Increased Gene Expression of the Cardiac Endothelin System in Obese Mice

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Abstract
Obesity is a well-known risk factor of atherosclerosis and heart failure. In the human heart, a local endothelin system containing prepro-endothelin-1, endothelin-converting enzyme-1, and endothelin receptors A and B has been described. The endothelin system is activated in heart failure; however, the impact of obesity on the cardiac endothelin system is unknown. In this study, 18-week-old male C57BL/6 mice fed either a control diet or a high-fat diet for 10 weeks were analyzed. High-fat diet significantly increased the body weight of the animals and augmented low-density lipoprotein, high-density lipoprotein, and cholesterol plasma levels, compared to control. The animal groups showed no significant differences in left ventricular size or function (heart rate, ejection fraction, fractional shortening, left ventricular posterior wall thickness, cardiac output) after control or high-fat diet. We did not observe signs of cardiac hypertrophy or changes in markers of cardiac fibrosis in these heart samples. The cardiac expression of prepro-endothelin-1 mRNA, endothelin-converting enzyme-1 mRNA, and protein and endothelin receptors A and B mRNA was increased in 18-week-old obese C57BL/6 mice compared to animals with normal weight (p<0.05 vs. control). Furthermore, endothelin-1 plasma levels showed an increasing trend. In conclusion, an increased expression of genes of the endothelin system was observed in the hearts of 18-week-old mice after high-fat diet, possibly contributing to later cardiovascular complications of obesity.

Introduction
Obesity, in addition to insulin resistance, hyperlipidemia, and hypertension, is a hallmark of the metabolic syndrome leading to type 2 diabetes and increasing the risk of cardiovascular disease [1]. Several cardiovascular risk factors are known to affect the endothelin system. Increased endothelin-1 (ET-1) synthesis is considered to be involved in the development and progression of heart failure [2]. In addition, circulating levels and cardiac tissue levels of ET-1 are upregulated in experimental models of heart failure and can predict the clinical outcome in cardiovascular diseases [3]. ET-1 synthesis includes proteolytic cleavage of precursor peptide big ET-1 by the metalloprotease endothelin-converting enzyme-1 (ECE-1). Inhibition of ECE-1 activity has been suggested as a therapeutic strategy in the treatment of cardiovascular diseases [4]. ET-1 binds to endothelin receptors A and B (ETA, ETB), thus mediating the regulation of vascular tone, positive inotropic effects, and cardiac hypertrophy. Local cardiac ET-1 synthesis has been shown in endocardium, myocardium, and coronary endothelium. Determinants of ET-1 biosynthesis are selectively regulated within this myocardial compartment in heart failure. Furthermore, both ETA and ETB receptors are expressed in murine and human myocardium. However, the impact of obesity on the cardiac endothelin system is currently unknown. Therefore, the aim of this study was to analyze the impact of high-fat diet on cardiac function and the endothelin system in obese mice.

Materials and Methods
Animals
Eight-week-old male C57BL/6 (n≥7/group) mice were fed control diet EF R/M CD88137 (as control) or high-fat diet EF R/M TD88137 (containing

Endocrine Care

GmbH, Mannheim, Germany.

content was analyzed by picrosirius red staining [6].

ature was stained by hematoxylin and eosin (HE). Collagen-

affinized and rehydrated through degraded ethanol. Heart struc-

tomery. The mRNA expression of components of the cardiac

many). RNA concentration was determined by UV spectropho-

lation. Cardiac biopsies of animals were dissected immediately

After explantation, snap-frozen and stored in liquid nitrogen

high-fat diet, hearts were rapidly excised and fixed in buffered

formalin (4 %), followed by rinsing in PBS (4 × 1 h), dehydration

and rinsing in PBS (4 × 1 h), dehydration and paraffin embedding. Serial 5 μm sections were cut, deparaffinized and rehydrated through degraded ethanol. Heart structure was stained by hematoxylin and eosin (HE). Collagen content was analyzed by picrosirius red staining [6].

Histology

Heart paraffin sections were generated as previously described [5]. In brief, after 18 weeks including 10 weeks of control diet or high-fat diet, hearts were rapidly excised and fixed in buffered formalin (4 %), followed by rinsing in PBS (4 × 1 h), dehydration and paraffin embedding. Serial 5 μm sections were cut, deparaffinized and rehydrated through degraded ethanol. Heart structure was stained by hematoxylin and eosin (HE). Collagen content was analyzed by picrosirius red staining [6].

