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DISSERTATION

# Transgenic Overexpression of Endothelin-2 Aggravates Diabetic Cardiomyopathy in Rats

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*I would like to dedicate this work to my lovely wife Gosia*

## Abbreviations

| | |
|---|---|
| °C | Degrees Celsius |
| ANP | Atrial natriuretic peptide |
| ATP | Adenosine triphosphate |
| BNP | Brain natriuretic peptide |
| bp | Base pair(s) in DNA |
| BP | Blood pressure |
| BG | Blood glucose |
| bpm | Beat per minute |
| cDNA | Complementary DNA |
| CNP | C-type natriuretic peptide |
| DAG | Diacylglycerol |
| dATP | Deoxyadenosine 5‘-triphophate |
| dCTP | Deoxycytidine 5‘-triphophate |
| DEPC | Diethylpyrocarbonate |
| dGTP | Deoxyguanosine 5‘-triphosphate |
| DM | Diabetes mellitus |
| DNA | Deoxyribonucleic acid |
| dTTP | Deoxythymidine 5‘-triphosphate |
| ECM | Extracellular matrix |
| ET-1 | Endothelin 1 |
| ET-2 | Endothelin 2 |
| ET-3 | Endothelin 3 |

| | |
|---|---|
| $ET_A$ | Endothelin A receptor |
| *et al.* | *Et alii* |
| $ET_B$ | Endothelin B receptor |
| ETs | Endothelins |
| fmol | Femtomol |
| *g* | Unit of centrifugal force |
| GFR | Glomerular filtration rate |
| hET-2 | Human endothelin 2 |
| *i.e.* | *Id est* |
| IE | International Unit |
| kg | Kilogram |
| LGR-7 | Leucine-rich repeat-containing G protein-coupled receptor 7 |
| LV | Left ventricle |
| MCSA | Media cross sectional area |
| mg | Milligram |
| min | Minutes |
| ML | Media/lumen ratio |
| ml | Millilitre |
| mM | Millimolar |
| mmHg | Millimetre of mercury |
| mRNA | Messenger ribonucleic acid |
| µl | Microlitre |
| NF-κB | Nuclear factor kappa B |

| | |
|---|---|
| nm | Nanometre |
| nmol | Nanomol |
| NO | Nitric oxide |
| PAI-1 | Plasminogen activator inhibitor-1 |
| PAS | Periodic acid Schiff |
| PCR | Polymerase chain reaction |
| PBS | Phosphate buffered saline |
| $PIP_2$ | Phosphatidylinositol 4,5-bisphoshate |
| PLC | Phospholipase C |
| RAAS | Renin-angiotensin-aldosterone system |
| RLX | Relaxin |
| rpm | Revolutions per minute |
| RT | Reverse transcription |
| RV | Right ventricle |
| s | Seconds |
| STZ | Streptozotocin |
| TGF-β1 | Transforming growth factor-β1 |
| TβR-I | Transforming growth factor-β receptor I |
| TβR-II | Transforming growth factor-β receptor II |
| TβR-III | Transforming growth factor-β receptor III |
| w | Weeks |

# 1 Introduction

Diabetes mellitus has emerged as one of the key public health problems of the 21st century world-wide. The major causes of morbidity and mortality in diabetic patients are alterations of the macro- and micro-vasculature. Cardiovascular events account for up to 80% of deaths in this population [1]. Characteristic structural changes in diabetic cardiomyopathy include myocardial hypertrophy, interstitial fibrosis and alterations of large and small heart arterial vessels [2,3]. Typical functional findings are progressive loss of heart function and increased incidences of arrhythmia [4].

Endothelins (ETs) are potent vasoconstricting [5] and growth-promoting factors [6,7,8] consisting of 21 amino acids. The peptides are produced and secreted mainly from vascular endothelial cells. There are three endothelin peptides, endothelin-1, -2 and -3, that act on two distinct cellular receptors, *i.e.* $ET_A$ and $ET_B$ receptor. With regard to diabetic cardiomyopathy, several inhibitory interventions using pharmacological ET receptor antagonists have described clearly beneficial morphological and functional effects in experimental diabetes models [9,10,11]. However, some other studies have reported contradictory findings of no or only modest actions of ET inhibition on diabetic heart myocardium and vessel changes [12,13].

In the present study, we used a stimulatory intervention, *i.e.* transgenic overexpression of the human ET-2 (hET-2) gene in rats, to further characterize the contribution of endothelins to the morphological and molecular changes in diabetic cardiomyopathy. The model employed, the ET-2 (TGR(hET-2)L37) rat, endogenously exposes the myocardium and its vessels to markedly increased circulatory endothelin levels, rather than through cardiac overexpression [14]. Diabetes mellitus was induced by streptozotocin injection into both hET-2-positive and -negative littermate animals. Non-diabetic transgene-positive and -negative rats served as controls to complete a 4-group study design. After 6 months of hyperglycaemia, we analyzed circulating endothelin levels, markers of heart hypertrophy and fibrosis, changes in large epicardial arteries and small intramyocardial arterioles, as well as heart ET-1, $ET_A$ and $ET_B$ expressions.

## 1.1 Diabetic Cardiomyopathy

Diabetic cardiomyopathy is a clinical condition, diagnosed when ventricular dysfunction develops in patients with diabetes in the absence of coronary atherosclerosis and hypertension [15,16]. The mechanisms of myocardial alteration in diabetes were investigated on the cellular and molecular levels.

### 1.1.1 Cellular Mechanisms Predisposing Towards Diabetic Cardiomyopathy

There are 3 characteristic metabolic disturbances in diabetic states: hyperlipidaemia, early hyperinsulinaemia, followed by pancreatic β-cell failure and leading to hyperglycaemia. In this study, the animal model of diabetes refers to type 1 diabetes, which differs principally from type 2 diabetes in that it is unaccompanied by a period of hyperinsulinemia and is characterized by early-onset hyperglycaemia.

Hyperglycaemia leads to increased glucose oxidation and mitochondrial generation of superoxide. In turn, excess superoxide leads to DNA damage and activation of poly (ADP ribose) polymerase (PARP) as a reparative enzyme [17]. PARP mediates the ribosylation and inhibition of glyceraldehydes phosphate dehydrogenase (GAPDH), which diverts glucose from its glycolytic pathway and into alternative biochemical pathways that are considered the mediators of hyperglycaemia-induced cellular injury. These consist of increases in advanced glycation end-products (AGEs), increased hexosamine and polyol flux and activation of classical isoforms of protein kinase C. The mechanism through which hyperglycaemia mediates tissue injuries and their consequences are summarized in Table 1.

Table 1: Alternative metabolic fates of glucose leading to cardiac structural and functional abnormalities in hyperglycaemia (Poornima e*t al*.).

| Mediators | Mechanisms of Action | Consequences |
|---|---|---|
| Increased AGE | Crosslink RyRs; crosslink type III collagen | Decreased SR calcium release and myocyte contractility; increased ventricular stiffness; impaired ventricular filling |
| Increased hexosamine flux | Sp1-specific O-GlcNAcylation of transcription factors decreasing SERCA 2a expression | Prolonged calcium transients; impaired relaxation |
| Increased polyol flux | Decreased regeneration of reduced glutathione leading to oxidative stress; increased DNA fragmentation; sorbitol-induced AGEs | Increased myocyte apoptosis; increased ventricular stiffness |
| Increased protein kinase C activation | Increased cardiac hypertrophy; increased extra cellular matrix; decreased SERCA 2a function | Impaired relaxation; increased ventricular stiffness |

RyRs indicates ryanodine receptors, SR – sarcoplasmic reticulum, Sp1 – transcription factor, O-GlcNAcylation – addition of O-linked N-acetylglucosamine residue, SERCA 2a – sarco(endo)plasmic reticulum $Ca^{2+}$-ATPase

Impaired contraction of the heart and loss of elasticity of the vascular system are associated with extracellular matrix (ECM) proteins, such as excess deposition of collagens and abnormal glycosylation/crosslinking [18]. Diabetes accelerates the mechanisms that underlie the development of fibrosis. Furthermore, the glycosylation produces very stable complex products and crosslinks known as AGEs (Figure 1). The formation of AGEs on vessels increases resistance to proteolysis, and thus results in fibrosis [19].

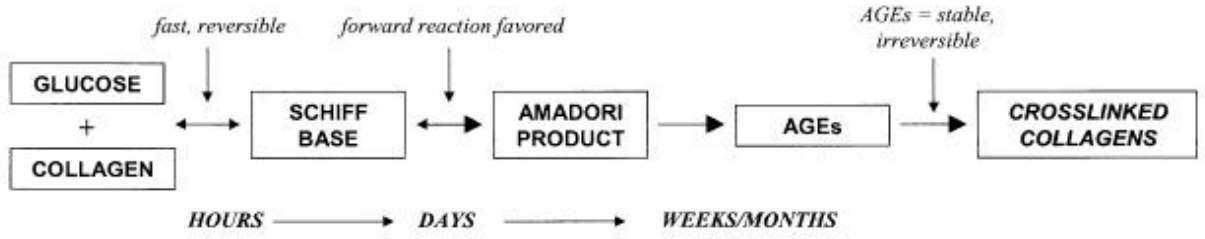


Figure 1: Production of collagen crosslinks.

Glucose interacts with collagen to form a Schiff base. The collagen Schiff base rearranges over a period of days to generate an Amadori product (*i.e.*, glycated collagen). This step occurs faster in the forward than in the reverse direction, and thus, glycated collagen accumulates. Glycated collagen undergoes further chemical modification to yield complex compounds and crosslinks known as advanced glycosylation end-products (AGEs). Collagen AGEs are more stable and more resistant to proteolysis (Asbun e*t al*.).

### 1.1.2 Molecular Changes

Many studies present various interactions of ETs with other molecular players in the development of diabetic cardiomyopathy. They are characterized as follows:

#### 1.1.2.1 *ETs are Upregulated in the Diabetic Heart*

In the heart of diabetic rats, ET-1, as well as $ET_A$ and $ET_B$, are upregulated [20]. ET-1 is produced by both cardiomyocytes and endothelial cells [21]. It is extracted by the failing heart, which is probably caused by the increase in myocardial $ET_A$ and $ET_B$ [22]. Some biochemical pathways, such as protein kinase C activation, nonenzymatic glycation, and activation of other vasoactive factors, may promote increased ET synthesis. ETs have been shown to produce fibrosis *via* activation of transcription factors NF-κB and activating protein 1 (AP-1) [23].

#### 1.1.2.2 *Additive Induction of Collagen Production by TGF-β and ET-1*

A key regulatory molecule in the control of the activity of fibroblasts is TGF-β, a 25-kDa dimeric protein, which influences cell growth and differentiation and exists as three isoforms. TGF-β acts through the following cell-surface receptors: TβR-I, -II and -III. TGF-β stimulates the production of ECM components, such as fibrillar collagen, fibronectin and proteoglycans, and inhibits matrix degradation by upregulating the synthesis of protease inhibitors and downregulating the synthesis of matrix-degrading proteases such as stromelysin and collagenase [24]. Both ET-1 and

TGF-β induce collagen I synthesis and their signaling pathways overlap partially [25]. Apart from this, ET-1 was shown to promote TGF-β release [26,27]. Furthermore, TGF-β facilitates the adhesion of inflammatory cells to the matrix. In sum, TGF-β contributes to the pathological cellular events that are responsible for myocardial fibrosis, hypertrophy and dysfunction [24].

#### 1.1.2.3 *ETs Activate Fibronectin Synthesis*

Diabetic cardiomyopathy is characterized by the deposition of extracellular matrix (ECM) proteins. The ECM maintains tissue architecture and contributes to a variety of developmental processes. It is composed mainly of collagens, proteoglycans and glycoproteins. In response to diabetes, ECM proteins, especially fibronectin and collagen, accumulate in the heart, the blood vessels and also in the kidney (Spiro *et al.*, 1995). Fibronectin is a large dimeric glycoprotein. It functions as an adhesive glycoprotein, aiding in branching morphogenesis, cellular proliferation, platelet-platelet cohesion and wound healing. Several factors, ETs for example, influence augmented fibronectin synthesis. High glucose in endothelial cell culture leads to the upregulation of fibronectin via an ET-dependent pathway involving activation of NF-κB and activation protein AP-1. Chen *et al.* demonstrated that diabetes-induced myocardial focal scarring can be prevented by ET antagonism [23].

#### 1.1.2.4 *ET-1 Enhances PAI-1 Production*

Plasminogen activator inhibitor 1 (PAI-1) is the major physiological inhibitor of plasminogen activators (tissue-type plasminogen activator and urokinase-type plasminogen activator). It is implicated in ECM accumulation by its inhibition of matrix degradation [28]. PAI-1 is synthesized by the endothelium and smooth muscle cells in normal and atherosclerotic arteries. ET-1 has been demonstrated to upregulate PAI-1 mRNA and protein levels [29]. Furthermore, its synthesis is regulated by such cytokines as platelet-derived growth factor, thrombin, interleukin-1, TGF-β, angiotensin II and endotoxin. Higher concentrations of PAI-1 have been found in patients with previous heart infarction and with type 2 diabetes. PAI-1 is also significantly elevated in obese non-diabetic patients [30].

#### 1.1.2.5 *ET-1 Promotes ADM Production*

Adrenomedullin (ADM), a 52-amino acid peptide structurally homologous with calcitonin gene-related peptide, is a marker of cardiac hypertrophy [31-34]. ADM has

been detected in the adrenal medulla, the heart, brain, lung, kidney and the gastrointestinal organs. It has powerful direct vasodilatory effects and is able to increase cardiac output, and it induces diuresis and natriuresis. Plasma ADM levels are reported to be increased in diabetes mellitus, hypertension, congestive heart failure and chronic renal failure. Hence, ADM levels have a statistically significant inverse relation with left ventricle function [35]. The potential role of ADM in the diabetic vascular complications is not clear. ADM may be either related to the existence of a generalized chronic inflammatory process or to endothelial damage [36]. ET-1 stimulates ADM production [37] and the same ADM may inhibit ET-1 expression [38].

#### 1.1.2.6 *ETs Induce ANP Secretion*

An important marker for cardiac hypertrophy is atrial natriuretic peptide (ANP). ANP is released from myocardial cells and circulates primarily as a 28 amino acid polypeptide. It is a direct vasodilator and it increases urinary natrium and water excretion by increasing the glomerular filtration rate (GFR) and reducing natrium reabsorption in the kidney. Both ANP and basic natriuretic peptide (BNP) are important in the control of body fluid homeostasis, blood pressure regulation and vascular remodelling [33]. ETs have been found to activate the release of ANP [39].

#### 1.1.2.7 *RLX Stimulates ET-1 Clearance*

The stimulation of ET-1 released in endothelial cells is inhibited by relaxin (RLX), known as a pregnancy hormone. RLX is a peptide hormone that belongs to the insulin family. It mediates its function through LGR-7, a member of the leucine-rich repeat family of G-protein-coupled orphan receptors [40]. RLX is expressed in human cardiovascular tissues, and the myocardial gene expression of RLX is remarkably upregulated in human congestive heart failure. The peptide affects cardiac function and participates in the regulation of blood pressure, blood flow and fluid balance. It acts in a positive inotropic way [41]. RLX causes vasodilatation of small-resistance arteries from the systemic circulation of humans, and it is a potent vasodilator of coronary blood vessels. RLX stimulates ANP peptide secretion in the perfused rat heart [42], as well as inducing upregulation of $ET_B$, which mediates ET-1 clearance and vasodilatation *via* NO and prostacyclin [31,32].

### 1.1.3 Experimental Models of Diabetic Cardiomyopathy

A wide array of experimental animal models is available to investigate the altered cardiac phenotypes associated with diabetic cardiomyopathy. The models representing similar influences on glycaemia, insulinaemia and lipidaemia are listed in Table 2 [43].

Table 2: Experimental animal models of diabetic cardiomyopathy divided into groups of similar influence on glycaemia, insulinaemia and lipidaemia.

| Groups of Animal Models | Models |
|---|---|
| Models of hyperglycaemia without hyperinsulinaemia | STZ, alloxan – rat or mouse |
| Models of hyperinsulinaemia with or without hyperglycaemia | $ob^-/ob^-$ – leptin-deficient mouse |
| | $db^-/db^-$ – leptin receptor-deficient mouse |
| | ZF – Zucker fatty rat |
| | ZDF – Zucker diabetic fatty rat |
| | OLETIF - Otsuka Long-Evans Tokushima fatty rat |

STZ-induced diabetes is characterized by profound hyperglycaemia, modest hypertriglyceridaemia, ketosis and markedly reduced plasma insulin levels. As such, the model is particularly useful in examining the effects of hyperglycaemia in the absence of hyperinsulinaemia. It represents animal models of type 1 diabetes.

## 1.2 The ET System

ETs are a family of peptides consisting of three isopeptides: endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3). They are 21 amino acid peptides. Yanagisawa *et al.* (1988) first isolated ET-1 from pig arterial endothelial cell cultures. ET-2 differs from ET-1 by 2 amino acids, and ET-3 differs from ET-1 by 6 amino acids (Figure 2). They all have two intrachain disulfide bridges and a hydrophobic C-terminal tail. The N-terminal disulfide loop differs among the ET-isoforms, and the C-terminal linear region is conserved [5,44].

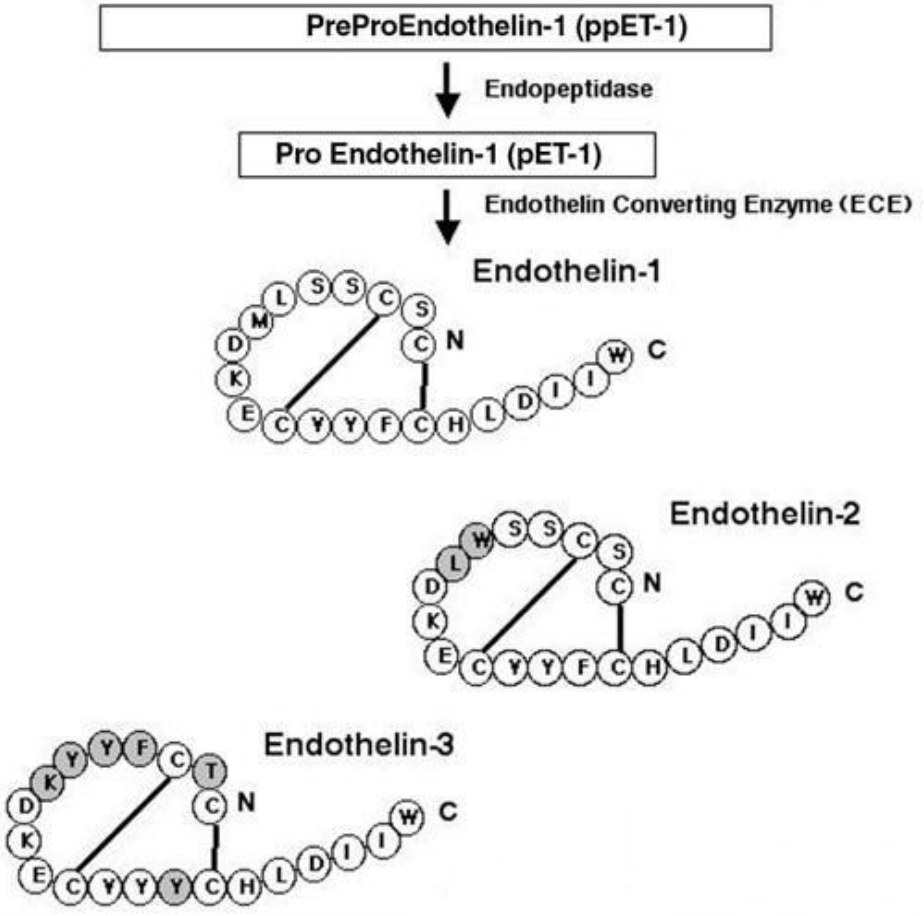


Figure 2: Biosyntheses, amino acid sequences and structures of endothelin-1, endothelin-2 and endothelin-3. ET-2 and ET-3 differ from ET-1 by 2 and 6 amino acids, respectively (Karen A. Fagan *et al.*).

