# Role of vasodilator mediators in endothelial dysfunction

Summary of Ph.D. Thesis

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## 1. Introduction

In recent decades, it has become evident that endothelium is not only a structural barrier between the circulation and surrounding tissue, but an active autocrine, paracrine and endocrine organ, which is indispensable for the maintenance of vascular homeostasis. Under physiological conditions, the endothelium prevents platelet and leukocyte adhesion, inhibits smooth muscle cell proliferation and favors fibrinolysis. It is also known that endothelium contributes to the regulation of blood flow and blood pressure by releasing vasodilator [nitric oxide – (NO), prostacyclin ($PGI_2$) and endothelium-derived hyperpolarizing factor (EDHF)] and vasoconstrictor [endothelin-1 (ET-1), thromboxane $A_2$ ($TxA_2$) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)] substances. In contrast, endothelial dysfunction is defined by impairment of endothelium-dependent relaxation, prothrombotic activation of endothelium, heightened leukocyte adhesion, increased smooth muscle cell proliferation and increased production of superoxide anion ($O_2^-$).

Accumulated data have revealed that major cardiovascular diseases, such as atherosclerosis, heart failure, diabetes mellitus and hypertension are all associated with endothelial dysfunction. Importantly, arterial endothelial dysfunction can be detected already in the presence of cardiovascular risk factors prior to any morphological changes in the vascular wall. Thus, endothelial dysfunction is an important and early step in the pathomechanism of cardiovascular diseases. Interestingly, it was reported that correction of these risk factors may restore the impairment of endothelium-dependent relaxation. Accordingly, the measurement of endothelium-dependent relaxation may represent a specific barometer of cardiovascular risk and the analysis of the phenotype of endothelial dysfunction may have preventive, diagnostic, prognostic and therapeutic significance.

In clinical practice endothelial function is almost exclusively measured as a decrease in stimulated (by pharmacological agonists or increased blood flow) NO-mediated vascular dilatation. However, the phenotype of endothelial dysfunction in cardiovascular diseases may involve not only the impairment of stimulated NO-dependent vasodilation, but also the impairment of basal NO release and reduced NO sensitivity of SMCs. Moreover, it is known that endothelium produces not only NO, but other vasodilator substances, therefore it is plausible that reduced $PGI_2$ and EDHF activity may contribute to the development of endothelial dysfunction. Consequently, the comprehensive analysis of stimulated NO-mediated vasodilation, basal NO production and the assessment of $PGI_2$- and EDHF-

mediated relaxation seems more reliable to diagnose endothelial dysfunction in cardiovascular diseases.

There are accumulating data suggesting that activated platelets are important players in the initiation and progression of atherosclerosis. It has been reported that activated platelets may induce endothelial inflammation leading to the acceleration of the atherosclerotic process. Surprisingly, our knowledge about the effects antiplatelet drugs, such as aspirin and thienopyridines, on atherogenesis and vascular function is limited and sometimes contradictory. Indeed, it was reported that aspirin at a dose that selectively inhibits COX-1 in plateletes blunt platelet activation, vascular inflammation and progression of atherosclerosis in $LDLR^{-/-}$, but not in $apoE^{-/-}$ mice. Furthermore, it is still not known whether the thienopyridine ticlopidine, an antagonist of the $P2Y_{12}$ subtype of the ADP receptor in platelets, yield protection against the progression of atherosclerosis in gene-targeted mice model of atherosclerosis.

**2. Major experimental goals**

The major aim of this work was to analyze the role of vasodilator mediators (NO, $PGI_2$ and EDHF) in endothelial dysfuncion in animal models of atherosclerosis, heart failure and diabetes mellitus. For that purpose we

1. investigated the progression of endothelial dysfunction in aorta along the development of atherosclerotic plaques in apoE/$LDLR^{-/-}$ mice;
2. examined the effect of ticlopidine on the progression of atherosclerosis and endothelial function in apoE/$LDLR^{-/-}$ mice;
3. investigated the phenotype of endothelial dysfunction in the thoracic aorta in rats at the early and late phase following coronary ligation in particular in relation to gender;
4. analyzed the development of endothelial dysfunction in the aorta in streptozotocin-induced diabetic rats.

**3. Methods**

**3.1. Animals**

*Animal model of atherosclerosis*

2-, 4-, 6- and 8-month-old female apoE/$LDLR^{-/-}$ (n=33) and age-matched C57BL/6J mice (n=20) kept on standard chow diet were used to investigate the relative contribution of NO-, $PGI_2$- and EDHF-pathways to endothelium-dependent relaxation in the thoracic aorta.



At the age of 8 weeks female apoE/$LDLR^{-/-}$ mice were put on a Western diet (consisting of 21% fat by weight, 0.15% cholesterol by weight and no cholic acid) for 4 months. 20 mice were randomly allocated to two groups: one receiving Western diet alone, the other receiving the same diet mixed with ticlopidine to yield a dose of 90 mg/kg/day. Vascular function was investigated in the thoracic aorta from 6-month-old mice.

*Animal model of heart failure*

Seven and 42 days after coronary ligation male (n=9) and female (n=8) Sprague-Dawley rats were used to analyze the alterations in NO-, $PGI_2$- and EDHF-dependent endothelial function in the thoracic aorta. Sham operated (8 male and 7 female) rats underwent the same surgical procedure, except coronary ligation.

