

Congestive heart failure is associated with pulmonary
inflammation [19]. We found strong ET-1-ir and preproET-1 mRNA in pulmonary inflammatory cells in lungs from rats in heart failure. We have previously reported that treatment with an ET receptor antagonist inhibits the inflammatory response in lungs of rats after sephadex instillation [20]. ET might, therefore, also have a local proinflammatory effect in the lung in congestive heart failure. A local proinflammatory effect would facilitate capillary leakage and edema formation. A pathophysiologi- cal role for ET in pulmonary edema formation in congestive heart failure is further supported by a study reporting increased microvascular permeability by activation of ET_A receptors [11]. Moreover, infusing ET-1 into animals have been shown to augment albumin escape into the lungs of rats [21] and raise pulmonary filtration rate [22].

The most common cause of pulmonary hypertension is congestive heart failure [12]. ET might contribute to pulmonary hypertension. Increased ET has been found in lungs of patients with pulmonary hypertension of different etiologies [23]. Patients with pulmonary hypertension secondary to congestive heart failure have an elevated circulating plasma ET-1 that correlates directly with mean pulmonary arterial pressure and pulmonary vascular resistance [12]. Furthermore, ET-1 augments proliferation of vascular smooth muscle cells [24,25]. Since both preproET-1 mRNA and mature peptide were localized to pulmonary vascular smooth muscle cells in the present study, it is tempting to speculate that ET is contributing to increased pulmonary resistance in congestive heart failure.

In conclusion, we have shown in the present study that preproET-1 mRNA is upregulated both in the heart and in the lungs of rats in heart failure following left coronary artery occlusion. In the failing heart, preproET-1 mRNA and the mature ET-1 peptide was located predominantly to the granulation tissue and inflammatory cells of the region subjected to myocardial infarction. In the lungs of rats in heart failure, the most abundant preproET-1 mRNA and ET peptide was located to bronchial and bronchiolar epithelial cells and to inflammatory cells. These findings strongly suggest a role for ET in tissue repair in congestive heart failure. Moreover, ET might exert a proinflammatory effect in the lung facilitating capillary leakage and edema formation.

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