Each isoform is encoded by a different gene: preproendothelin-1 on chromosome 6p23-24, preproendothelin-2 on chromosome 1p34 and preproendothelin-3 on chromosome 20q13.2-13.3 [45].

### 1.2.1 Physiology and Properties of ETs

All ETs have vasoconstrictive potency [5]. ET is ten times more potent than angiotensin II, vasopressin, or neuropeptide Y. As shown in Figure 3, apart from vasoconstriction, ETs modulate inotropy and chronotropy, bronchoconstriction and neurotransmission, regulating other hormones and cytokines. These vasoconstrictors occur in all vascular beds, but the renal vasculature has a sensitivity to ETs about ten times higher than that of the other vascular beds [46]. ETs constrict the renal microcirculation, and this vasoconstriction is particularly long-lasting. They contribute

to decreasing the glomerular plasma flow rate and filtration, because the afferent arteriole constricts more in response to ETs, namely ET-1, than the efferent arteriole. ETs also regulate tubular function. In the inner medullary collecting duct, ET-1 can directly influence tubular function in an autocrine manner. The direct effect of ET-1 on tubules is reduction in fluid re-absorption [47].

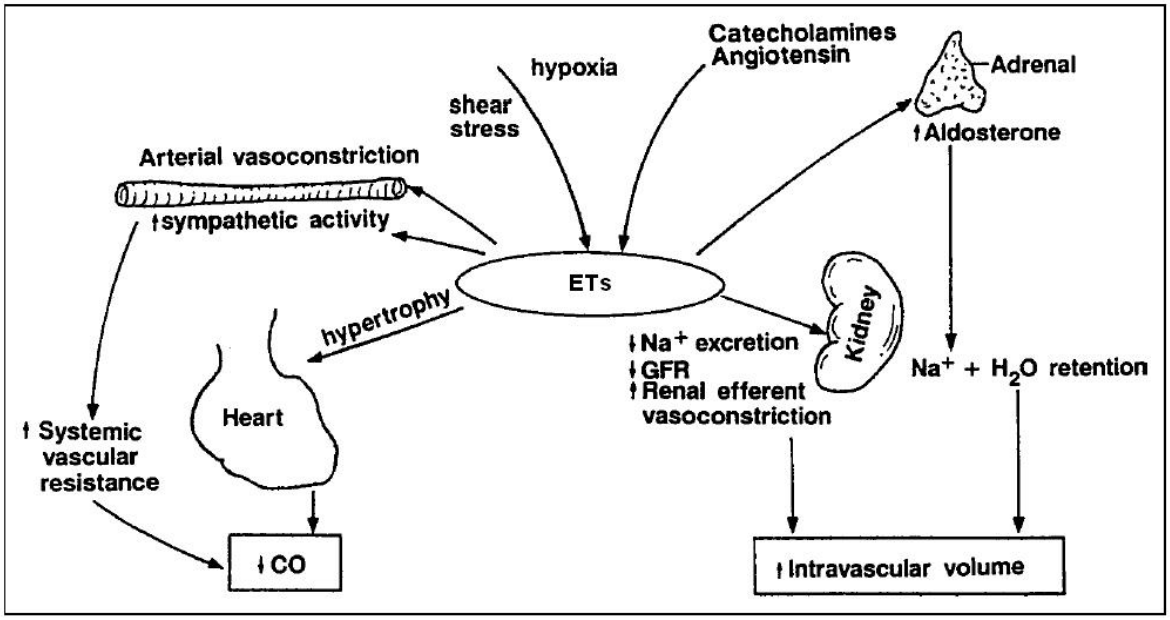


Figure 3: Physiological actions of endothelins (ETs) (Endothelin ELISA manual, Biomedica Immunoassays).

The three human isoforms of ETs are distinct from each other in both structure and pharmacological activity. When assayed on porcine coronary artery strips, the vasoconstrictor activity in terms of maximum tension induced is ET-2 > ET-1 > ET-3. The time required for the recovery of arterial pressure after injection of the bolus of the peptide in rats is also ET-2 > ET-1 > ET-3. However, in terms of molar potency, ET-1 is the most potent ET peptide [44]. Figure 4 shows typical tracings for the blood pressure changes in response to a bolus injection of one of the peptides into anesthetized rats *in vivo*.

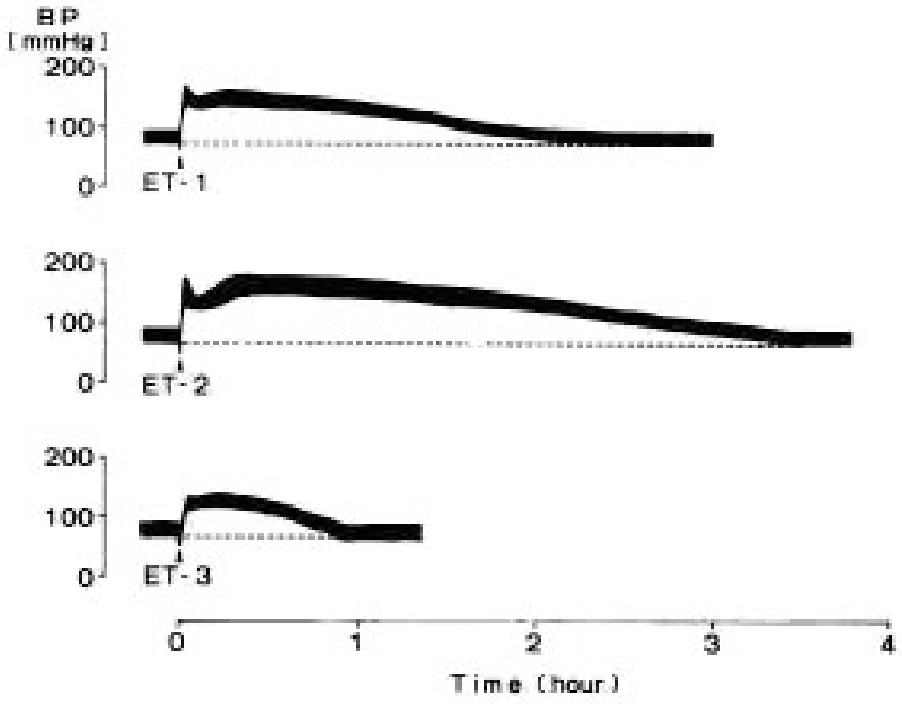


Figure 4: Typical recordings for blood pressure responses of anesthetized chemically denervated rats to intravenous bolus injections of ET-1, ET-2 and ET-3 (1 nmol/kg). BP – blood pressure (Inoue *et al.*)

### 1.2.2 Production and Distribution of ETs

Two-step proteolytic processing is required for the production of biologically active ETs (Figure 1). The 212-residue preproendothelins are first cleaved by a furin-like processing protease. The results are biologically inactive intermediates called big ETs. They are then proteolytically activated at the common Trp21 residue by the highly specific ET converting enzymes (ECEs) [48,49]. There are three isoenzymes of ECE: ECE-1, ECE-2 and ECE-3 [48,50,51].

ECEs belong to the type II membrane-bound metalloprotease family. *In vitro* ECE-1 and ECE-2 catalyze the conversion of big ET-1 to ET-1 most efficiently among the three big ETs. ECE-3 is totally different from ECE-1 or ECE-2 in that the enzyme is highly specific for big ET-3 [51]. ECE-1 and ECE-2 are thought to regulate the production of active ETs. However, as shown by Yanagisawa *et al.* [49], there probably are other proteases responsible for the production of mature ETs.

The isoforms of ET are widely distributed among organs. The best-studied and most widely-distributed ET is ET-1, which is produced by endothelial, mesangial, glomerular and tubular epithelial cells. Circulating cells also secrete ET-1, as, for

example, macrophages. Neutrophils can convert exogenous big ET-1 into the mature form [52,53].

ET-2 mRNA has been detected in renal tissue and is abundant in renal adenocarcinoma [54]. ET-2 mRNA was detected by O'Reilly *et al.* in the human amnion, chorion, endometrium, left ventricle, cultured human vascular smooth muscle and in human umbilical vein endothelial cells [55].

ET-3 predominates in the brain and is also found in various parts of the kidney [51].

### 1.2.3 Regulation of ET Levels

ETs are not stored and their synthesis is regulated at the level of gene transcription with *de novo* synthesis and release. There are a lot of factors that stimulate ET production, for example: angiotensin II, vasopressin, adrenaline, insulin, cortisol, IL-1, TGF-β, thrombin, glucose, low density lipoprotein (LDL), hypoxia, endotoxin, low-shear stress, ET itself and platelet-derived growth factor (PDGF). ET is downregulated by prostacyclin, NO, ANP, heparin and high-shear stress [45]. As shown for the first time by Kohno *et al.*, ADM inhibits the ET production stimulated by PDGF [38].

### 1.2.4 The Function of ET Receptors

There are two ET receptors, $ET_A$ and $ET_B$, which are extensively distributed. They are homologous with other heptahelical receptors of the rhodopsin superfamily. $ET_A$ contains 427 amino acids and binds with the order of affinities of ET-1=ET-2>>ET-3 [56]. $ET_B$, with 442 amino acids, is a relatively non-selective receptor and binds with the order of affinities of ET-1=ET-2=ET-3. The $ET_A$ receptor is found in vascular smooth muscle cells. It is the predominant subtype in many organs, notably the heart. It is not detected in liver or in endothelial cells. $ET_B$ is found in endothelial cells and in vascular smooth muscle cells. It is prominent in the aorta, the brain and the lung. The two receptors co-exist in some regions of the kidney, such the as arterioles, glomerular capillaries and inner medullary collecting ducts. The $ET_A$ receptor modulates vasoconstriction, cellular proliferation and matrix deposition. The function of $ET_B$ receptor is not as obvious. This receptor mediates the release of prostaglandin $I_2$ and NO in endothelial cells, indirectly reducing the vasoconstrictive and mitogenic effects of ET-1. On the other hand, $ET_B$ receptor also has direct vasoconstrictory actions, including vasoconstriction of the renal circulation [50].

The intracellular signaling pathways of ET-1 are shown in Figure 5. Occupation of $ET_A$ receptor activates phospholipase C (PLC), which is central to the generation of cellular responses to ET-1. PLC cleaves phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to form diacylglycerol (DAG) and D-myo-inositol-,4,5-trisphosphate ($IP_3$). DAG binds to and activates protein kinase C (PKC). $IP_3$ binds to the $IP_3$ receptor on the endoplasmic reticulum (ER) and stimulates $Ca^{2+}$ release from the ER stores. Increases in the concentration of intracellular $Ca^{2+}$ are crucial for eliciting responses to ETs. Furthermore, the $ET_A$ receptor is linked to a $Ca^{2+}$ channel (CaCh) in the plasma membrane that opens in response to receptor occupation by ET-1 or its agonists, causing further increase of the intracellular $Ca^{2+}$ content. ETs induce cellular depolarization, increasing influx of $Na^+$ and efflux of $Cl^-$, by opening non-selective cation channels (NsCh) or chloride channels (ClCh), respectively. ETs also open potassium channels (KCh), which leads to the passive efflux of $K^+$ and hyperpolarization, that, in turn, inhibits the CaCh and $Ca^{2+}$ influx [57,58].

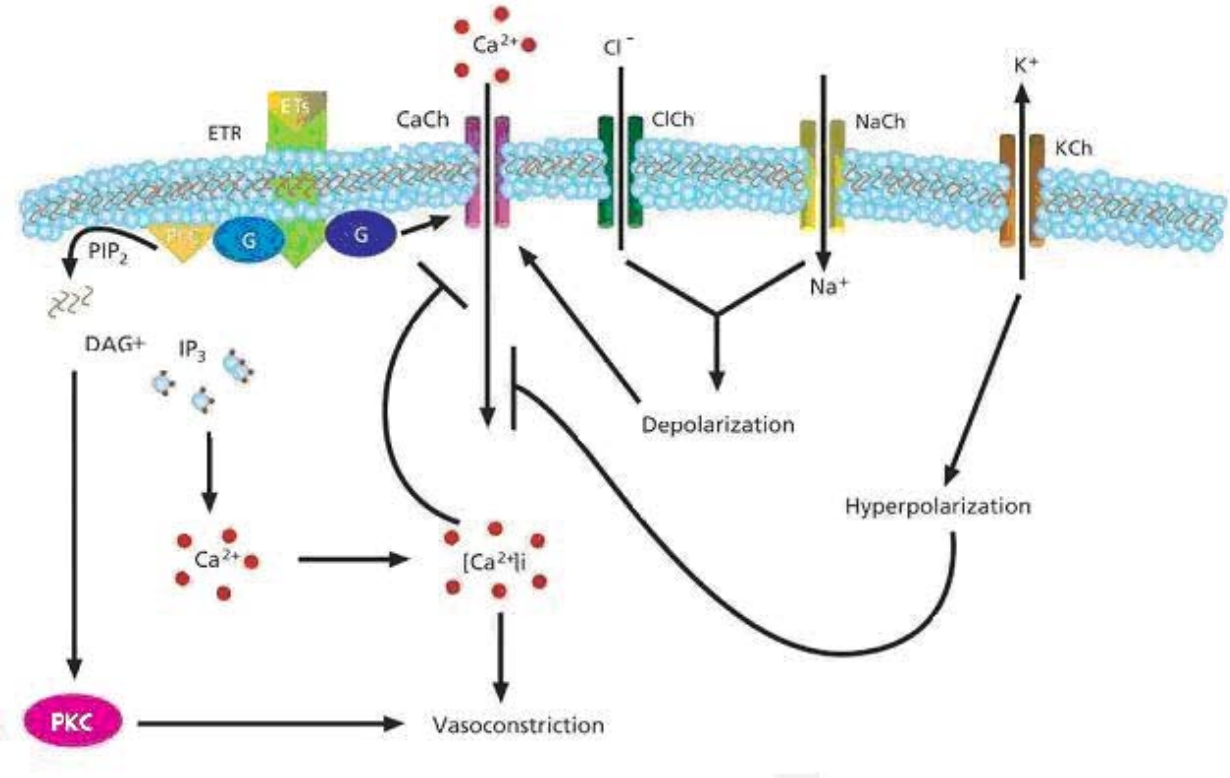


Figure 5: Intracellular signaling pathways activated by ETs (Sigma-Aldrich). For explication, see text above.

### 1.2.5 Recent Reports on the Role of ETs in Diabetic Cardiomyopathy

Patients with diabetes mellitus have significantly increased serum ET-1 levels [59]. ETs are known to act as mitogen and to stimulate cellular hypertrophy, proliferation and collagen deposition. ET-1 interacts synergistically with epidermal growth factor (EGF), TGF-$\alpha$, TGF-$\beta$ and PDGF to potentiate mitogenic and proliferative processes. Furthermore, ETs can increase expression of mRNA for collagen types I, II, and IV and laminin [44]. All these processes are involved in the development of diabetic cardiomyopathy. Thus, ETs were assumed to play a role in hyperglycaemia-induced cardiomyopathy.

Previously, the evidence for the importance of the ET system in the pathogenesis of diabetic cardiomyopathy was mainly derived from inhibitory interventional studies using non-selective or selective ET receptor antagonists. In these investigations, ET receptor blockade has been shown to improve diabetic heart function (stroke volume and ejection fraction [10], systolic and diastolic compliance [60,61] and electrophysiological dysfunction [11,62]), myocardial hypertrophy and fibrosis (cardiomyocyte hypertrophy [9], injury [63] and apoptosis [20], as well as increased extracellular matrix mRNA: fibronectin, collagen α1 [20]). Furthermore, ET receptor antagonists prevented exaggerated coronary reactivity to ET-1 [64] and improved coronary perfusion pressure in diabetes [62]. Accordingly, this is circumstantial evidence showing that ET-1 mRNA and receptor binding is elevated in the rat heart in diabetes [65,66], and that ET-1 is associated with myocardial cell death and focal scarring [20]. However, these reports were not entirely convincing, since several opposing investigations have found no or only modest actions with ET inhibition. This applies to such effects as diabetic hearth hypertrophy, myocardial tissue fibrosis, microangiopathy and coronary vessel stiffness [12,13,61].

### 1.2.6 Transgenic Models for ET-System Components

To define the role of ET in physiology and pathophysiology, the transgenic approach has been used. This experimental system consists of adding a specific gene to the animals' DNA make-up. There are several models for ET-system components.

**Transgenic rats expressing the human ET-2 gene** were characterized by Liefeldt *et al.* [14]. They were generated by micro-injection of fertilized oocytes from Sprague-Dawley rats [67]. The transgene was expressed predominantly in the kidney

(almost exclusively within the glomeruli), the gastrointestinal tract, adrenal gland, spleen, lung and in several brain regions, whereas non-transgenic littermates showed no ET-2 mRNA signal in the kidneys. Plasma ET levels rose 2-fold. Despite these changes, the expression of human ET-2 in transgenic rats did not result in hypertension. Using a competitive reverse transcription-polymerase chain reaction (RT-PCR), no significant differences of $ET_A$ und $ET_B$ receptor expressions were evident. Normotension in the transgenic animals is independent of ET receptor regulation [68,69]. One of the mechanisms influencing the effects of ET on blood pressure may be the nitric oxide system, since infusion of an inhibitor of nitric oxide synthase resulted in a greater blood pressure response than in non-transgenic littermates [68]. The normal blood pressure level may also be due to the fact that the rate of transgene expression in extrarenal vascular beds and the heart is very low [70]. Glomerular transgene expression resulted in a significantly increased glomerular injury score. Male rats were proven to be more susceptible to ET actions. Perivascular infiltration of mononuclear cells was detectable in the kidneys, while glomerular filtration rate was not altered. Creatinine clearance was within a normal range, however, protein excretion was significantly increased [71]. In sum, the elevation of ET-2 expression in this transgenic model does not induce hypertension but leads to changes at the end-organ level. It causes the development of glomerulosclerosis with increased protein excretion.

Other transgenic models are shown in Table 3.

Table 3: Genetic models for ET-system components.

| Name of the model | Genetic modification |
|---|---|
| Human ET-1 transgenic mice | Animal model containing the human ET-1 gene |
| Knockout transgenic mice | Animals without gene function of one or more ET-system components |
| Animals with genetic mutation | Animals with natural mutation of gene, for example with deletion in $ET_B$ receptor |

## 1.3 The Aim of the Study

Several inhibitory interventional studies using pharmacological ET receptor antagonists to prevent diabetic cardiomyopathy have described marked beneficial morphological and functional effects in experimental diabetes models. However, some others have reported conflictingly, observing no or only modest actions of ET inhibition on diabetic heart myocardium and vessel changes.