RNA isolation and quantification of mRNA expression

At 18 weeks of age, hearts were excised for RNA and protein iso-

lation. Cardiac biopsies of animals were dissected immediately

after explantation, snap-frozen and stored in liquid nitrogen

until RNA preparation. Total RNA from myocardial tissue was

isolated using the TRIFAST protocol (Peqlab, Erlangen, Ger-

many). RNA concentration was determined by UV spectropho-

tometry. The mRNA expression of components of the cardiac

endothelin system (prepro-endothelin-1 or ppET-1, ECE-1, ET_A

and ET_B) was quantified by quantitative reverse transcription-

polymerase chain reaction (RT-PCR) and normalized to 18 S

rRNA in the RNA from murine hearts. The following primer were

used: ppET-1 sense (5′-AAC TCA GGG CCC AAA GTA GC-3′), anti-

sense (5′-TGG GCA ACA CGA AAA GAT GC-3′) (annealing tem-

terature: 60 °C, 30 cycles, fragment size: 169 bp); ECE-1 sense

(5′-CAT GGA CCC CAG AGT AGA CC-3′), antisense (5′-CTC CCA

tCT CAA TCA GC-3′) (annealing temperature: 66 °C, 30 cycles,

fragment size: 281 bp); ET_A sense (5′-TGC ACG CCT ATT

GCC CAC AG-3′), antisense (5′-AGA GGG AAC CAG CAC AGG

GC-3′) (annealing temperature: 68 °C, 30 cycles, fragment size:

772 bp); ET_B sense (5′-CGA GGT GTT CCT TGG AGT C-3′),

antisense (5′-CCG GAA GGT TTC ATC TGG TTC ATC-3′) (annealing

temperature: 69 °C, 30 cycles, fragment size: 702 bp); and 18S

rRNA sense (5′-GTT GTT GGA GCG ATT TCT CGT G-3′), anti-

sense (5′-AGG GCA GGG ACT TAA TCA CTA GC-3′) (annealing

temperature: 60 °C, 11 cycles, fragment size: 346 bp).

In additional experiments, mRNA expression of genes involved

in cardiac fibrosis was quantified by real-time PCR in the Applied

Biosystems 7500 Fast Real-Time PCR system (Applied Biosys-

tems, Foster City, CA, USA). Specific oligonucleotide primers

were synthesized by TIB MolBiol SyntheseLabor (Berlin, Ger-

many). The reactions were carried out in 20 μl reaction volumes

containing corresponding cDNA, gene specific primers (0.5 μM
each) and Power SYBR Green PCR Master Mix (Applied Biosys-

tems, Foster City, CA, USA). Primer sequences: collagen, type I,

alpha 1 (Col1a1) sense (5′-GAG CGG CGG AGA GTA CTG CAG GC-3′),

antisense (5′-GTT CCG GGG CCT GTA CCA GT-3′); collagen, type

III, alpha 1 (Col3a1) sense (5′-GCA CAG CAG GCC TCC AAC GAT

G-3′), antisense (5′-TCT CCA AAT GGG ATC TCT GGC-3′); and β-2-

microglobulin (B2m) sense (5′-GAA GCT GTT GCT TCT TGG AGT

C-3′), antisense (5′-GGG AGC GAA GAT CTT CCC GAT-3′); and 18 S

rRNA sense (5′-AGG ATG AAT CTT CAG ACG ATG-3′), anti-

sense (5′-CAA ATG AAT CTT CAG ACG ATG-3′). Reaction

conditions included a 2-min initial activation step at

50 °C and a 10-min denaturing step at 95 °C, followed by 40

cycles of 15 s at 95 °C, and 1 min at 60 °C. Specificity of the reac-

tion was verified by performing melting curve analysis at

the end of each series of assays. The relative amount of the tran-

script was calculated by the cycle threshold method using the

Applied Biosystems 7500 System v.1.2.3 software and normal-

ized to the endogenous reference gene (B2m).

Protein isolation and Western blot analysis

Protein from myocardial tissue was isolated using the TRIFAST

protocol. The protein concentration was determined with Pierce

BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham,

MA, USA).

Equal amounts of protein were separated by SDS-PAGE (10 %)

and transferred to nitrocellulose membranes (Schleicher &

Schuell, Dassel, Germany). Membranes were incubated with

polyclonal antibodies generated against ECE-1, ET_A, ET_B (Santa

Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and secondary

horseradish peroxidase-linked anti-rabbit IgG (GE Healthcare,

Freiburg, Germany). The protein expression was detected with

Western Lightning Chemiluminescence Reagent Plus (Perkin-

Elmer Life Sciences, Boston, MA, USA) and quantified using

AIDA Image Analyzer software (Raytest, Berlin, Germany). The

protein expression was normalized to glyceraldehyde-3-phos-

phate dehydrogenase (GAPDH) using a monoclonal antibody

(HyTest, Turku, Finland) and a secondary horseradish peroxi-

dase-linked anti-mouse IgG (GE Healthcare, Freiburg, Germany).
Endothelin-1 plasma levels
Endothelin levels were quantified in plasma of 18-week-old mice fed a control diet or high-fat diet for 10 weeks using the Endothelin-1 Quantikine ELISA Kit (R & D Systems, Inc., Minneapolis, MN, USA).