The aim of this study was to provide independent and complementary results on the role of the ET system in diabetic cardiomyopathy. Thus, a stimulatory intervention was used, *i.e.* transgenic overexpression of the human ET-2 gene in rats, to further characterize the contribution of ETs to the morphological and molecular changes in diabetic cardiomyopathy. The applied model exposes the myocardium and its vessels to markedly increased circulatory ET levels endogenously, rather than through cardiac overexpression. Diabetes mellitus was induced by STZ-injection into both hET-2-positive and -negative littermate animals. Non-diabetic transgene-positive and -negative rats served as controls to complete the following 4 group study design:

1. Non-diabetic non-transgenic group (non-diabetic wild-type)
2. Non-diabetic transgenic group (non-diabetic hET-2 TG+)
3. Diabetic non-transgenic group (diabetic wild-type)
4. Diabetic transgenic group (diabetic hET-2 TG+)

After 6 months of hyperglycaemia, the following questions were addressed:

1. How is the function of the myocardium altered, according to hemodynamic results (blood pressure and pulse)? Will hyperglycaemia abolish the phenomenon of normotension in hET-2 transgenic rats? Both blood pressure and pulse were measured at the end of the study.
2. How is the structure of the myocardium affected? How are the absolute and relative heart weights changed? How do the left and right heart ventricle wall thicknesses relate to the heart weight? How do the cardiomyocyte diameter and molecular markers of heart hypertrophy (ANP and ADM) correlate with ventricle wall morphometry? What is the extension of fibrosis in the heart in this diabetic model, as reflected by volume density of interstitial tissue and molecular fibrosis markers (TGF-β1, fibronectin and PAI-1)? The central feature of myocardial remodelling in diabetes is an increase in ventricular mass on the cellular level, hypertrophy of individual cardiomyocytes and diffuse interstitial fibrosis.

3. What is the characteristic of the remodelling of vessels, including the aorta, large epicardial arteries and small intramyocardial arterioles? Will aortic media thickness correlate with blood pressure? Will diabetes and hET-2 expression influence the morphometry of heart vessels, regardless of their diameter? Vascular complications are a critical factor in the prognosis of diabetes patients.
4. What are the main changes of ET-system components expression? Do they explain the functional and structural findings of diabetic cardiomyopathy? Will any well-known interactions of ETs with other molecular players in the development of diabetic cardiomyopathy be confirmed by this study? Will hET-2 overexpression in this transgenic model promote diabetic cardiomyopathy, despite the absence of hET-2 expression in the myocardium? How will hET-2 overexpression influence the expression of other ET-system components? mRNA was isolated from the heart apex for further analysis of ET-system components, fibrosis and cardiovascular markers.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Tools, Instruments and Chemicals

Table 4 contains the tools that were used during the experiments.

Table 4: Tools and instruments used during the experiments.

| Tools and instruments | Name of producer |
|---|---|
| Blood pressure monitor | Infraton Tensiomat, Boucke, Germany |
| Camera Polaroid | Polaroid GmbH, United Kingdom |
| Centrifuge 5417R | Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany |
| Cryo Tube™ Vials | NUNC™ Brand Products, Denmark |
| Eppendorf pipette/Multipette | Eppendorf AG, Hamburg, Germany |
| Eppendorf tips | Eppendorf AG, Hamburg, Germany |
| Eppendorf tubes 0,5/1,5/2,0 ml | Eppendorf AG, Hamburg, Germany |
| Fluorescence detector – Typhoon 8600 Variable Mode Imager | Amersham Pharmacia Biotech, USA |
| Glucometer | Medi Sense® Bedford, USA |
| Homogenizer | Labortechnik, ART, Müllheim, Germany |
| LightCycler™ | Roche Diagnostic GmbH, Mannheim, Germany |
| LightCycler™-Kapillaren | Boehringer Mannheim, Mannheim, Germany |
| Megafuge 2.0R | Heraeus Instruments, Hanau, Germany |
| Microscope | Leica Microscopie & Systeme GmbH, Wetzlar, Germany |

| | |
|---|---|
| Microscope | Axio Imager.A1, Zeiss, United Kingdom |
| Microwave | Bosch, Germany |
| Microtiter plate shaker TITRAMAX 101 | Heidolph Instruments, Schwabach, Germany |
| Microtome | Microm International GmbH, Walldorf, Germany |
| NONC-Immuno 96-Well shape plates | NUNC A/S, Roskilde, Denmark |
| Pipette 2, 5 and 25 ml | Greiner Bio-one GmbH, Frickenhausen, Germany |
| pH Meter | Wissenschaftlich Technische Werkstätten, Weilheim, Germany |
| RNA-DNA-Calculator | Pharmacia Biotech Ltd, Cambridge Science Park, England |
| S-Monovette 9 ml with EDTA | Sarstedt, Nümbrecht, Germany |
| Vortex-2-Genie | Scientific Industries (SI), Bohemia, N.Y.USA |

Unless otherwise stated, all of the chemicals used, came from Sigma Chemical-Aldrich Co. (Taufkirchen, Germany).

### 2.1.2 Computer and Software

- ELISA measurements and data analysis were done using BioLinx$^{TM}$ (Dynatech Laboratories, Inc, Sullyfield Circle, USA).
- Analyses of the real-time polymerase chain reaction (PCR) were carried out using LightCycler 3.5.3 (Roche, Mannheim, Germany).
- Morphometric analyses were carried out with specific software ImageJ 1.31v (National Institutes of Health, USA).
- Statistical analyses were done using SPSS 12.01 (SPSS Inc. Chicago, USA) and Excel 2002 (Microsoft Corporation, USA).

- Graphics were done using Excel 2002 and Power Point 2002 (Microsoft Corporation, USA).
- The dissertation was written using Word 2003 (under Windows XP Professional, Microsoft Corporation, USA)

## 2.2 Animals

Sixty-eight male Sprague-Dawley (SD) rats with an initial weight of 197-345 g were used in this study. Thirty-four of the animals were heterozygous for the transgene human ET-2 named TGR(hET-2)37 [71], the other 34 littermates were transgene-negative. In this model, transgenic hET-2 overexpression results in significantly increased circulatory endothelins concentrations. Predominant source of hET-2 is kidney, gastrointestinal tract, adrenal gland, spleen, lung and several brain regions, but not heart or aorta [14]. Animals were housed in Forschungszentrum für Experimentelle Medizin in Berlin (Krahmerstr.6, 12207 Berlin). They were kept in cages (four rats per cage) and maintained under a 12-h dark/light cycle and environmental temperature of 21 to 23°C (75 ± 10% humidity). The rats had free access to food and tap water throughout the entire study.

The procedures were performed in accordance with the standard principles of laboratory animal care. The study has been approved by Landesmat für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit (LAGetSi).

## 2.3 Experimental Design

### 2.3.1 Diabetes Induction

Hyperglycaemia was induced by intraperitoneal administration of STZ (S-0130 SIGMA-Aldrich CHEMIE GmbH, Steinheim, Germany) at 60 mg/kg body weight in 17 randomly chosen animals from both the transgenic and non-transgenic group. The remaining animals were left non-diabetic. STZ was stored as a powder at -21°C. Immediately before use, the material was dissolved at a concentration of 40mg/ml in 50mM sodium citrate (pH 4.5). Rats with a blood glucose level greater than 250 mg/dl in the early morning were considered diabetic.

STZ, an antibiotic produced by a strain of Streptomyces achromogenes, is described as a Trojan horse-like molecule. It is N-methylnitrosocaramoyl-D-glucosamine. STZ is glucose linked to a reactive nitrosourea moiety, and as such it is internalized through the cell's glucose transporters. Once the molecule is inside, the

nitrosourea moiety is released and poisons the cell by cross-linking vital structures. The most sensitive cells to STZ poisoning are β cells of the pancreas, because they are more active than other cells in taking up glucose. That is why STZ preferentially kills β cells and causes diabetes [72].

### 2.3.2 Insulin Treatment

Diabetic rats were treated with insulin subcutaneously (Protaphane, 100 IE/ml, Novo Nordisk Pharmaceuticals Ltd, Denmark, 2–8 IE/day). The dose was adjusted individually according to body weight change and blood glucose concentration to maintain blood glucose concentration in the range of 250 to 600 mg/dl and thereby to prevent mortality of the rats due to metabolic complications. Blood samples were taken from the tail vein and blood glucose levels were measured by the glucose oxidase method using glucometer Precision$^{TM}$ PCx$^{TM}$. Insulin treatment was initiated 2 weeks after diabetes was confirmed and was administered every second day. After 12 weeks of diabetes, insulin was injected daily.

### 2.3.3 Metabolic Study

After diabetes induction, all rats were housed separately in metabolic cages for 24 hours once a month. Body weight, daily intake of water and food, and excretion of urine were measured individually.

### 2.3.4 Systolic Blood Pressure and Heart Rate

The systolic blood pressure and heart rate were measured before the end of the study by non-invasive tail cuff plethysmography. After a one week training period, 7 measurements were taken for each rat and the values were averaged.

### 2.3.5 Harvesting of Materials

After 6 months of continuous hyperglycaemia, the animals were sacrificed and hearts were harvested for molecular and structural analyses. Animals were anesthetised with Ketamine 0.5mg/kg body weight and Rompun (Xylazin) 2% 0.05mg/1kg body weight. A midline incision was made and the renal segment of the aorta exposed. To assess plasma endothelin level the blood samples were drawn from the aorta *via* an intra-aortic cannula into tubes containing chilled potassium ethylenediamine-tetra-acetic acid (EDTA) and immediately placed on ice until it was centrifuged for 10 min at 3000 x *g* at 4°C. Plasma samples were kept frozen at -70°C. The animals were perfused *in vivo* at arterial pressure with ice-cold sterile PBS

(Phosphate Buffered Saline – Dulbecco, without $Ca^{2+}$, $Mg^{2+}$, Biochrom AG, Berlin, Germany) *via* the same cannula. The aorta and the inferior caval vein were clamped together proximal to renal arteries and veins, the inferior caval vein was opened distal to renal veins for drainage so that the kidneys could be perfused with 60 ml PBS. Both kidneys were then excised from the animal, adhering tissue was trimmed and the left kidney was weighed. Apex of the left kidney was cut off and frozen in liquid nitrogen. One slice of the kidney was put into 10% formaldehyde for histological examination. The remaining tissue was used to isolate glomeruli. The eyes were removed. One of them was put into 10% formaldehyde, the second one was frozen in liquid nitrogen. Thoracotomy was performed (dissection of the sternum) and apex of the heart was clamped, removed and immediately frozen in liquid nitrogen. For perfusion the left ventricle was punctured and the right atrium was incised. The perfusion was performed with 20 ml Rheomacrodex® (Pharmalink, Germany) and 20 ml 3% glutaraldehyde for structural analyses [2,3,13]. The heart was excised and weighed together with the apex. Results are expressed as absolute and relative heart weight in relation to the corresponding body mass of the animal in order to adjust for differences in the mean body weights between the groups.

Samples of kidney and heart for RNA isolation were stored at -70°C.

## 2.4 Measurements

### 2.4.1 Evidence of hET-2 Expression

Transgenic state was confirmed by PCR analysis of isolated tail tissue DNA, RT-PCR of isolated kidney RNA and by ELISA measurements of ET plasma level.

#### 2.4.1.1 *Tail Tissue DNA Isolation*

DNA was isolated from snap-frozen tail tissue using TRIzol® Reagent (Total RNA Isolation Reagent, Invitrogen™ Life Technologies, USA).

Approximately 250 mg frozen material was transferred to a tube with 1 ml TRIzol reagent. Then it was frozen in liquid nitrogen and homogenised for one min (Art-Micra D-8, n = 39000 $min^{-1}$, ART, Müllheim, Germany). The homogenised sample was incubated for 5 min at room temperature. After adding 0.2 ml chloroform and mixing for 30 s, this suspension was incubated at room temperature for 2 min and centrifuged at 12000 x *g* for 10 min at 4°C. After this procedure the aqueous phase was completely removed and the DNA remaining in the interphase was

precipitated with 0.3 ml of 100% ethanol and stored at room temperature for 3 min. DNA was then sedimented by centrifugation at 2000 x *g* for 5 min at 4°C. After removing the phenol-ethanol supernatant, the DNA pellet was washed twice in 1 ml of a solution containing 0.1 M sodium citrate in 10% ethanol. At each wash, the DNA pellet was stored in the washing solution for 30 minutes at room temperature and centrifuged at 2000 x *g* for 5 min at 4°C. Following these two washes, the DNA pellet was suspended in 2 ml of 75% ethanol and stored for 20 min at room temperature with periodic mixing and centrifuged at 2000 x *g* for 5 min by room temperature. The DNA was then air dried for 10 min in an open tube and dissolved in 8 mM NaOH such that the concentration of DNA was 0.2 – 0.3 µg/µl.

2.4.1.2 *Conventional PCR*

PCR was created using PCR-Script® Amp Cloning kit (Stratagene, Canada). The amplification was carried out using the primers for human ET-2 (sense: 5'-AGCGTCCTCATCTCATGCCC-3'; antisense: 5'-TCTCTTCCTCCACCTGGAATG-3') with a predicted product size of 435 bp. Reactions were performed in 20 µl volumes containing the following reaction components:

Table 5: Mixture of PCR Components.

| **Components** | **Volume (µl)** |
|---|---|
| PCR-buffer | 2 |
| $MgCl_2$ | 2 |
| dNTP mix | 0.1 |
| Tag polymerase | 0.25 |
| RT product | 1 |
| Primer SE | 1 |
| Primer AS | 1 |
| DEPC water | 12.65 |

PCR was carried out using the following templates:

Table 6: Templates of PCR.

| | **First (initial) cycle** | | **Subsequent cycles** (x38) | | **Last cycle** (final extension) | |
|---|---|---|---|---|---|---|
| | Temp.(°C) | Time (s) | Temp.(°C) | Time (s) | Temp.(°C) | Time (s) |
| Denaturation | 95 | 630 | 95 | 30 | 95 | 30 |
| Annealing | 59 | 45 | 59 | 45 | 59 | 45 |
| Extension | 72 | 120 | 72 | 120 | 72 | 480 |

40 cycles of amplification were used. The products of PCR were qualitatively examined by the staining of the samples separated by gel electrophoresis.

2.4.1.3 *Kidney RNA Isolation*

RNA was isolated from snap-frozen sections of kidney using TRIzol® Reagent (Total RNA Isolation Reagent, Invitrogen™ Life Technologies, USA). The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement on the single-step RNA isolation method developed by Chomczynski and Sacchi [2440339].

Approximately 250 mg of frozen material was transferred to a tube with 1 ml TRIzol reagent. Then it was frozen in liquid nitrogen, homogenised for one min (Art-Micra D-8, n = 39000 $min^{-1}$, ART, Müllheim, Germany) and stored for 24 hours at -70°C. The homogenised sample was incubated for 5 min at room temperature. After adding 0.2 ml chloroform and mixing for 30 s, this suspension was incubated at room temperature for 2 min and centrifuged at 12000 x *g* for 10 min at 4°C. After this procedure the aqueous phase was transferred to a clean tube. A second chloroform extraction was performed (adding of 0.2 ml chloroform, mixing for 30 s, incubating at room temperature for 2 min and centrifuging at 12000 x *g* for 10 min at 4°C), followed by a precipitation with 0.5 ml isopropyl alcohol and centrifuging at 12000 x *g* for 10 min at 4°C. The resulting pellet was then washed in 1 ml 75% cold ethanol, mixed and centrifuged at 7500 x *g* for 5 min by 4°C. The air dried pellet was dissolved in 50 µl DEPC– water and stored at -70°C.

2.4.1.4 *First-Strand cDNA Synthesis*

First-strand complementary DNA was prepared using RNA PCR Core KIT (GeneAmp®, Applied Biosystems by Roche Molecular Systems Inl., Branchburg, New Jersey USA). Reverse transcription (RT) was carried out by the addition of 2 µl of isolated RNA as template, 4 µl of 25mM $MgCl_2$, 2 µl of 10X PCR Buffer II, 2µl each of dATP, dCTP, dGTP, dTTP, 1µl each of RNase Inhibitor, MuLV Reverse Transcriptase, Random Hexamers and DEPC – water. The reaction profile was 21°C for 10 min, followed by 42°C for 60 min. The reaction was terminated by a 5-min incubation at 99°C. The resulting RT products were stored at -21°C.

PCR analysis using cDNA and hET-2 primers was carried out as described in 2.4.1.2.

2.4.1.5 *Endothelin Plasma Level*

Plasma endothelin concentration was measured with a commercial enzyme immunoassay kit (Biomedica Medizinprodukte BmbH & Co KG, Austria). The cross-reactivity was 100% toward ET-1, 100% toward ET-2, less than 5% toward ET-3 and less than 1% toward Big Endothelin. The minimal level of detection determined for this assay was 0.05 fmol/ml. Endothelin plasma levels were defined from 2 repeated measurements.

One ml of each plasma sample was placed in a tube containing 1.5 ml of diluted PAA (Precipitating Agent Additive), mixed for 30 s and centrifuged for 20 min at 3000 x g at 4°C. The resulting supernatant was transferred into another tube, dried for 3 hours by 30°C, re-dissolved in 500 µl of assay buffer and mixed for 30 s. After that standards (serial dilution: 10 / 5 / 2.5 / 1.25 / 0.625 fmol/ml) for standard curve were prepared using endothelin stock (synthetic human ET-1) and ready to use buffer. This buffer was used as a zero standard. 200 µl of each sample, standards, and a control were added in duplicate into respective wells of a microtiter plate. Then 50 µl of detection antibody (monoclonal mouse anti endothelin antibody) was added into each well. After incubation at room temperature for 16 hours the plate wells were washed 5 times with 300 µl diluted wash buffer. The remaining wash buffer was removed by hitting the plate against a paper towel after the last wash. After that, 200 µl of conjugate (anti mouse IgG antibody coupled to the substrate-modifying enzyme) was added to each well. The plates were covered and incubated 3 hours at 37°C. The same washing procedure used after the first incubation. After that, 200 µl of

substrate solution was added to each well. After incubation at room temperature for 30 min in the dark, the reaction was stopped by adding 50 µl of stop solution into each well. The absorbance was measured immediately at 450 nm.

### 2.4.2 Morphometric Analyses

Heart samples were fixed in 4% formalin and embedded in paraffin. Individual blocks of this material contained the tissue in a certain orientation. Each tissue was sliced at 4 µm. The sections were stained with periodic acid Schiff (PAS) and examined under a light microscope. Every morphometric analysis was carried out by an observer blinded to the experimental treatment.

#### 2.4.2.1 *PAS Staining*

This method, used for the detection of glycogen, required the following solutions:

1. Alcoholic periodic acid solution: 1 g periodic acid was dissolved in 30 ml distilled water and then 70 ml 100% ethanol was added.

2. Alcoholic disulphide solution: 0.5 g potassium disulphide was dissolved in 30 ml distilled water and then 70 ml 100% ethanol was added.

3. Schiff reagent commercial mixture (Merk, Darmstadt, Germany).

The slides were deparaffinized and transferred into the descending alcohol row as follows: twice xylene (for 10 min), twice 100% ethanol (for 2 min), twice 96% ethanol (for 2 min), once 80% ethanol (for 1 min), once 50% ethanol (for 1 min). Then they were brought into 1% periodic acid for 10 min and washed in running tap water for 5 min. The slides were then placed in the Schiff reagent warmed to 40°C and incubated for 20 min. After briefly dipping in disulphide solution the slides were washed in running tap water for 10 min. Then the slides were placed in Meyer‘s hematoxylin (Merk, Darmstadt, Germany) and washed in running tap water again for 10 min. They were then put into the following ascending alcohol row: distilled water (for 1 min), ethanol 50% (for 1 min), ethanol 80% (for 1 min), twice ethanol 96% (for 2 min), twice ethanol 100% (for 2 min) and twice xylene (for 5 min) and covered up with Corbit-Balsam (R. Langenbrink, Emmendingen, Germany). The stain‘s results were: connective tissue – blue, cytoplasm – pink, nucleus – blue or black.

2.4.2.2 *Thickness of Heart's Walls*

The thickness of the heart's wall was measured using the optical microscope supplied with a graduated eyepiece micrometer and a 20x/0.75 objective. The right ventricle wall thickness was measured at four different sites and the left ventricle wall thickness was assessed at two different sites. The results were averaged.

2.4.2.3 *Cardiomyocyte Diameter*

Cardiomyocyte size was determined by digital measuring diameters of fifteen randomly selected cells of right and left ventricle. Images were captured by a high-resolution video camera connected to a light microscope with the 20x/0.5 objective. Diameters of cardiomyocytes were determined by use of ImageJ 1.31v (National Institutes of Health, USA). Data was shown as a mean of diameter of right and left ventricle cardiomyocytes together and as the ratio of right/left ventricle myocyte diameter.

2.4.2.4 *Coronary Arteries*

All morphometric analyses of coronary arteries were carried out digitally. Light microscope, high resolution camera and specific software were used as described above. Areas were determined by manually tracing the media contour on a digitized image.

The media cross-sectional area was obtained from the area of artery without adventitia and the area of lumen, calculated as:

$$MCSA = A - L$$

MCSA – media cross sectional area

A – area of artery without adventitia

L – area of lumen

Using the calculated MCSA and the lumen area, the media/lumen ratio of coronary arteries (ML) was then calculated:

$$ML = \frac{MCSA}{L}$$

ML – media to lumen ratio

MCSA – media cross sectional area

L – area of lumen

The media width was measured at four different sites along the wall and the results were averaged. The lumen diameter obtained was the mean of the two measurements at the wide and narrow sites of lumen.

### 2.4.3 Stereological Analyses

Heart tissue samples and sections were obtained and stained according to the orientator method [73]. Briefly, uniformly random sampling of the myocardium was achieved by preparing a set of equidistant slices of the left ventricle and the interventricular septum with a random start. Two slices were selected by area weighted sampling and processed accordingly. Eight pieces of the left ventricular muscle including the septum were prepared and afterwards embedded in Epon-Araldite. Semi-thin sections (0.8 µm) were stained with methylene-blue and basic fuchsin and examined by light microscopy with oil immersion and phase contrast at a magnification of 1000:1.

All investigations were performed in a blinded manner, *i.e*. the observer was unaware of which study group the animal belonged to. Stereological analysis was performed on 8 random samples of differently orientated sections of the left ventricular myocardium per animal according to the orientator method [73]. Volume density ($V_V$) of capillaries, interstitial tissue and myocytes was obtained using the point counting method according to the equation $P_P = V_V$ (with $P_P$ is point density). Using the point counting method and a magnification of x 1000 these structures can be easily identified and counted on semi-thin sections using standard criteria [74]. Reference volume was the total myocardial tissue (exclusive of non-capillary vessels, *i.e.* arterioles and veins, and tissue clefts). Vascular geometry of intramyocardial arterioles, *i.e*. vessels with lumen diameters between 30 and 150 µm and at least one muscular layer, was analysed using planimetry and a semiautomatic image analysis system (Analysis, SIS, Münster, Germany) as described in detail [74] to determine the mean wall thickness, lumen diameter, media and lumen area in every arteriole that was present in the 8 semi-thin sections per animal.

### 2.4.4 Heart RNA Analyses

RNA was isolated from the heart apex using the method described above (2.4.1.3).

2.4.4.1 *Quantitative Determination of RNA*

One µl of isolated RNA was dissolved in 999 µl of DEPC–water. Absorbance of RNA was measured at 260 nm using spectrophotometer (GeneQuant II RNA/DNA Calculator, Pharmacia Biotech Ltd, Cambridge Science Park, England). The calculation of the RNA-concentration was done using the formula:

$$C = A \times 40$$

C – RNA concentration (µg/µl)

A – absorbance of sample

The purity of RNA was assessed using the ratio of absorbances: $A_{260}/A_{280}$.For pure RNA in amounts from 1.8 to 2.

2.4.4.2 *First-Strand cDNA Synthesis*

First-strand cDNA was synthesised from isolated heart RNA using the method described above (2.4.1.4).

2.4.4.3 *Real-Time PCR*

Real-time PCR was carried out with the LightCycler™. At the beginning the PCR conditions were established. The concentration of $MgCl_2$ and annealing temperature were optimized. In order to asses the presence of contaminating traces of DNA, RT-minus control was always carried out.

PCR reactions were performed in microcapillary tubes with a final volume of 20 µl using LightCyler FastStart DNA, Master SYBR Green I (Roche Diagnostics GmbH, Penzberg, Germany). The reaction mixture consisted of 11.4 µl $H_2O$, 1.6µl $MgCl_2$, 1.5 µl each of forward and reverse 10 µM primers (Tib-Molbiol, Berlin, Germany), 2 µl "Hot Start" reaction mix and 2 µl of cDNA template. For temperature profiles and primer sequences for PCR reactions see Tables 7, 8 and 9.

Table 7: Templates of real-time PCR.

| **Template** | **Sequence** | **Annealing/Signal Temperature** |
|---|---|---|
| ADM | | 60°C/88°C |
| sense | 5’-ATGAAGCTGGTTTCCATCGC-3’ | |
| antisense | 5’-CCATGCCGTCCTTGTCTTTG-3’ | |
| ANP | | 60°C/80°C |
| sense | 5’-GCCATATTGGAGCAAATCCC-3’ | |
| antisense | 5’-CTACCGGCATCTTCTCCTCC-3’ | |
| β-actin | | 62°C/87°C |
| sense | 5’-GGCATCCTGACCCTGAAGTACCCCA-3’ | |
| antisense | 5’-GTGCAACAAGACAGCACTGTGTTG-3’ | |
| ET-1 | | 62°C/88°C |
| sense | 5’-TTCTCTCTGCTGTTTGTGG-3’ | |
| antisense | 5’-CTGAGTTCTTTTCCTGCTTGGC-3’ | |
| ET-2 | | 59, 60, 62, 64°C/ no signal |
| sense | 5’-GTGTCCTCTCCAGCTTTCCC-3’ | |
| antisense | 5’-CTTCTCACTGGGAGGGTCCC-3’ | |
| $ET_A$ | | 60°C/85°C |
| sense | 5’-GGACCTGCATGCTCAATGCC-3’ | |
| antisense | 5’-GCATCTCACAGGTCATGAGG-3’ | |

| | | |
|---|---|---|
| $ET_B$ | | 65°C/78°C |
| sense | 5’-GGGTCTGCATGCTTAATCCC-3’ | |
| antisense | 5’-GTAGAAACTGAACAGCCACC-3’ | |
| Fibronectin | | 64°C/84°C |
| sense | 5’-GGTCCAAATCGGTCATGTTCCCA-3’ | |
| antisense | 5’-CGTAATGGGAAACCGTGTAAGGG-3’ | |
| LGR-7 | | 65°C/82°C |
| sense | 5’-AACATCAGTAAGTGCCTGCC-3’ | |
| antisense | 5’-GTCCTCATCAGCTTGGTTCC-3’ | |
| PAI-1 | | 64°C/86°C |
| sense | 5’-CAGCATGTGGTCCAGGCCTCCAAA-3’ | |
| antisense | 5’-TGTGCCGCTCTCGTTCACCTCGATCT-3’ | |
| RLX | | 65°C/82°C |
| sense | 5’-CTTCTCTCCTTTCAGTTCCC-3’ | |
| antisense | 5’-AAGTCTGAGTACTGGGGACC-3’ | |
| TGF-β1 | | 64°C/86°C |
| sense | 5’-GGTGGCAGGCGAGAGCGCTGA-3’ | |
| antisense | 5’-GGCATGGTAGCCCTTGGGCT-3’ | |

Table 8: PCR Parameters.

| | |
|---|---|
| Denaturation | 95°C – 10s<br>20°C/s |
| Annealing | X*–5s<br>20°C/s |
| Extension | 72°C – 20s<br>20°C/s |
| Signal | Y**<br>20°C/s |

Table 9: Melting-Curve Analysis.

| | |
|---|---|
| Step 1 | 95°C – 0s<br>20°C/s |
| Step 2 | 65°C – 15s<br>20°C/s |
| Step3 | 95°C – 0s<br>0,1°C/s |
| Signal | Continuous |

* Annealing temperature in Table 7

** Signal temperature in Table 7

2.4.4.4 *Relative Quantification*

Real-time PCR monitoring on the LightCycler, offers a convenient way to identify and measure the amount of starting material (cDNA). Data analysis was done in the phase of constant amplification efficiency. The target concentration was calculated relative to the house-keeping gene β-actin, which serves as internal reporter for data normalization [75]. This method, which uses an endogenous control as a reference, has the advantage that it corrects for factors influencing the PCR.

## 2.5 Statistics

The data are expressed as means ± standard error of the mean (SEM). Comparisons between groups were performed using the Kruskal-Wallis test and Mann-Whitney-U-Test with SPSS, Windows Version 12.01. A p value $<0.05$ was considered statistically significant.

## 2.6 Supplement

The analysis of volume density of interstitial tissue and morphometry of intramyocardial arterioles were carried out in a blinded fashion (the investigator was unaware of group of the respective animals) by F. Walcher under supervision of Prof. K. Amann (Department of Pathology, University of Erlangen-Nürnberg, Erlangen).

# 3 Results

## 3.1 The Experimental Model

### 3.1.1 Confirmation of STZ-Induced Diabetes

#### 3.1.1.1 *Blood Glucose Level*

As shown in Table 10, serum glucose concentrations confirmed successfully developed experimental diabetes 48 h after STZ injection in diabetic groups.

Table 10*:* Glycaemia 48 h after STZ injection among non-transgenic (Wild-type) and transgenic (hET-2 TG+) rats. Rats with a blood glucose level greater than 250 mg/dl in the early morning were defined as diabetic animals.

| | Non-diabetic rats | | Diabetic rats | |
|---|---|---|---|---|
| | Wild-type | hET-2 TG+ | Wild-type | hET-2 TG+ |
| Glycaemia (mg/dL) | 117±3 | 122±6 | 382±25*** | 389±28$^{§§}$ |

***p<0.001 *vs.* non-diabetic wild-type

$^{§§}$p<0.01 *vs.* non-diabetic hET-2 TG+

#### 3.1.1.2 *Body Weight*

As shown in Figure 6, body weight increase over time was strongly down regulated by diabetes and hET-2 expression. During the first 3 months diabetic groups failed to follow the weight gain pattern displayed by their transgene state related non-diabetic groups (wild-type groups: diabetic 327±12 g *vs.* non-diabetic 489±11 g, p<0.001; transgenic groups: diabetic 285±23 g *vs.* non-diabetic 411±10 g, p<0.001).

At the end of the study, due to intensified insulin treatment for the last 3 months of experiment, while transgenic groups reached the similar body weight (diabetic 400±8 g *vs.* non-diabetic 423±15 g), diabetic wild-type animals remained significantly lighter than non-diabetic (435±8 g *vs.* 515±15 g, p<0.001).

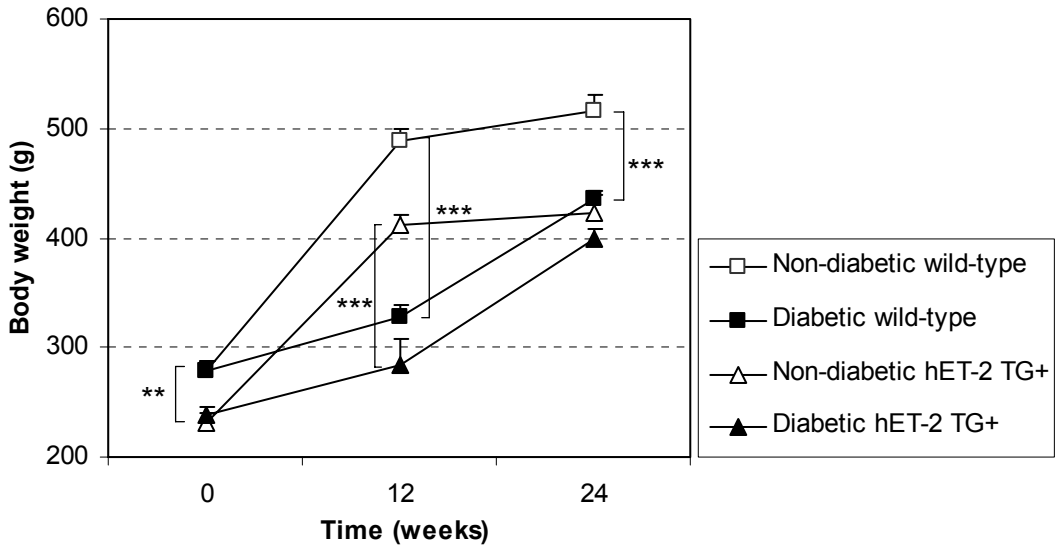


Figure 6: Effect of hET-2 expression (hET-2 TG+) on body weight at the beginning of the study (0 week), 12 weeks and 24 weeks after induction of diabetes. White and black figures represent body weight of non-diabetic and diabetic rats, respectively. Squares represent wild-type groups and triangles are attributed to transgenic groups. ** $p<0.01$, *** $p<0.001$

As anticipated, at the beginning of the study transgenic animals were significantly lighter than wild-type animals (non-diabetic: 231±10 g *vs.* 278±9 g, $p<0.01$; diabetic: 239±8 g *vs.* 279±8 g, $p<0.01$), confirming previous reports in the hET-2 model [14]. During the whole study, body weights of transgenic rats remained lower than those in wild-type diabetes state related groups.

3.1.1.3 *Water Intake and Urine Volume*

As shown in Table 11, daily intake of water and excretion of urine, which was much greater among diabetic rats, confirmed the development of diabetes, indicating polydipsia and polyuria, typical symptoms of hyperglycaemia. Interestingly, transgenic rats drank two times less water and voided two times less urine.

Table 11: Effects of hET-2 expression (hET-2 TG+) on daily water intake and urine volume 1 month after induction of diabetes. Rats had free access to water. Urine was collected for 24 hours using metabolic cages.

| | Non-diabetic rats | | Diabetic rats | |
|---|---|---|---|---|
| | Wild-type | hET-2 TG+ | Wild-type | hET-2 TG+ |
| Water intake (ml/day) | 34.6±6.9 | 17.1±4.5 | 152.8±19.6*** | 68.3±23.5$^{\S\#}$ |
| Urine volume (ml/day) | 15.1±1.8 | 8.5±1.3** | 132.3±15.9*** | 65±19.4$^{\S\S\S\#}$ |

**p<0.01 *vs.* non-diabetic wild-type

***p<0.001 *vs.* non-diabetic wild-type

$^{\S}$p<0.05 *vs.* non-diabetic hET-2 TG+

$^{\S\S\S}$p<0.001 *vs.* non-diabetic hET-2 TG+

$^{\#}$p<0.05 *vs.* diabetic wild-type

### 3.1.2 Evidence of hET-2 Expression

Transgenic state was confirmed by PCR analysis of isolated tail tissue DNA, RT-PCR of isolated kidney mRNA and by ELISA measurements of ET plasma level.

#### 3.1.2.1 *hET-2 Gene in Tail Tissue*

Detailed analysis of genomic DNA by PCR revealed that the hET-2 gene was present in tail tissue of transgenic rats, but not in tail tissue of wild-type animals (Figure 7).


Figure 7: Expression of hET-2 in tail tissue. Rats number 22, 23, 26, 27, 28 and 29 belong to wild-type groups. Rats 24 and 25 are hET-2 TG+. K+ and K- are positive and negative control, respectively. The PCR product size equals 435 bp. The last lane shows a DNA size ladder.

### 3.1.2.2 *hET-2 mRNA in the Kidney*

It was demonstrated by RT-PCR that all transgenic rats exhibited expression of hET-2 mRNA in the kidney, whereas wild-type animals showed no hET-2 mRNA signal (Figure 8).

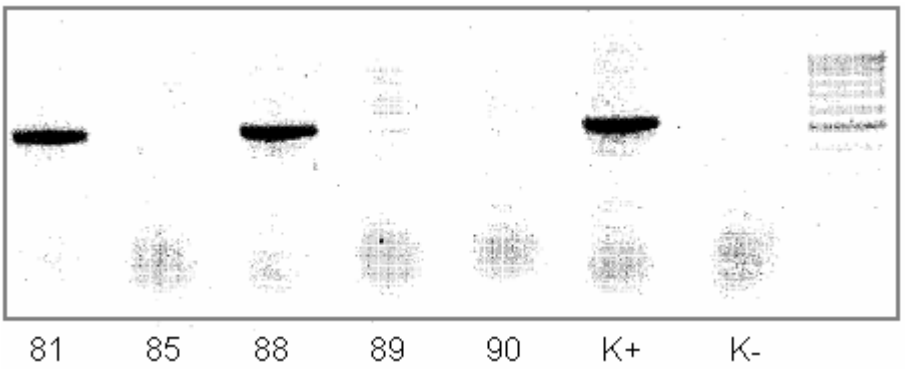


Figure 8: Expression of hET-2 in the kidney. Rats number 81 and 88 are hET-2 TG+. Rats 85, 89 and 90 belong to the wild-type group. K+ and K- are the positive and negative control, respectively. The PCR product size equals 435 bp. The last lane shows a DNA size ladder.

### 3.1.2.3 *ET Plasma Level*

As anticipated, plasma endothelin concentrations were significantly higher in both transgenic groups than in corresponding wild-type rats (non-diabetic: 3.5±0.4 fmol/ml *vs.* 2.1±0.2 fmol/ml, $p<0.05$; diabetic: 4.5±0.4 fmol/ml *vs.* 2.5±0.4 fmol/ml, $p<0.01$, Figure 9). Hyperglycaemia leads to an independent increase in endothelin plasma levels in both the wild-type and hET-2 transgenic animals, however, this effect did not reach significance.

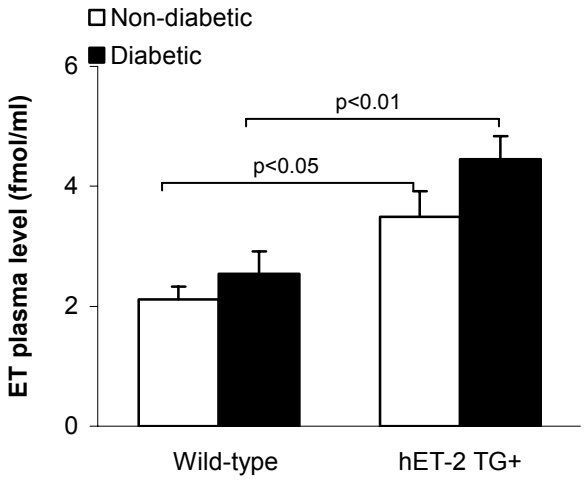


Figure 9: Effect of hET-2 expression (hET-2 TG+) on ET plasma level 24 weeks after induction of diabetes. White and black columns represent ET plasma level of non-diabetic and diabetic rats, respectively.

## 3.2 Diabetic Cardiomyopathy

### 3.2.1 Myocardial Structural Changes

#### 3.2.1.1 *Heart Weight*

As shown in Figure 10, chronic hyperglycaemia led to increased relative heart weights in both the wild-type and the hET-2 transgene groups, indicating cardiac hypertrophy (wild-type: 3.5±0.1 mg/g *vs.* 3±0.1 mg/g, $p<0.001$; transgenic: 3.1±0.1 mg/g *vs.* hET-2 TG+ 2.9±0.1 mg/g, $p<0.05$). This effect was more pronounced in diabetic wild-type animals. Overexpression of the ET-2 transgene alone did not significantly alter relative heart weight.