Statistical analysis
Data are shown as mean±SEM. Statistical analysis was performed with Student’s t-test (SigmaStat, Systat Software, Erkrath, Germany). A p-value of < 0.05 was considered to be statistically significant.

Results

Lipid parameters
After receiving a high-fat diet for 10 weeks, the body weight of the animals was significantly increased from 24.5±0.6 g of the control diet group to 34.2±1.9 g of the high-fat diet group (p < 0.05 vs. control) (Fig. 1a). This was accompanied by augmented blood glucose, low-density lipoprotein and cholesterol plasma levels in the obese animals. These data and additional parameters are summarized in Table 1.

Cardiac function
In a separate set of experiments, cardiac function was analyzed by echocardiography in 18-week-old male C57BL/6 mice fed a control diet or high-fat diet for 10 weeks (n = 12/group). A typical M-Mode picture of the left ventricle of a C57BL/6 mouse (control diet) was taken from left apical 2-chamber view. The echocardiographic data are summarized in Table 2. The animal groups showed no significant differences in left ventricular size or function (heart rate, ejection fraction, fractional shortening, left ventricular posterior wall thickness, cardiac output) after 10 weeks of control diet or high-fat diet (18 weeks of age). We did not observe signs of heart failure in these animals.

Cardiac structure and fibrosis
In an independent set of experiments, we analyzed the size and structure (by HE staining) and the collagen content (by picrosirius red staining, collagen in red on a pale yellow background) of cardiac sections in mice fed a control diet or a high-fat diet for 10 weeks (Fig. 2). We did not observe increased signs of hypertrophy or fibrosis in these cardiac biopsies. In additional experiments, we analyzed mRNA expression of markers of cardiac fibrosis collagen, type I, alpha 1 (Col1a1) and collagen, type III, alpha 1 (Col3a1) by real-time PCR (Fig. 3). The cardiac mRNA expression of Col1a1 and Col3a1 was not different in 18-week-old wild-type mice fed a control diet or high-fat diet for 10 weeks.

Gene expression of endothelin system
In obese C57BL/6 mice, cardiac expression of prepro-endothelin-1 mRNA was significantly increased compared to the control
group (Fig. 4a). Endothelin-1 plasma levels in 18-week-old mice fed a control diet (n = 12) or a high-fat diet (n = 11) for 10 weeks showed a trend to be increased (124 ± 19% of control) without reaching significance (Fig. 4b). High-fat diet induced the endothelin-converting enzyme-1 mRNA (Fig. 4c) and protein (Fig. 4d) expression in hearts from C57BL/6 mice compared to mice fed control diet (p < 0.05 vs. control). Furthermore, mRNA expression of cardiac endothelin receptor A and B was upregulated by high-fat diet in the murine heart (Fig. 4e, f). The protein expression of cardiac endothelin receptors A (135 ± 56% of control) and B (158 ± 74% of control) in 18-week-old mice on high-fat diet showed a trend to be increased without reaching statistical significance.

**Discussion**

Wild-type mice might not reflect all lipid parameters observed in clinical studies. The increase in total cholesterol and LDL-cho-
lesterol in our mice is in agreement with previous data [7]. Other studies showed increased total cholesterol and LDL-cholesterol, but no increase in plasma triglycerides in mice after high-fat diet as well [8, 9]. Furthermore, discrepancies between different studies might have been due to metabolic and/or endocrine adaptations to the long-term high-fat diet and the different genetic backgrounds between mouse models.

The increased HDL-cholesterol levels in our mice are in agreement with previous studies feeding a Western diet to C57BL/6 mice [7]. The HDL-cholesterol is important in the lipid transport of these mice. The cholesterol content of Western diets is known to play a major role in the paradoxical increase in HDL [8]. A high-saturated fatty acid- and cholesterol-containing diet can promote the fecal excretion of macrophage- and HDL-derived cholesterol. This change in reverse cholesterol transport may constitute a compensatory mechanism to protect macrophages from cholesterol accumulation [8]. In addition, both dietary saturated fat and cholesterol intake are known to raise plasma high-density lipoprotein cholesterol (HDL-C) levels. Several epidemiological studies and 1 meta-analysis of 60 controlled trials showed a positive correlation between high saturated fat intake and HDL-C as well [10, 11].