Absolute heart weight of transgenic rats was significantly decreased when compared to corresponding wild-type rats (non-diabetic 1.22±0.05 g *vs.* group 1.49±0.03 g, $p<0.001$; diabetic 1.19±0.03 g *vs.* 1.38±0.07 g, $p<0.001$). This finding was in line with significantly reduced body weight in transgenic groups when compared with non-transgenic groups. Diabetes did not influence the absolute heart weight significantly.

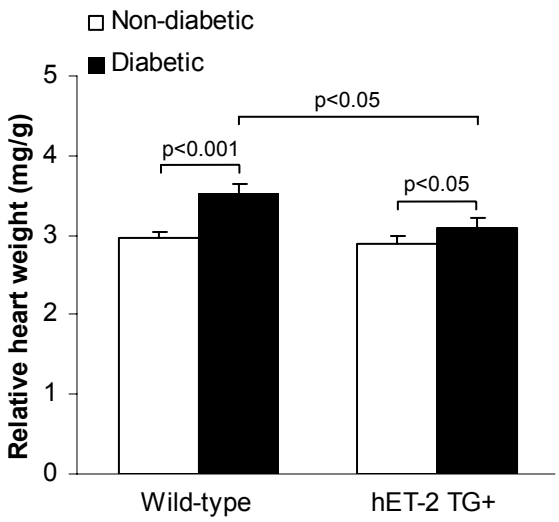


Figure 10: Effect of hET-2 expression (hET-2 TG+) on relative heart weight 24 weeks after induction of diabetes. Black and white columns represent heart weight of non-diabetic and diabetic rats, respectively. Relative heart weight is shown as a ratio of organ weight/body weight (mg/g).

3.2.1.2 *Ventricle Wall Morphometry*

In line with the hyperglycaemia-induced increased relative heart weight was the left ventricle wall thickness (Figure 11). Both diabetic groups revealed greater left ventricle wall thickness compared with non-diabetic groups (wild-type: 112±5% *vs.* 100±5%; transgenic: 100±1% *vs.* 85±4%, p=0.1) and transgene expression in diabetes led to markedly reduced left ventricle wall thickness. Theses changes did not reach significance.

As shown in Figures 11, 12 and 13, right ventricular hypertrophy was observed in the non-diabetic transgenic animals (122±8%), although the differences to the non-diabetic wild-type (100±9%, p=0.1), diabetic wild-type (111±7%) and diabetic transgenic groups (95±2%, p=0.07) did not reach statistical significance.

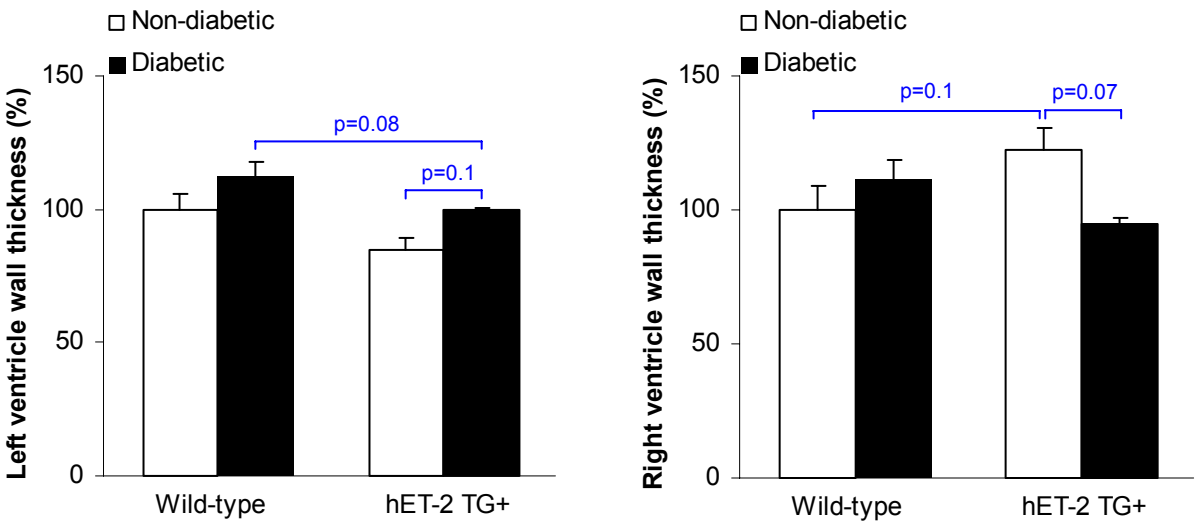


Figure 11: Effects of hET-2 (hET-2 TG+) expression on heart's walls thickness 6 months after diabetes induction. Measurements of thickness were carried out 4 times in each heart. White and black columns represent ventricle wall thickness of non-diabetic and diabetic rats, respectively. Wall thickness is shown as a percentage of the non-diabetic wild-type group.

The relative thickness of the right ventricle wall (*i.e.* the ratio of right ventricle wall thickness/heart weight) confirmed the data of right heart hypertrophy in the non-diabetic transgenic group. As shown in Figure 12, relative right ventricle wall thickness was significantly ($p<0.01$) greater in the non-diabetic transgenic group (152±16%) compared to the non-diabetic wild-type group (100±9%), indicating distinct right ventricle hypertrophy in this group. The heart ventricle wall thickness divided by heart weight that still remained enlarged is a strong indication of this ventricle hypertrophy.

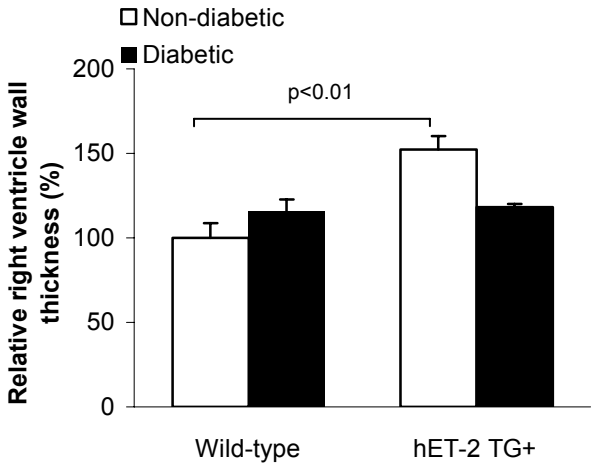


Figure 12: Effects of hET-2 (hET-2 TG+) expression on relative right ventricle wall thickness 24 weeks after diabetes induction. Measurements were carried out 4 times on each heart. Right ventricle wall thickness was divided by heart weight. White and black columns represent the relative right ventricle wall thickness of non-diabetic and diabetic rats, respectively. Relative right ventricle wall thickness is shown as a percentage of the non-diabetic wild-type group.

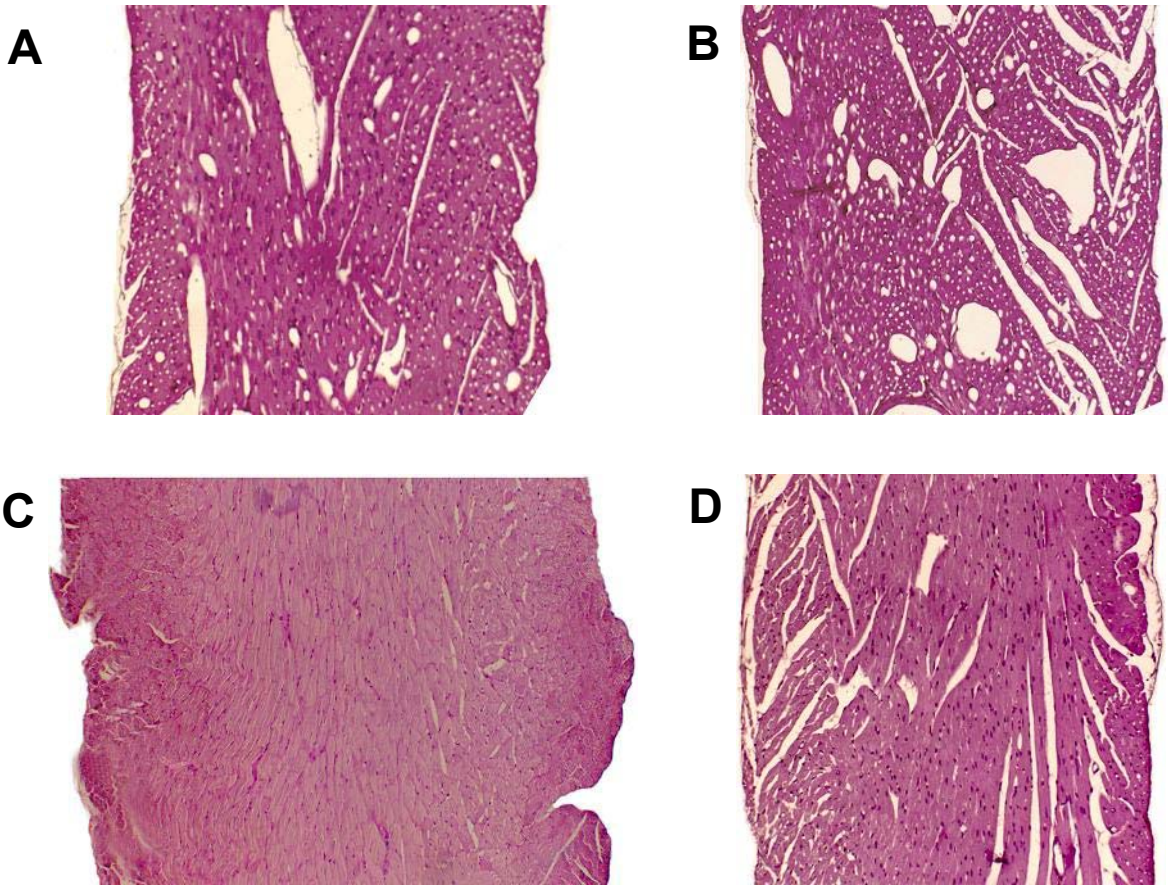


Figure 13: Representative examples of right ventricle sections in light microscopy 24 weeks after induction of diabetes in (A) non-diabetic wild-type, (B) diabetic wild-type, (C) non-diabetic hET-2 TG+ and (D) diabetic hET-2 TG+ groups. A marked right ventricle hypertrophy in non-diabetic transgenic group (C) is noted. Sections were stained with PAS.

#### 3.2.1.3 *Cardiomyocyte Diameter*

As shown in Figure 14, there were no significant differences in cardiomyocyte size in any group (non-diabetic: wild-type 12±0.4 µm and transgenic 12.2±0.4 µm; diabetic: wild-type 11.3±0.3 µm and transgenic 12.4+0.5 µm), however diabetic transgenic group revealed the greatest cardiomyocyte diameter.

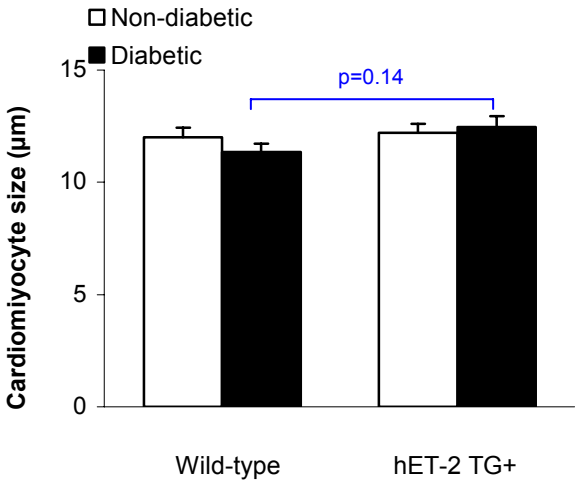


Figure 14: Effects of hET-2 (hET-2 TG+) expression on cardiomyocyte diameter 6 months after induction of diabetes. Measurements were carried out on 10 different cardiomyocytes in each heart. White and black columns represent cardiomyocyte diameter of non-diabetic and diabetic rats, respectively.

In addition, hyperglycaemia led to significantly higher right to left ventricle myocyte diameter ratio. As shown in Figure 15, the highest significant right/left ventricle myocyte diameter ratio was found in the hearts of animals with both diabetes and hET-2 overexpression (1.13±0.04 µm/µm) compared to non-diabetic wild-type (0.89±0.06 µm/µm, $p<0.05$), diabetic wild-type (1.05±0.05 µm/µm) and non-diabetic transgenic rats (1.03±0.05 µm/µm). Right to left ventricle cardiomyocyte ratio was already elevated in the two one-hit groups, *i.e.* diabetic wild-type ($p<0.05$) and non-diabetic hET-2 animals ($p>0.1$).

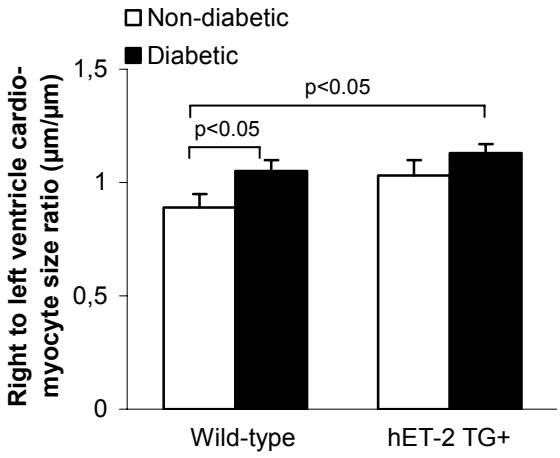


Figure 15: Effects of hET-2 (hET-2 TG+) expression on ratio of right to left cardiomyocytes diameter 24 weeks after induction of diabetes. White and black

columns represent data of non-diabetic and diabetic rats, respectively.

### 3.2.1.4 *Volume Density of Interstitial Tissue*

Interstitial tissue volume density served as main marker of myocardial fibrosis and was calculated by computer-based histomorphometry. As shown in Figure 16, the highest significant interstitial tissue volume density was found in the hearts of animals with both diabetes and hET-2 overexpression (1.5±0.07%) compared to non-diabetic wild-type (0.8±0.01%, p<0.001), diabetic wild-type rats (1.1±0.03%, p<0.001) non-diabetic transgenic (1.1±0.03%, p<0.01). Myocardial interstitial volume density was already significantly elevated in the two one-hit groups, *i.e.* diabetic wild-type (p<0.01) and non-diabetic hET-2 (p<0.001) animals.

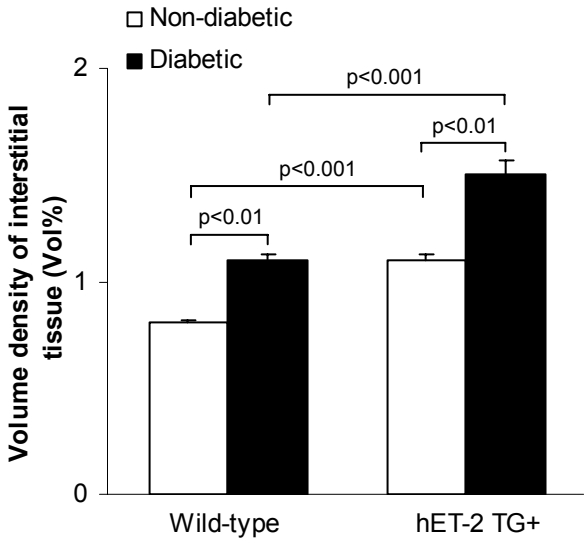


Figure 16: Effects of hET-2 (hET-2 TG+) expression on volume density of interstitial tissue 6 months after induction of diabetes. Non-transgenic rats (Wild-type) showed no hET-2 expression. White and black columns represent non-diabetic and diabetic rats, respectively.

### 3.2.1.5 *Molecular Fibrosis Markers*

In line with the significantly more severe myocardial fibrosis in the diabetic hET-2 than diabetic wild-type animals were mRNA expressions of the molecular fibrosis mediator TGF-β1 and the matrix protein fibronectin (Table 12). While major changes of TGF-β1 and fibronectin mRNA expression were not detectable in the diabetic wild-type animals, TGF-β1 expression was 2.1-fold and fibronectin expression was 3.2-

fold higher in the diabetic ET-2 transgenic rats, respectively. On the other hand, PAI-1 expression was higher in both diabetic groups (1.5-fold and 1.8-fold in wild-type and transgenic animals, respectively). Due to high variability in the individual animals, theses changes did not reach significance.

Table 12: Effects of hET-2 (hET-2 TG+) expression on fibrosis markers expression, including fibronectin, TGF-β1 and PAI-1, 24 weeks after induction of diabetes. mRNA was analyzed by a real-time PCR method using β-actin as housekeeping gene. Fibrosis markers mRNA levels are shown as a percentage of the non-diabetic non-transgenic group.

| | Wild-type | | hET-2 TG+ | |
|---|---|---|---|---|
| | Non-diabetic | Diabetic | Non-diabetic | Diabetic |
| TGF-β1/β-actin (%) | 100±21 | 96±17 | 60±16 | 126±29 |
| Fibronectin/β-actin (%) | 100±30 | 87±34 | 36±9* | 115±38 |
| PAI-1/β-actin (%) | 100±12 | 155±30 | 79±15 | 146±36 |

*p<0.05 *vs.* non-diabetic wild-type

### 3.2.2 Vessel Parameters

#### 3.2.2.1 *Aorta*

As shown in Figure 17, aortic media thickness tended to be higher in animals with both diabetes and hET-2 overexpression compared to diabetic wild-type group (83±1 µm *vs.* 80±1 µm, p=0.07). Major changes of aortic media thickness in other groups were not found (non-diabetic wild-type 79±2 µm and non-diabetic hET-2 TG+ 80±2 µm).

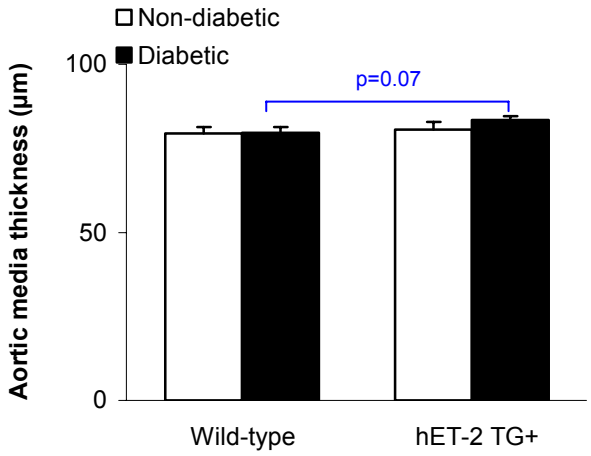


Figure 17: Effects of hET-2 (hET-2 TG+) expression on aortic media thickness (ascending thoracic aorta) 24 weeks after induction of diabetes. White and black columns represent aortic media thickness in non-diabetic and diabetic rats, respectively.

### 3.2.2.2 *Coronary Arteries*

As shown in Figures 18 and 20, the larger coronary arteries showed marked vessel wall hypertrophy in the presence of transgene expression and diabetes, but not with either condition alone. The highest significant media cross sectional area was observed in the diabetic transgenic animals (648±208 $\mu m^2$) as compared to non-diabetic transgenic (148±26 $\mu m^2$, $p<0.01$) and diabetic wild-type animals (301±105 $\mu m^2$, $p<0.05$), respectively. In line with this, the media width was highest in diabetic transgenic animals (32±5 µm), although the differences to the non-diabetic wild-type (24±2 µm), diabetic wild-type (27±2 µm) and non-diabetic transgenic group (25±2 µm) did not reach statistical significance.

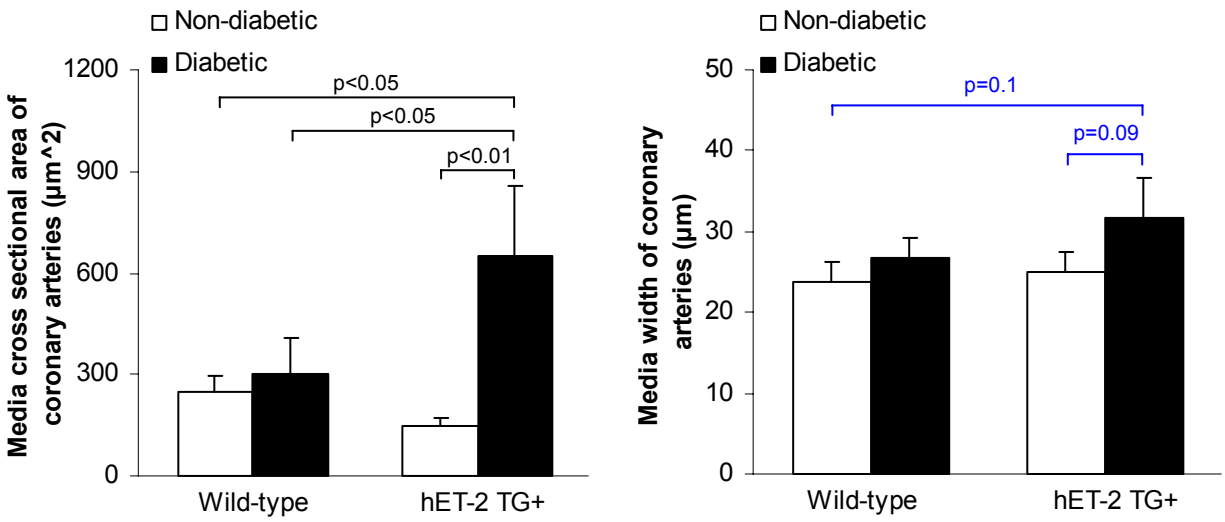


Figure 18: Effects of hET-2 (hET-2 TG+) expression on measurements of media of coronary arteries 24 weeks after induction of diabetes. White and black columns represent media' measurements of coronary arteries of non-diabetic and diabetic rats, respectively.

A similar pattern, *i.e.* the most severe changes in the diabetic transgenic animals, was observed for lumen diameter. As shown in Figure 19 and 20, the lumen diameter was smallest in diabetic transgenic group (82±9 µm), although the differences to the non-diabetic wild-type (117±18 µm), diabetic wild-type (123±16 µm) and non-diabetic transgenic group (96±14 µm) did not reach statistical significance. Consequently media/lumen ratio was highest in diabetic transgenic animals (3.8±0.8 $\mu m^2/\mu m^2$) when compared to non-diabetic wild-type (3±0.6 $\mu m^2/\mu m^2$), diabetic wild-type (2.6±0.7 $\mu m^2/\mu m^2$) and non-diabetic transgenic (2.6±0.6 $\mu m^2/\mu m^2$) groups. Due to high variability in the individual animals, theses changes did not reach significance.

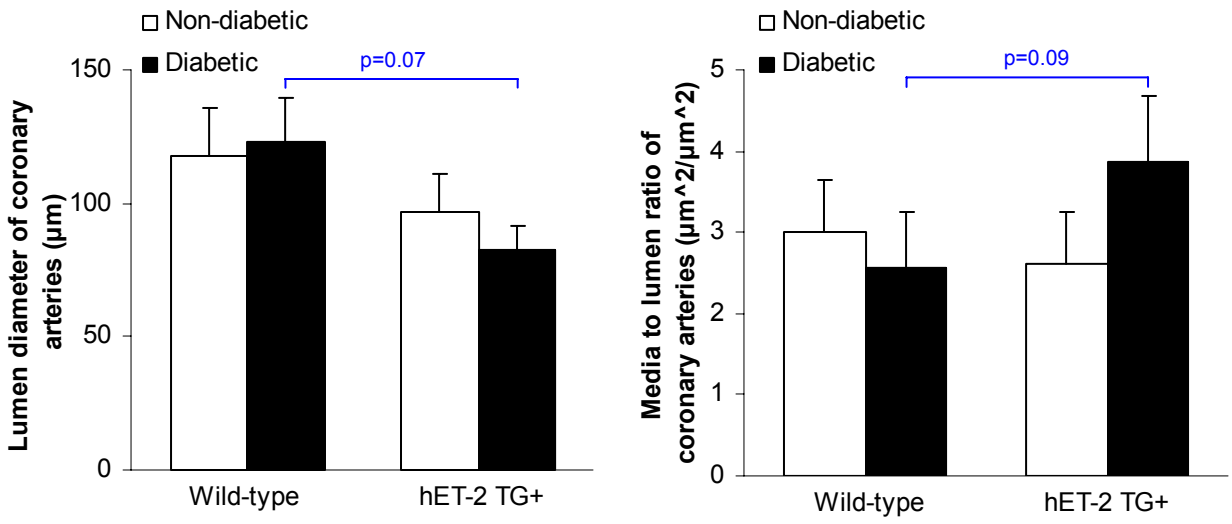


Figure 19: Effects of hET-2 (hET-2 TG+) expression on dimensions of coronary arteries 24 weeks after induction of diabetes. White and black columns represent media parameters of coronary arteries of non-diabetic and diabetic rats, respectively.

There were no significant changes of MCSA, media weight, lumen diameter and media/lumen ratio observed in the two one-hit groups, *i.e.* diabetic wild-type and non-diabetic hET-2 animals.

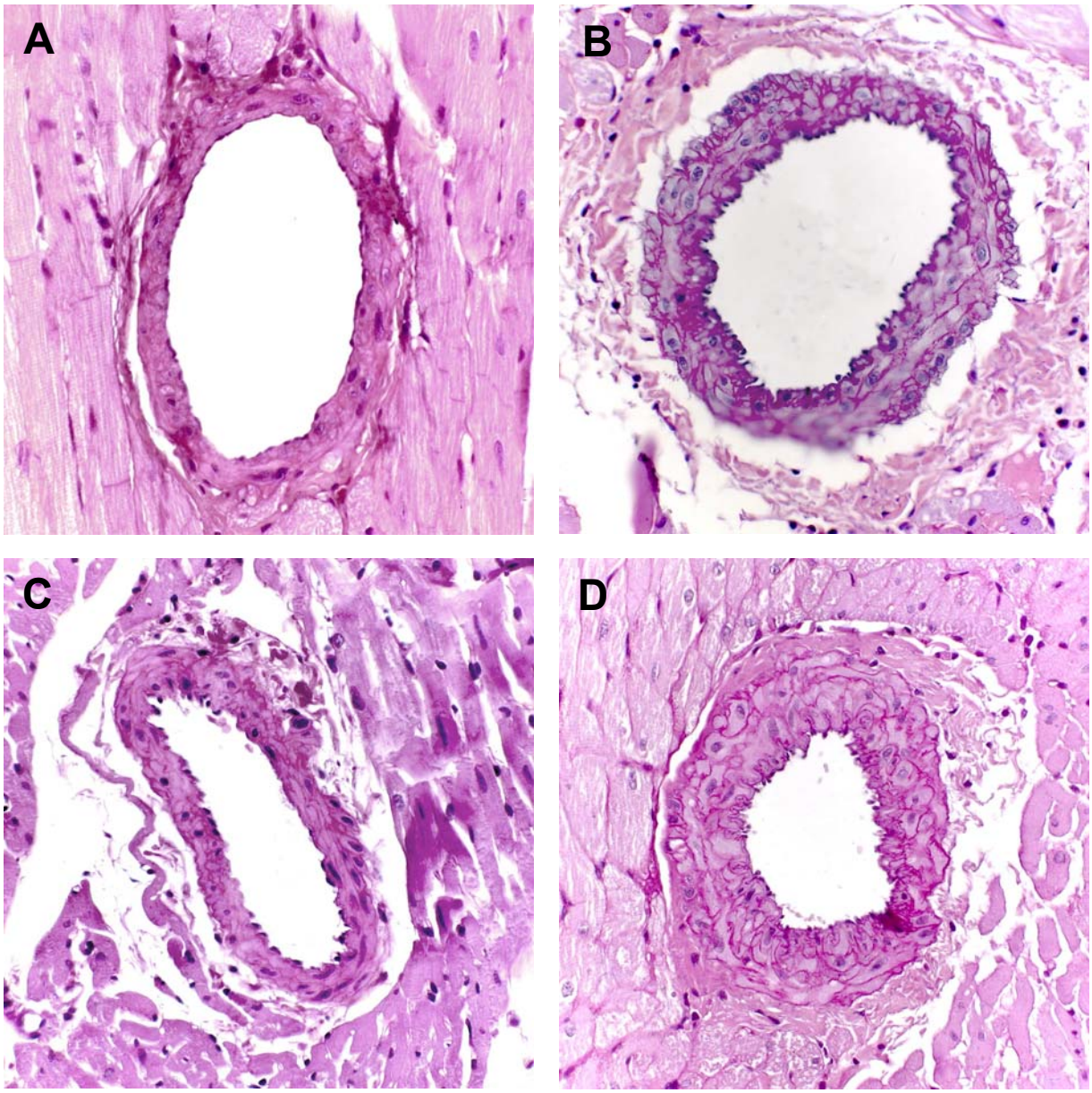


Figure 20: Representative examples of coronary arteries in light microscopy 6 months after induction of diabetes in (A) non-diabetic wild-type, (B) diabetic wild-type, (C) non-diabetic hET-2 TG+ and (D) diabetic hET-2 TG+ groups. A marked increase in media cross sectional area, media width and media to lumen ratio in the diabetic transgenic group (D) is noted. Sections were stained with PAS.

#### 3.2.2.3 *Intramyocardial Arterioles*

As shown in Figure 21, expression of the hET-2 transgene alone resulted in significant wall thickening of intramyocardial smaller arterioles. This was seen in the non-diabetic (transgenic 2.6±0.1 µm *vs.* wild-type 1.9±0.1 µm, $p<0.01$) as well as in the diabetic animals (transgenic 2.5±0.1 µm *vs.* wild-type 2.1±0.1 µm, $p<0.01$).

Similar to the larger coronary arteries, media to lumen ratio of intramyocardial arterioles was highest in the hET-2 transgene and diabetic group (98±5 nm/nm, p≤0.05 *vs.* all other groups). Chronic hyperglycaemia on its own increased myocardial arterioles media to lumen ratio in the wild-type (diabetic 77±3 nm/nm *vs.* non-diabetic 67±2 nm/nm, p<0.05) and the transgenic animals (diabetic 98±5 nm/nm *vs.* non-diabetic 84±2 nm/nm, p<0.05), respectively.

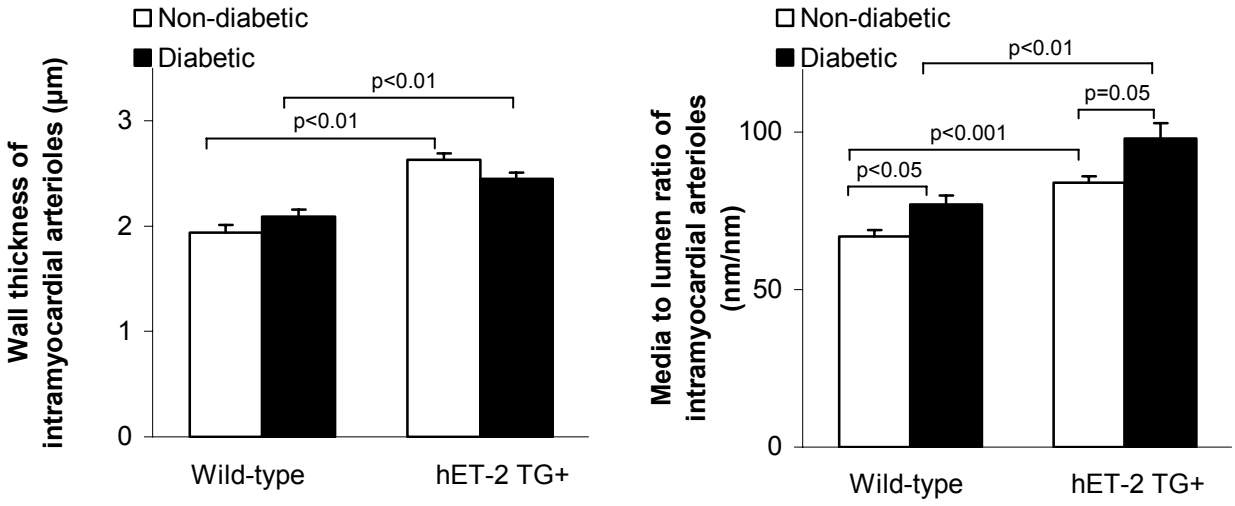


Figure 21: Effects of transgenic hET-2 (hET-2 TG+) expression on dimensions of myocardial arterioles 6 months after induction of diabetes. White and black columns represent measurements of non-diabetic and diabetic rats, respectively.

### 3.2.3 Hemodynamic Results

Systolic blood pressure at the end of the six months study period did not significantly differ between all four groups (Table 13). The diabetic hET2 transgenic group showed the relative highest blood pressure corresponding to the highest aortic media thickness in this group (see 3.2.3.1). Heart rates were comparable in all groups expect in the non-diabetic wild-type animals, in which they were higher.

Table 13: Effects of hET-2 (hET-2 TG+) expression on systolic blood pressure (SBP) and heart rate (bpm – beat per minute) 6 months after induction of diabetes. Blood pressure and heart rate were measured in conscious rats using tail cuff.

| | Non-diabetic rats | | Diabetic rats | |
|---|---|---|---|---|
| | Wild-type | hET-2 TG+ | Wild-type | hET-2 TG+ |
| SBP (mmHg) | 145,9±2,2 | 151,5±5,6 | 150,7±2,9 | 155,6±3,1 |
| Heart rate (bpm) | 502,9±6,7 | 482,5±14,7 | 446,6±11,3** | 484,8±27,1 |

**p<0.01 *vs.* non-diabetic wild-type

### 3.2.4 Molecular Basis

Representative example of real-time PCR results is shown in Figure 22.

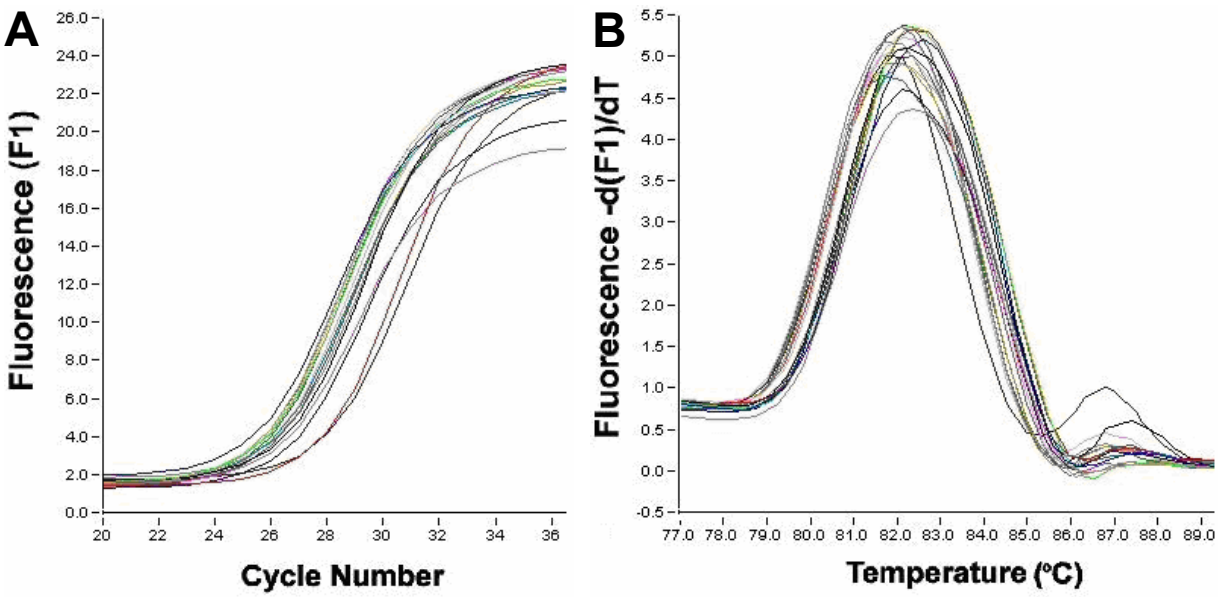


Figure 22: $ET_B$ mRNA quantification in rat heart by real-time PCR. A: PCR amplification plot of $ET_B$. $ET_B$ mRNA was subjected to real time PCR. Crossing point (CP) value, obtained from SYBR Green I signal, was used to compute relative concentration of $ET_B$ mRNA from $ET_B$ standard curve. B: PCR products were also subjected to melting-curve analysis to determine specificity of the products. All samples showed a specific product, with melting temperature of 81°C.

### 3.2.4.1 *ET System*

Expression of ET-1 in myocardium was lowered by 41% by diabetes in wild-type animals (diabetic 59±36% *vs.* non-diabetic 100±25%). In contrast, chronic hyperglycaemia increased myocardial ET-1 mRNA expression 4.1-fold in the hET-2 transgenic group (diabetic 228±120% *vs.* non-diabetic 55±19%). Due to high variability in the individual animals, theses changes did not reach significance. Confirming previous reports in the hET-2 model [14], real-time PCR analysis detected no hET-2 gene expression in myocardium of the transgenic animals.

As shown in Figure 23, diabetes increased $ET_A$ mRNA expression significantly by a factor of 2.4 (diabetic 170±43% *vs.* non-diabetic 70±23%, $p<0.05$) in the hET-2 transgenic animals, while its expression was only slightly and not significantly changed in the wild-type group (diabetic 141±49% *vs.* non-diabetic 100±20%). Expression of the $ET_B$ receptor mRNA was significantly down-regulated in the presence of the hET-2 transgene (non-diabetic transgenic 46±8% *vs.* non-diabetic wild-type 100±16%, $p<0.05$). Chronic diabetes increased $ET_B$ expression by a factor of 2.0 in the transgenic animals, while it was almost unchanged in the wild-type animals.

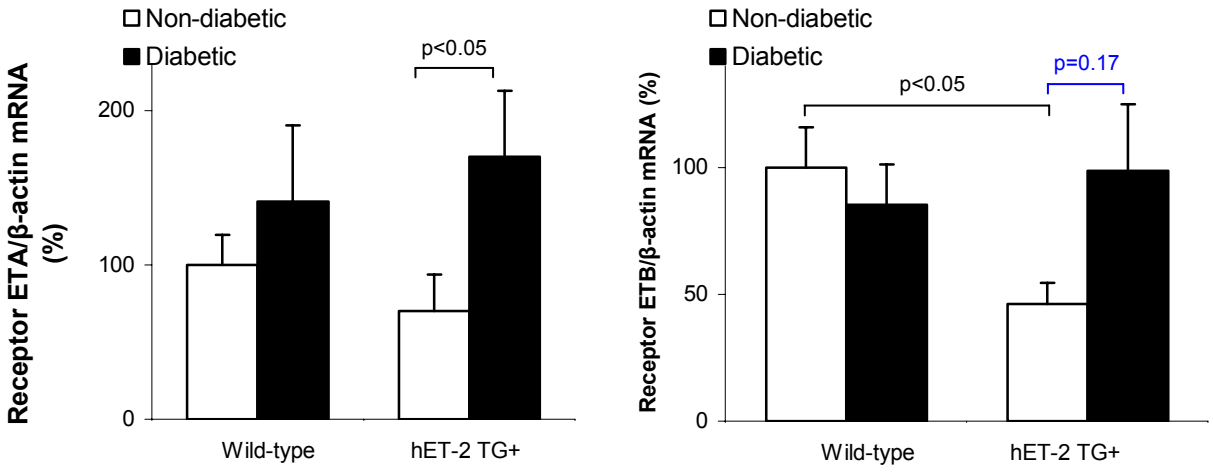


Figure 23: Effects of hET-2 (hET-2 TG+) expression on ET system components mRNA levels in the heart, including $ET_A$ and $ET_B$ receptors, 6 months after induction of diabetes. mRNA was analyzed by a real-time PCR method using β-actin as a housekeeping gene. White and black columns represent the levels of ET mRNA receptors in non-diabetic and diabetic rats, respectively. Receptors mRNA levels are shown as a percentage of the non-diabetic wild-type group.