The blood glucose levels in our control mice appear to be quite high after overnight fasting. C57BL/6 is the most widely used inbred strain and wild-type control. C57BL/6J mice fed a high-fat diet develop obesity and mild to moderate hyperglycemia (The Jackson Laboratory, Bar Harbour, ME, USA). Therefore, we have chosen this strain as a murine model to study some of the diet-induced changes seen in patients with metabolic syndrome. In agreement with previous findings, we observed in our mice further elevated blood glucose levels after high-fat diet. A putative explanation could be a naturally occurring deletion in nicotinamide nucleotide transhydrogenase (Nnt) exons 7–11 occurred in C57BL/6J sometime prior to 1984. This deletion results in the absence of the NNT protein, and is associated with impaired glucose homeostasis control and reduced insulin secretion [12]. Furthermore, the type and duration of diet, duration of fasting and collection of blood samples might affect the blood glucose levels as well [13].
We did not observe significant changes in cardiac function at 18 weeks of age. The cardiac parameters were in the normal physiological range as described in the literature [14, 15]. Therefore, the animals did not display functional signs of heart failure in response to high-fat diet at the time point of our study. In additional experiments, we did not find signs of cardiac hypertrophy and fibrosis in heart sections of animals from both experimental groups. Furthermore, we did not observe changes in the cardiac gene expression of collagens. They are well-established markers of cardiac fibrosis [16]. This further supports the view that both groups did not differ in cardiac structure or function.

This study demonstrates an upregulation of the mRNA expression of genes of the cardiac endothelin system in C57BL/6 mice after 10 weeks of high-fat diet. Obesity can be associated with an ET-1-dependent activation of renal angiotensin-converting enzyme and an enhanced vascular contractility to angiotensin II [17]. Furthermore, endothelin might modulate increased endothelium-dependent vasoconstriction in obesity [18]. Our findings are supported by the recently described increased serum ET-1 levels and augmented myocardial ppET-1 mRNA expression in mice fed a high-fat diet [19]. Interestingly, this study suggested an involvement of leptin in the upregulation of ET-1. The endothelin-1 peptide plasma levels and the endothelin receptor A and B protein expression in our mice showed a trend to be increased without reaching significance. The circulating plasma ET-1 levels might rather reflect a spill-over of the vascular ET-1 production and has been used as predictor of prognosis in patients with heart failure [20]. Even while the ET-1 plasma level might not be directly linked to the local cardiac expression of components of the endothelin system, the slightly increased ET-1 level supports an activation of the endothelin synthesis in these animals. As increased plasma endothelin-1 levels were found in normotensive and hypertensive obese subjects compared with a lean healthy group [21], our findings could also have clinical implications. We could previously show that endothelin–converting enzyme-1 mRNA and ET-1 peptide levels were increased in failing myocardium of patients and partially normalized by ACE inhibition [22]. The endothelin receptor A was 2-fold higher expressed in human atrium, compared to ventricle [23]. Furthermore, endothelin receptor A was markedly upregulated in failing human myocardium and normalized by ventricular assist device unloading [24].

We could not determine whether upregulation of the endothelin system was due to augmented gene expression in cardiac myocytes or nonmyocytes (e.g., endothelial cells, smooth muscle cells, or fibroblasts). ECE-1 protein expression was in previous studies more widespread compared to its mRNA, being present in endothelial cells, mesenchymal cells and myocytes, and particularly enriched in the trabeculae and nascent ventricular conduction system [25]. The 2 endothelin receptors were found to be expressed in cardiomyocytes as well as in the cardiac vessels [26]. Ventricular ET-1 receptors have a cell-specific distribution: the majority of ET receptors in cardiomyocytes are of the ETα subtype, whereas fibroblasts had a nearly equal proportion of the ETα and ETβ subtypes [27]. The ET receptor number on smooth muscles and fibroblasts does not exceed ET receptor number on myocytes. Endothelial cells express ETβ receptors only. Therefore, the increased cardiac mRNA expression of the endothelin receptors most likely reflects an increased mRNA expression in cardiomyocytes. A putative mechanism of activation of the endothelin system in obesity could involve LDL. In agreement with clinical data obesity increased the LDL-cholesterol level in our study as well. We could show in previous in vitro studies that native and oxidized LDL increases the ppET-1 and ECE-1 mRNA expression and the ET-1 peptide formation in human endothelial cells [28]. Furthermore, the endothelial ETβ receptor mRNA was induced by both lipoproteins [29]; therefore, the increased LDL levels of the obese mice could contribute to the accelerated expression of genes of the endothelin system, suggesting a novel mechanism explaining how obesity supports the development of cardiac hypertrophy and heart failure. This might have also implications for the development of novel therapeutic strategies in heart failure [30].

In summary, our data show that the elevated expression of genes of the endothelin system in obese animals is independent from histological or functional signs of heart failure or cardiac fibrosis. An activated endothelin system might represent an early marker of compensatory efforts to maintain cardiac function in obesity and might contribute to later cardiovascular complications of obesity.

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Conflicts of Interest

The authors declare that they have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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