3.2.4.2 *Cardiovascular Markers*

In line with more severe myocardial structural changes and myocardial vessels remodelling in the diabetic hET-2 than diabetic wild-type animals were mRNA expressions of the molecular cardiovascular damage markers (Table 14). While chronic hyperglycaemia alone did not increase ADM, ANP, RLX and LGR-7 expression in the diabetic wild-type animals, ADM expression was 2.8-fold, ANP 2.5-fold, RLX 3.5-fold and LGR-7 3.5-fold higher in the diabetic ET-2 transgenic rats, respectively. Due to high variability in the individual animals, theses changes did not reach significance.

Table 14: Effects of hET-2 (hET-2 TG+) expression on cardiovascular markers mRNA levels, including ADM, ANP, RLX and LGR-7, 24 weeks after induction of diabetes. mRNA was analyzed by a real-time PCR method using β-actin as a housekeeping gene. Cardiovascular markers mRNA levels are shown as a percentage of the non-diabetic wild-type group.

| | Wild-type | | hET-2 TG+ | |
|---|---|---|---|---|
| | Non-diabetic | Diabetic | Non-diabetic | Diabetic |
| ADM/β-actin % | 100±18 | 58±27 | 53±15 | 152±66 |
| ANP/β-actin % | 100±17 | 84±34 | 45±16* | 114±42 |
| RLX/β-actin % | 100±29 | 40±22 | 35±17 | 124±66 |
| LGR-7/β-actin % | 100±29 | 39±19 | 39±19 | 137±73 |

*p<0.05 *vs.* non-diabetic wild-type

# 4 Discussion

The aim of this study was to provide independent and complementary results on the role of the ET system in diabetic cardiomyopathy by using an ET transgene overexpressing approach. Six months of hyperglycaemia resulted in heart changes in the non-transgenic wild-type animals that were characterized by a marked heart hypertrophy and a slight increase in myocardial interstitial tissue density, without an increase of fibrosis marker (fibronectin and TGF-β1) and cardiovascular damage marker (ADM, ANP, RLX and LGR-7) mRNA levels. By contrast, chronic hyperglycaemia in the presence of transgenic hET-2 expression induced more advanced diabetic cardiac changes, *i.e.* a significant increase in interstitial tissue density along with increased fibrosis and cardiovascular damage markers and a remarkable remodelling of large epicardial arteries and of small intramyocardial arterioles.

## 4.1 Critical Evaluation of the Animal Model Employed

The animal model used in this study consisted of the fusion of two other models: the STZ-induced diabetic rat model and the transgenic rat model with hET-2 overexpression.

### 4.1.1 Model of Diabetes

A single intraperitoneal injection of STZ into both non-transgenic and transgenic rats caused the death of beta cells in the pancreas. The rapidly occurring lack of beta cells, which produce insulin, led to hyperglycaemia. STZ rarely is the cause of diabetes in humans, and so the diabetes produced by this toxin is not an analogue to the pathogenesis of human diabetes, either type 1 or type 2. The STZ model of diabetes is an intrinsic model, which, unlike analogue models, does not have to mimic reality. This model serves as an analogue to the complications of uncontrolled diabetes [72]. It is most comparable with type 1 diabetes characterized by hyperglycaemia in the absence of hyperinsulinaemia.

In this study, rats with an early morning blood glucose level greater than 250 mg/dl one day after STZ-injection were considered diabetic. A state of diabetic dysmetabolism was confirmed by polydipsia and polyuria, which are typical symptoms of hyperglycaemia, and by significantly reduced body weight gain over time.

### 4.1.2 Model of hET-2 Overexpression

ET-2 has a similar peptide sequence and comparable pharmacological properties to ET-1, but different tissue expression. In this model, transgenic hET-2 overexpression results in significantly increased circulatory endothelin concentrations. The predominant sources of hET-2 are the kidney, the gastrointestinal tract, adrenal gland, spleen, lung and several brain regions, but not the heart or the aorta [14]. An important factor for the evaluation of diabetic complication pathogenesis in this transgenic model is normotension, despite hET-2 overexpression and its strong vasoconstrictive properties, which is explained by a balance maintained between vasoconstrictors and vasodilators [71].

In this study, the transgenic state was confirmed by PCR analysis of isolated tail tissue DNA, RT-PCR of isolated kidney mRNA and by ELISA measurements of ET plasma level. All these methods confirmed expression of hET-2 in transgenic animals. Real-time PCR using cDNA synthesized from isolated heart mRNA revealed no hET-2 expression.

The results from this hET-2 overexpressing intervention provide strong and complementary evidence for the importance of the ET system in the pathogenesis of diabetic cardiomyopathy, which previously mainly derived from inhibitory interventional studies using non-selective or selective ET receptor antagonists. In these investigations, ET receptor blockade has been shown to improve diabetic heart function [10,60,61], electrophysiological function [11,62] and myocardial remodelling (cardiomyocyte hypertrophy [9], injury [63] and apoptosis [20], as well as increased extracellular matrix mRNA: fibronectin, collagen α1 [20]). Furthermore, ET receptor antagonists improved coronary perfusion pressure in diabetes [62] and prevented exaggerated coronary reactivity to endothelin-1 [64]. However, these reports were not entirely convincing. Several opposing investigations have found no or only modest benefits of ET inhibition on diabetic heart hypertrophy, myocardial tissue fibrosis, microangiopathy and coronary vessel stiffness [12,13,61]. Accordingly, it is of value that a study with targeted ET overexpression provides a novel and independent line of substantiation for a detrimental role of the ET system in diabetic changes of the heart.

## 4.2 Diabetic Cardiomyopathy

Diabetic cardiomyopathy refers to a disease process which affects the myocardium in diabetic patients, causing structural abnormalities eventually leading to left ventricular hypertrophy, diastolic and systolic dysfunction and coronary artery disease. The concept of diabetic cardiomyopathy is based upon the idea that diabetes is the factor which promotes changes at the cellular level, resulting in structural abnormalities [76].

Not all components of ETs exist within the heart. Confirmation of the absence of endogenous and hET-2 expression in cardiovascular tissue in non-transgenic and transgenic animals strengthened the concept of a putative systemic action of ETs on the development of diabetic cardiomyopathy. In this model, the different regulation of ET system components was believed to influence diabetic cardiac changes.

Diabetic cardiomyopathy advance was assessed according to the findings on myocardial remodelling, morphometrical and stereological analyses of heart and heart vessels, hemodynamic results and changes in fibrosis and cardiovascular damage molecular markers.

### 4.2.1 hET-2 Expression and Hyperglycaemia Reduce Body Weight

As demonstrated in the present study, the body and heart weights of transgenic rats were significantly lower when compared to non-transgenic animals. It remained unclear whether reduced body weight was a consequence of the presence of hET-2 in the intestine or of an increased metabolism in the presence of the transgene.

Takizawa *et al.* 2005 [77] determined for the first time the cellular distribution of ET-2 mature peptide in the mouse intestinal tract. Basal localization of ET-2 indicated that ET-2 might be secreted from the basement membrane of epithelial cells into the *lamina propria*, where it could modulate the mucosal immune system. On the other hand, another report demonstrated that ET-2 was a chemoattractant for macrophages (Grimshaw *et al.* 2002). ET-2 and ET-1 were expressed most highly in M-cells in Peyer‘s patch, which was a center of mucosal immunity. These findings suggest that ET-2 might play a crucial role in antigen presentation, and that it might be involved in the apoptosis of colonic epithelial cells. Therefore, hET-2 expression in the intestinal tract may modulate intestinal function.

Previous reports showed that ETs regulate contractile activity in the gastrointestinal tract. Huang *et al.* (2004) suggested that ETs may play an important

role in the control of lower oesophageal sphincter motility, demonstrating that ETs caused contraction as well as relaxation of the guinea-pig lower oesophageal sphincter through interaction with $ET_A$ and $ET_B$ receptors, respectively [78].

Taking all of these findings together, it is safe to assume that the ETs' modulating of immune cells in the gastrointestinal tract, as well as their regulating of its motility, may influence the quantity of absorbed substances in the intestine.

All diabetic animals displayed reduced body weight as compared to their age-matched controls, which was associated with increased blood glucose levels. These observations were in keeping with previous studies and indicated a state of diabetic dysmetabolism [10,79,80]. After 12 weeks of diabetes, because of poor condition and high mortality of the diabetic rats, the frequency of insulin treatment was changed. Insulin treatment was initiated 2 weeks after diabetes was confirmed and continued every second day. After 12 weeks, insulin was injected every day. This intensive treatment with insulin led to a significant increase in body weight.

### 4.2.2 Myocardial Structural Changes

Previous clinical studies showed that myocardial dysfunction frequently occurs in diabetic patients. The general process by which the ventricular myocardium experiences changes in structure and function is often referred to as myocardial remodelling [81]. The central features of this process are an increase in ventricular mass on the cellular level, hypertrophy of individual cardiomyocytes and diffuse interstitial fibrosis.

#### 4.2.2.1 *Less Pronounced Diabetic Heart Hypertrophy in the Presence of hET-2*

As demonstrated in this study, chronic hyperglycaemia led to increased relative heart weights in both the wild-type and the hET-2 transgene groups, indicating cardiac hypertrophy. Interestingly, the heart hypertrophy in the diabetic wild-type group was significantly more evident in comparison to the diabetic transgenic group. It has been reported that STZ-induced diabetes heart remodelling may be associated with myocardial atrophy [82]. This is related to loss of contractile proteins and myocyte apoptosis, resulting from the absence of the mitogenic and pro-survival effects of insulin [83]. In this study, elevated ET level was believed to potentiate the effect of hypoinsulinaemia, since ETs are known to promote cardiomyocyte apoptosis [20]. This feature of ET may explain the less pronounced diabetic heart hypertrophy observed in the hET-2 transgenic animals as compared to the diabetic wild-type

animals. Secondly, the data of metabolic study revealed a 2-fold decrease in water intake and urine volume in diabetic transgenic rats when compared to diabetic non-transgenic rats. Thus, it may be speculated that diabetic heart hypertrophy is less pronounced in the presence of hET-2 overexpression due to dehydration and decreased blood volume.

The findings on absolute heart weight indicate significantly reduced heart weight in the transgenic groups, corresponding to the reduced body weight of transgenic animals. In addition, no significant absolute heart weight changes were observed after 6 months of chronic hyperglycaemia. This is explained by reduced body weight in diabetic rats due to advanced diabetes and the consequent reduction of absolute organ weights. For better assessment of heart hypertrophy in a state of diabetic dysmetabolism, results are expressed as relative heart weight in relation to the corresponding body mass of the animal in order to adjust for differences in the mean body weights between the groups.

#### 4.2.2.2 *Left Ventricle Wall Morphometry Confirmed Diabetic Cardiac Hypertrophy*

Left ventricle wall thickness corresponded to the hyperglycaemia-induced increased relative heart weight. Both diabetic groups revealed greater left ventricle wall thickness when compared to non-diabetic groups. This important observation, points out firstly the heart hypertrophy under hyperglycaemia, and, secondly, the diastolic ventricle abnormalities like impaired relaxation or increased stiffness [84]. These impairments in diastolic function have been studied in isolated perfused hearts *in vivo* in rats with STZ-induced diabetes [83]. Notably, these functional disorders occurred in the absence of significant changes in myocardial perfusion. Equally important was the observation that administration of insulin in type 1 diabetic rats partially rectified these abnormalities, even though modest hyperglycaemia persisted [85]. This study provided complementary results, confirming the absence of significant changes of epicardial artery geometry under hyperglycaemia, but indicating hypertrophic remodelling of intramyocardial arterioles. The second key finding was the high severity of hypertrophic remodelling of both epicardial and intramyocardial vessels in animals with diabetes and hET-2 overexpression.

#### 4.2.2.3 *Cor Pulmonale is Promoted by hET-2 Overexpression*

Also of note was the observation that, according to the data on heart wall thickness, the non-diabetic transgenic group, in contrast to other groups, revealed

right ventricle hypertrophy. In this transgenic model for ET-system components, hET-2 is expressed predominantly in the kidney and also in the lung. Pulmonary release of ETs contributes to increased plasma ET levels and vasoconstriction in congestive heart failure [86]. Therefore, it may be speculated that hET-2, a vasoactive contractor, caused pulmonary hypertension and *cor pulmonale* to occur. This hypothesis is in accordance with a study showing that the $ET_A$ antagonist YM598 ameliorates the progression of cardiopulmonary changes in rats with *cor pulmonale* [87]. Furthermore, according to Namasivayam *et al.*, $ET_A$ blockade prevents and partially reverses pulmonary vascular remodelling induced by hypoxemia in human neonates [88], indicating a possible role of ETs in the pathogenesis of arterial structural changes in pulmonary hypertension.

According to the data on the right to left ventricle cardiomyocyte ratio, right ventricle cardiomyocyte hypertrophy was most pronounced in the diabetic transgenic group. The lack of right ventricle hypertrophy despite cardiomyocyte hypertrophy in the diabetic transgenic group may be explained by the reduced cardiomyocyte number, since streptozotocin-induced diabetes leads to a significant increase in cardiac cell apoptosis [89] through the mitogenic action of ETs [44]. On the other hand, diabetic transgenic rats revealed marked dehydration and, as a consequence, decreased blood volume. This could be a compensatory factor for the development of *cor pulmonale*.

#### 4.2.2.4 *Cardiomyocyte Hypertrophy in Diabetic Transgenic Animals*

Diabetic cardiomyopathy is associated with an increase in cardiomyocyte diameter and cardiomyocyte volume. These changes lead to an increase in oxygen diffusion distance and impede the diffusion of oxygen to the center of the cardiomyocyte, contributing to myocardial ischemia [90]. A number of factors have been identified as potential causes of cardiomyocyte hypertrophy, including the ET system, the RAAS [91], the sympathetic nervous system [92], growth factors and inflammatory cytokines [93]. However, in this study there was no significant difference in cardiomyocyte diameters, the largest size of cardiomyocytes was found in diabetic hET-2 transgenic rats. Increased cardiomyocyte diameter is explicable, since it is known that ETs act as mitogens and stimulate cellular hypertrophy. ET-1 interacts synergistically with epidermal growth factors (EGF), TGF-$\alpha$, TGF-$\beta$ and PDGF to potentiate mitogenic and proliferative processes [44]. Cardiomyocyte

hypertrophy in the diabetic transgenic group could be supposed to be a compensatory mechanism for increased myocyte apoptosis in this group. Heart hypertrophy with components of myocardial atrophy constitutes strong evidence of advanced hyperglycaemia-induced cardiomyopathy in the diabetic transgenic group.

In conclusion, three factors seem to be responsible for cardiomyocyte hypertrophy:

1) hyperglycaemia-induced cardiac cell apoptosis and cell hypertrophy as compensatory mechanism,

2) enhanced ET plasma level and its mitogenic action - other studies showed that ET-1 promoted the growth of cardiomyocytes [10,94],

3) enhanced ET level in the lung and pulmonary hypertension as a result, which are the reasons for cardiomyocyte hypertrophy in the right ventricle.

#### 4.2.2.5 *Myocardial Interstitial Fibrosis*

One of the main findings was the most pronounced myocardial fibrosis in the diabetic hET-2 transgenic rats, as expressed by the highest significant histological interstitial volume density in this group. However, myocardial interstitial volume density was already significantly elevated in the two one-hit groups, *i.e.* diabetic wild-type and non-diabetic hET-2 animals. Nevertheless, the most severe myocardial interstitial fibrosis was found in the diabetic hET-2 transgenic group, which in parallel revealed the highest endothelin plasma level. Corresponding to the changes of TGF-β and fibronectin expression in this group, other reports document increased collagen I and III synthesis and reduced collagenolytic activity of endothelins *in vitro* [28].

#### 4.2.2.6 *TGF-β1, Fibronectin and PAI-1 as Molecular Markers of Cardiac Fibrosis*

Many studies in humans and experimental models have shown increased myocardial TGF-β1 expression during cardiac hypertrophy and fibrosis [95,96]. TGF-β1 is expressed particularly in the hypertrophic myocardium during the transition from stable hypertrophy to heart failure in experimental models [97]. This peptide induces the production of ECM components, including fibrillar collagen, fibronectin and proteoglycans by cardiac fibroblast [98].

While major changes in TGF-β1 mRNA expression were not detectable in the diabetic wild-type animals used in this study, TGF-β1 expression was 2.1-fold higher in the diabetic ET-2 transgenic rats. The fact that the apex of the heart, which

consisted almost exclusively of left ventricle cardiomyocytes, was used for mRNA isolation, may explain why there were no molecular changes in the non-diabetic transgenic group. In this group, hypertrophy was observed only in the right ventricle, and the left ventricle remained unchanged.

Since overproduction of fibronectin is known to decrease the motility and replication of many cells, including endothelial cells [99], and to be a major component of the ECM [100], the fibronectin mRNA level in the heart was used as a fibrosis marker. Similar to TGF-β1, fibronectin expression was 3.2-fold higher in the diabetic ET-2 transgenic rats, and there was no fibronectin mRNA increase in diabetic wild-type animals.

PAI-1 is known as a marker of coronary artery disease and ECM accumulation. In this study, PAI-1 expression in the heart was independent of hET-2, however, ETs were demonstrated to upregulate PAI-1 mRNA and protein levels [29]. PAI-1 showed a trend towards increasing under hyperglycaemia, independently of the presence of the transgene. This finding is in accordance with data stating that higher concentrations of PAI-1 are found in patients with type 2 diabetes [30]. Similar to TGF-β and fibronectin, PAI-1 confirmed cardiac impairment in the diabetic transgenic group. Interestingly, it was also elevated in diabetic wild-type rats with histologically proven enhanced interstitial fibrosis, showing that PAI-1 is a more sensitive marker of cardiac fibrosis than TGF-β and fibronectin.

### 4.2.3 Heart Vessel Remodelling

Since the involvement of ET peptides in the pathogenesis of hypertension has already been discussed [101], we hypothesized that in spite of normotension in the transgenic model employed, local hypertension in some organs cannot be excluded. The data on heart vessel geometry provided evidence of most severe hypertensive remodelling in the heart vessels of diabetic transgenic rats.

The morphological changes that occur in small arteries in hypertension have been explored for many years. Small arteries present either hypertrophic remodelling, in which the media/lumen ratio is increased but the media cross-sectional area is also augmented, which indicates the presence of net growth, or eutrophic remodelling, in which the media/lumen ratio is increased and the external diameter of the vessel is reduced but the media cross-sectional area is maintained, with no net growth. There are different reasons for the growth of the media, such as

hyperplasia or hypertrophy of smooth muscle cells, or the deposition of extracellular matrix typical for diabetes [102]. Increases in collagen may occur in the media [103], causing widening and the typical increase in media/lumen ratio. The functional consequences are impaired vasodilatation, reduced blood flow, decreased oxygen supply and, ultimately, cardiac ischemia.

#### 4.2.3.1 *Remodelling of Epicardial Arteries in Diabetic Transgenic Animals*

Since the increased media/lumen ratio is the common denominator found in all forms of small-artery hypertensive remodelling, we investigated the arterial structure of the heart in this study. Both hyper- and eutrophic patterns of arterial remodelling are involved in the pathogenesis of hypertension, which constitutes strong evidence of local hypertension, and both of them were observed in epicardial arteries, but only in those of diabetic transgenic animals. Significantly increased MCSA and markedly increased media width and media/lumen ratio in the diabetic transgenic group indicated hypertrophic remodelling of epicardial arteries. On the other hand, markedly increased media/lumen ratio and decreased lumen diameter in the diabetic transgenic group demonstrated eutrophic remodelling of arteries. The reduction in lumen diameter in the diabetic transgenic group presumably was not due to changes in mechanical properties, but to remodelling. The nature of the changes in the media that result in this eutrophic remodelling, by which the vessel is restructured so that smooth muscle cells are more tightly wrapped around a smaller lumen, is unclear. It has been suggested that the changes occur in large part at the level of the extracellular matrix with increased collagen deposition [103]. The same process may occur in diabetes in the context of hET-2 overexpression, considering the overexpression of fibrosis markers, as shown above.

It is important to emphasize that neither 6 months of hyperglycaemia nor transgene overexpression alone were sufficient to develop advanced epicardial coronary structure disturbance. Only in the presence of these two factors combined (in the diabetic transgenic group) was the structure of coronary arteries significantly altered.

#### 4.2.3.2 *Synergistic Action of Hyperglycaemia and ETs on the Intramyocardial Arterioles*

Hypertrophic remodelling of intramyocardial arterioles was present in both one-hit groups, *i.e.* diabetic wild-type and non-diabetic hET-2 animals. Small arteries seemed to be more sensitive to diabetes and the ET system than coronary arteries.

Furthermore, the hypertrophic remodelling of intramyocardial arterioles was most intense in the diabetic transgenic group, indicating a synergistic action of hyperglycaemia and ETs on the heart vessels.

#### 4.2.3.3 *ETs Contribute to Heart Vascular Damage*

However, recent studies about the influence of hypertensive remodelling of arteries on agonist-stimulated contractions in hypertension have revealed that an increased media/lumen ratio does not provide a means for a contractile response amplifier, yet may do so for a vascular resistance amplifier [104]. Increased media/lumen ratio enhances resistance to blood flow and contributes to the elevation of resistance typical of essential hypertension. This might be an explanation for the mechanism of diabetic cardiomyopathy in the presence of hET-2 overexpression. Endothelins are believed to play an important role in the hypertrophy of smooth muscles [105], since they have growth-promoting properties [61,64,106]. Thus, ETs may contribute to vascular damage and consequently to diabetic cardiomyopathy through coronary artery remodelling.

### 4.2.4 Normotension Despite hET-2 Expression

Since ETs are known to have strong vasoconstrictive properties [77], it still remains unclear why rats with the transgene hET-2 do not reveal hypertension. This was also the case in this study. In spite of significantly elevated ET plasma levels, the mean arterial blood pressure did not significantly differ between the groups. This might be due to the low expression of the transgene in the vascular bed, considering the paracrine rather than the systemic action of ETs and the counterregulation by other vasoactive systems, for example, the nitric oxide system [99].

The data from this study revealed a significantly increased heart rate in non-diabetic wild-type rats, as a consequence of stress during blood pressure measurement. This effect is absent in other groups, which became accustomed to more frequent contact with the author during insulin injection and to more frequent blood glucose and weight measurement.

### 4.2.5 The Molecular Basis of Diabetic Cardiomyopathy

To answer the question as to the main changes of ET-system component expression and the interactions of ETs with other molecular players in the

development of diabetic cardiomyopathy, heart RNA was isolated and investigated using real-time PCR.

#### 4.2.5.1 *The Role of the ET System in the Pathogenesis of Diabetic Cardiomyopathy*

ETs are involved in diabetic heart disease [107]. Previous studies have pointed out that hearts from diabetic animals showed a significant increase in ET-1 mRNA expression. $ET_A$ and $ET_B$ mRNA expressions were higher in the hearts after 6 months of diabetes [10]. These findings were confirmed in culturing heart cells from human ventricular biopsy, indicating that heart cells subjected to hyperglycaemia generated more ET-1 [63]. The data on plasma ET-1 in diabetes are discrepant. In uncomplicated type 1 diabetes, reduced values have been reported (Malamitisi-Puchner *et al.*, 1996), whereas other authors have found significantly increased levels in patients with end-stage vascular complications (Kirilov *et al*., 1994; Sarman *et al.* 2000). These results can probably be explained by the different stages of diabetic cardiomyopathy [36]. Furthermore, studies with the ET antagonist showed that ETs promote diabetic heart dysfunction [10,60,61], left ventricular hypertrophy and dilatation [10,20], growth of cardiomyocytes and collagen synthesis in cardiac fibroblasts [94,108] and myocardial hypoperfusion [62]. Thus, it is of value that a study with targeted ET overexpression provides a novel and independent line of substantiation for a detrimental role of the ET system in diabetic changes of the heart.

This study reports on phenotypic characterization of transgenic rats expressing the entire hET-2 gene and subjected to chronic hyperglycaemia. In addition to heart hypertrophy, myocardial fibrosis and large- and small-vessel remodelling, diabetes and transgenic hET-2 overexpression altered heart expressions of ET-1, $ET_A$ and $ET_B$ receptors, although not in a uniform and systematic manner.

Myocardial ET-1 expression was lowered by diabetes, but markedly increased in the hET-2 transgenic group thereby partially mediating the heart effects seen in the hET-2 animals. Consistent with previous reports [13], diabetes increased myocardial ET receptor expression in our experiment, although only modestly [65,66]. In the presence of diabetes, hET-2 overexpression increased myocardial $ET_A$ and $ET_B$ receptor expressions. This interaction may have indirectly intensified the negative actions of hET-2 overexpression on diabetic cardiomyopathy.

Whereas $ET_A$ modulates vasoconstriction, cellular proliferation and matrix deposition, $ET_B$ receptor indirectly reduces the vasoconstrictive and mitogenic effects of ET-1 [50]. Significantly downregulated $ET_B$ receptor in non-diabetic transgenic rats, and the resulting lack of its ET-1-inhibitive action, may be an explanation for the extremely elevated volume density of interstitial tissue in the heart (which reflects fibrosis) and the hypertrophic remodelling of intramyocardial vessels.

Summing up, the findings of this stimulatory intervention support the important role of ETs in diabetic cardiomyopathy, showing that enhanced ETs may contribute to cardiac damage by at least two distinct pathways of injury, one mediated by myocardial hypoperfusion (as a result of hypertrophic remodelling of myocardial capillaries) and the other based on the enhanced extra-capillary matrix deposition. These changes were observed in both one-hit groups, *i.e.* non-diabetic transgenic and diabetic wild-type animals, however, myocardial capillary remodelling was even more advanced in transgenic non-diabetic rats. Another noteworthy observation was that in the presence of both factors, *i.e.* diabetes and hET-2 overexpression, in the diabetic transgenic group, the myocardial remodelling, the morphometrical and stereological findings from the heart and heart vessels, the hemodynamic results and the changes in fibrosis and cardiovascular damage molecular markers indicated the most advanced diabetic cardiac changes. Hence, this study provided independent insights as evidence for a detrimental role of ETs in the pathogenesis of diabetic cardiomyopathy.

#### 4.2.5.2 *ETs Activate the Release of ANP*

According to Brenner *et al.* (1982), hypersecretion of ANP is promoted by the rise in blood osmotic pressure and the increase in circulating blood volume with hyperglycaemia. Since the main actions of natriuretic peptides include natriuresis and diuresis, the increase of ANP apparently is an attempt to overcome the water retention, thereby maintaining the physiological volume homeostasis [80]. This point was underlined by this study's finding that myocardial ANP mRNA reached the highest level in the diabetic transgenic group, which revealed the greatest glomerular filtration rate (data not shown).

Increased myocardial mRNA expression of the natriuretic peptide ANP is used as an indirect marker for cardiac hypertrophy and failure [80]. ANP is released from myocardial cells and contributes to vascular remodelling [33]. In this study, 2.5-fold

increased ANP mRNA expressions were in line with more severe myocardial structural changes and myocardial vessel remodelling in the diabetic hET-2 group when compared to the diabetic wild-type animals. This may be also explained by other data, substantiating that ETs activate the release of ANP [39].

#### 4.2.5.3 *Complex Regulation of ADM in Diabetic Cardiomyopathy*

ADM is another cardiovascular marker. Plasma ADM is elevated under pathological conditions associated with overhydration, hypertension, ischemia and endocrine and metabolic disorders [109]. Several stimulants that make up a complex ADM control system are known to raise the plasma concentration of ADM *in vivo*: ET-1, ANP, hyperglycaemia, *etc.* [110]. Plasma ADM levels in heart failure reflect a systemic or peripheral response to cardiac impairment and may be mediated by a variety of mechanisms, including induction of endothelial production of ADM, elevated levels of ET, or other humoral and neural mechanisms [111].

Similar to ANP, ADM mRNA expression was 2.8-times higher in the diabetic hET-2 group as compared to diabetic wild-type animals, confirming histomorphometric findings which indicated more advanced diabetic cardiomyopathy in the presence of hET-2 overexpression. This study has shown that ETs, ANP and hyperglycaemia also stimulated ADM expression in cardiovascular tissue.

#### 4.2.5.4 *Protective Action of Relaxin in Diabetic Cardiomyopathy*

Known for many years as a hormone of human reproduction, RLX has been shown to represent a potentially compensatory mediator in human heart failure. Plasma concentration of RLX and myocardial expression correlated with the severity of disease [112]. In this study, RLX and its receptor (LGR-7) were downregulated in the presence of hET-2 as well as, separately, under hyperglycaemia. It may be speculated that in the early stage of cardiomyopathy, one of the mechanisms of cardiac failure is downregulation of the relaxin system. Increased myocardial fibrosis in RLX-deficient mice confirms this hypothesis [112]. In more advanced cardiomyopathy (in the diabetic transgenic group), upregulation of the RLX system by a factor of 3.5 was a compensatory reaction to very poor heart condition. One of the mechanisms of the compensatory RLX response is upregulation of $ET_B$, which mediates ET-1 clearance and vasodilatation *via* NO and prostacyclin [31,32]. This mechanism of the protective action of RLX was confirmed by this study, since there was a 2.0-fold increase in $ET_B$ expression in the diabetic hET-2 animals.

# 5 Summary

Cardiovascular complications are the major causes of morbidity and mortality in patients with diabetes mellitus. Inhibitory interventions using endothelin receptor antagonists have suggested that the endothelin system plays an important role in diabetic cardiomyopathy, although some studies observed no or only a modest effect. In the present study, a stimulatory intervention, *i.e.* transgenic overexpression of the human endothelin-2 gene in rats, was used to further characterize the morphological and molecular contributions of endothelins to diabetic cardiomyopathy.

Diabetes mellitus was induced by streptozotocin injection into male rats expressing the human endothelin-2 transgene and into transgene-negative littermate controls. Non-diabetic transgene-positive and -negative animals were included as well to form a 4-group study design. Insulin treatment was begun 2 weeks after diabetes was confirmed and was administered every second day. After 12 weeks of diabetes, insulin was injected daily. The aim of the insulin treatment was to prevent a blood glucose concentration of higher than 600 mg/dl and rat mortality due to metabolic complications. Heart morphological and molecular changes were analysed following 6 months of hyperglycaemia.

Transgenic overexpression of the human endothelin-2 gene significantly intensified diabetic cardiomyopathy in the rats. The most prominent pathological changes were observed in epicardial and intramyocardial vessel hypertrophy and myocardial interstitial fibrosis. These changes were paralleled by a modest, but complex regulation of myocardial endothelin-1, endothelin A and B receptor expression. Six months of diabetes alone (*i.e.* without endothelin-2 overexpression as co-factor) were insufficient for revealing advanced cardiomyopathy. In diabetic wild-type rats, when compared with diabetic transgenic animals, the myocardial fibrosis markers were significantly lower and there was no hypertrophic remodelling of large coronary arteries.

This interventional animal study, overexpressing human endothelin-2 in rats, provides strong complementary support for, and mechanistic insights into, a detrimental, blood pressure-independent role of endothelins in diabetic cardiomyopathy. The endothelin system seems to promote the development of diabetic cardiomyopathy by altering the arterial vessels and through profibrotic action.

In conclusion, this study has demonstrated the usefulness of a transgenic model for evaluating the endothelin system's role in the pathogenesis of diabetic end-organ damage. The use of an endothelin antagonist has previously been shown to have beneficial consequences in experimental diabetes. The findings of this stimulatory interventional study correspond to this assessment, as they show the negative effects of endothelin overexpression on diabetic cardiomyopathy. Effective intervention in diabetes, however, has to take into account the complex regulation of the components of the endothelin system on the molecular level, and the observation that endothelins seem to interact with other molecular players in the development of diabetic cardiomyopathy. Thus, further studies are warranted in order to analyze whether endothelins play a role in other diabetic complications, and whether the contribution of endothelins in diabetic cardiomyopathy mechanisms may be pharmacologically modulated in order to yield a long-term beneficial effect on the evolution of this diabetic end-organ damage.

# 6 Zusammenfassung

Kardiovaskuläre Komplikationen sind die wichtigste Ursache von Morbidität und Mortalität der Patienten mit Diabetes mellitus. Interventionen mit Endothelin-Antagonisten wiesen auf die mögliche Bedeutung des Endothelin-Systems bei der diabetischen Kardiomyopathie hin, obwohl die Ergebnisse entsprechender Studien nicht konsistent waren. In dieser Studie wurde ein transgenes Überexpressionsmodell für humanes Endothelin-2 verwendet, um morphologische und molekulare Auswirkungen von Endothelin auf die diabetische Kardiomyopathie zu charakterisieren.

Der Diabetes mellitus wurde mittels Streptozotocin-Injektion induziert. Verwendet wurden männliche Ratten mit transgener Expression von humanem Endothelin-2 und nicht-transgene Kontrolltiere. Nicht-diabetische transgene und nicht-transgene Tiere wurden ebenfalls in die Studie eingeschlossen. Eine Insulintherapie wurde 2 Wochen nach Manifestation des Diabetes begonnen. Nach initialer Insulin-Applikation alle 2 Tage, wurde nach 12 Wochen beginnend Insulin täglich injiziert. Ziel der Insulintherapie war es, den Blutzucker unter 600 mg/dl einzustellen und Mortalität infolge metabolischer Komplikationen zu vermeiden. Die Herzen der Tiere wurden nach sechsmonatiger Hyperglykämie histologisch und molekular analysiert.

Die transgene Expression des Gens für humanes Endothelin-2 in Ratten führte zu einer signifikant ausgeprägteren diabetischen Kardiomyopathie. Die deutlichsten Unterschiede betrafen die Hypertrophie epikardialer und intramyokardialer Gefäße und die Fibrose des myokardialen Interstitiums. Parallel zu diesen Veränderungen fand sich eine komplexe transkriptionelle Regulation der myokardialen Expression von Endothelin-1, Endothelin A- und B-Rezeptoren. Sechs Monate Diabetes mellitus alleine (ohne Endothelin-Überexpression als Co-Faktor) waren nicht in der Lage, eine fortgeschrittene Kardiomyopathie auszulösen. In der diabetischen nicht-transgenen Gruppe waren die myokardialen Fibrosemarker signifikant schwächer als in der diabetischen transgenen Gruppe. Diabetes alleine war nicht in der Lage, ein hypertrophes Remodelling der großen Koronargefäße zu bewirken.

Die Überexpression von humanem Endothelin-2 bei Ratten mit Diabetes mellitus liefert neue Einblicke in die Bedeutung von Endothelinen für die Pathophysiologie der diabetischen Kardiomyopathie. Das Endothelinsystem

begünstigt die Entwicklung der diabetischen Kardiomyopathie durch vaskuläres Remodelling und profibrotische Wirkungen unabhängig vom arteriellen Blutdruck.

Die Ergebnisse dieser Arbeit zeigen den Nutzen eines transgenen Modells bei der Untersuchung der Rolle des Endothelin-Systems in der Pathogenese der diabetischen End-Organ-Schäden. Eine positive Wirkung von Endothelin-Antagonisten wurde bereits in experimentellen Arbeiten nachgewiesen. Diese Studie bestätigt diese Arbeiten durch den Nachweis negativer Effekte der Überexpression von Endothelin auf die diabetische Kardiomyopathie. Die komplexe Regulation des Endothelin-Systems und dessen Interaktionen mit anderen molekularen Faktoren der diabetischen Kardiomyopathie müssen berücksichtigt werden, wenn es um pharmakologische Interventionen bei Patienten mit Diabetes mellitus geht. Zukünftige Studien sollten analysieren, ob auch bei anderen diabetischen Komplikationen Endothelin eine Rolle spielt und ob das Fortschreiten der diabetischen Kardiopathie durch pharmakologische Intervention verzögert werden kann.

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# 9 Publications and Abstracts

The findings of this study were partially published and presented as follows:

Liefeldt, L.; Rylski, B.; Walcher, F.; Manhart, J.; Kron, S.; Wang-Rosenke, Y.; Paul, M.; Neumayer, H.-H.; Amann, K.; Peters, H. (2007): Transgenic overexpression of endothelin-2 aggravates diabetic cardiomyopathy in rats, J.Mol.Med. (submitted)

Rylski, B.; Walcher, F.; Manhart, J.; Kron, S.; Wang-Rosenke, Y.; Paul, M.; Neumayer, H.-H.; Amann, K.; Peters, H.; Liefeldt, L. (2007): Overexpression of human ET-2 aggravates diabetic cardiomyopathy, Tenth International Conference on Endothelin, Bergamo, Italy (submitted)

Rylski, B.; Liefeldt, L.; Walcher, F.; Manhart, J.; Kron, S.; Wang-Rosenke, Y.; Paul, M.; Neumayer, H.-H.; Amann, K.; Peters, H. (2007): Overexpression of human ET-2 aggravates diabetic cardiomyopathy, American Society of Nephrology – Renal Week, San Francisco, USA (submitted)

Rylski, B.; Manhart, J.; Walcher, F.; Amann, K.; Paul, M.; Neumayer, H.-H.; Peters, H.; Liefeldt, L. (2006): Diabetic cardiomyopathy but not diabetic nephropathy is promoted by human ET-2 overexpression in rats, Kongress für Nephrologie, Essen, Deutschland, pp. 133

Liefeldt, L.; Manhart, J.; Rylski, B.; Paul, M.; Neumayer, H.-H.; Peters, H. (2005): The renal endothelin system in diabetic endothelin-transgenic rats, Ninth International Conference on Endothelin, Utah, USA, pp. 41

Liefeldt, L.; Manhart, J.; Rylski, B.; Paul, M.; Neumayer, H.-H. (2005): Das renale Endothelin-System bei Diabetes mellitus – Untersuchungen an einem Endothelin-transgenen Tiermodell, Kongress für Nephrologie, Saarbrücken, Deutschland

Liefeldt, L.; Manhart, J.; Rylski, B.; Paul, M.; Peters, H.; Neumayer H.-H. (2003): Severe glomerulosclerosis in male but not in female rats overexpressing endothelin. World Congress of Nephrology, Berlin, Deutschland

# 10 Erklärung

„Ich, Bartosz Rylski, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: Transgenic overexpression of endothelin-2 aggravates diabetic cardiomyopathy in rats - selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Berlin, den 12. Juni 2007 Unterschrift

# 11 Lebenslauf

Mein Lebenslauf wird aus Datenschutzgründen in der elektronischen Version meiner Arbeit nicht mit veröffentlicht.