*Animal model of diabetes mellitus*

Male Sprague-Dawley rats were randomly divided into to groups: diabetic group (n=16) receiving a single iv injection of streptozotocin (65 mg/kg body weight), the control group (n=16) receiving vehicle (sodium citrate). We analyzed diabetes-induced alterations in NO-, $PGI_2$- and EDHF-dependent relaxation in the aorta 4 and 8 weeks after the injection.

**3.2. Model quantifications**

*Atherosclerosis*

Total cholesterol, HDL, LDL and triglycerides were assayed after fasting for 8 hours in apoE/$LDLR^{-/-}$ mice using commercially available kits. We quantified the area covered by atherosclerotic lesions in the aortic root (cross section analysis) and in the whole descending aorta (en face analysis).

*Heart failure*

Before the coronary artery ligation and 42 days after myocardial infarction *in vivo* transthoracic echocardiography was used to determine fractional shortening. Seven or 42 days after coronary ligation the heart was rapidly removed, blotted dry and the total heart weight, right ventricule, left ventricle (LV) and the infarcted region was weighed. Infarct-size was expressed as the ratio of the infarct region to total LV mass. Importantly, animals with an infarct size more than 30% of LV were used for the experiments.

*Diabetes mellitus*

Four or 8 weeks after streptozotocin injection, plasma glucose and $HbA_{1c}$ levels were determined after 8 hours of fasting. Furthermore, the development of diabetic state was validated by body weight measurement and 24-hour urine collection.

### 3.3. Analysis of eicosanoids in aortic rings

*Determination of basal prostacyclin production by enzyme immunoassay*

Prostacyclin released from the aortic tissue was determined as its stable metabolite 6-keto-$PGF_{1\alpha}$. The aortic rings were incubated in Krebs-Henseleit solution and the samples of supernatant were collected after 30 min of incubation. $PGI_2$ production was expressed as pg/mg of dry weight of the aortic rings. The enzymatic source of $PGI_2$ was analyzed by non-selective COX or selective COX-2 inhibitors such as indomethacin or rofecoxib, respectively.

*Determination of eicosanoid production by 1-[$^{14}$C]-arachidonic acid*

Aortic rings (15 mg wet weight/in each sample) were preincubated at 37 $^{o}$C for 10 min in 1 ml Medium 199 tissue culture. The enzyme reaction was started by the introduction of tracer substrate, 1-[$^{14}$C]-arachidonic acid (3.7 kBq, 0.172 pmol), into the incubation mixture. Thirty minutes later, the enzyme reaction was stopped and the samples were then extracted with ethyl acetate (2 x 3 ml) and the organic phases were pooled and evaporated to dryness under nitrogen. The residues were reconstituted in 2 x 100 µl ethyl acetate and quantitatively applied to silica gel G thin-layer plates. Each 3-mm band of the chromatograms was then scraped off and the radioactivity was determined by liquid scintillation analyzer. Radioactivity was expressed in disintegrations per minute (dpm). The radiolabeled products of arachidonic acid were identified with unlabeled authentic standards, which were detected by anisaldehyde reagent. Prostacyclin and $TxA_2$ were determined as their stable metabolites (6-keto-$PGF_{1\alpha}$ and $TxB_2$, respectively).

### 3.4. Protocol of experiments in isolated aortic rings

Following sacrifice, the descending thoracic aorta was quickly removed, and after washing with ice-cold saline it was placed in cold, freshly-prepared Krebs–Heinseleit buffer of the following composition (in mmol/l): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, $KH_2PO_4$ 1.2, glucose 10 and EDTA 0.03 (mouse) or 0.5 (rat). After removal of connective tissue, the aorta was cut into 3 (mouse) or 6 (rat) rings, each approximately 3 mm in length. The rings were set up in organ baths and perfused with Krebs-Heinseleit solution at 37°C, pH 7.4 that was continuously bubbled with 95% $O_2$/5% $CO_2$ mixture. After 1-h equilibration under a resting tension of 0.75 g (mouse aorta) or 4 g (rat aorta), viability of the vessels was verified as the magnitude of vasoconstriction induced by potassium chloride (KCl, 30 or 60 mM for mice or rat aortic rings, respectively).



*NO-dependent relaxation*

Aortic rings were precontracted with phenylephrine (Phe, $5x10^{-8}$ to $2x10^{-7}$ M) and after reaching a stable plateau phase, acetylcholine (Ach, $10^{-9}$ to $10^{-5}$ M) was added cumulatively.

The endothelium-independent vasorelaxation was evoked by sodium nitroprusside (NaNP, $10^{-9}$ to $10^{-5}$ M) or *S*-nitroso-*N*-acetylpenicillamine (SNAP, $10^{-9}$ to $10^{-6}$ M). Basal NO production was assessed on mildly preconstricted rings (20% of KCl-induced contraction) on the basis of contraction induced by the nitric oxide synthase inhibitor $N_{\omega}$-nitro-L-arginine methyl ester (L-NAME, 300 µM, 15 min).

*COX-dependent endothelial function*

In some experiments, non-selective COX or selective COX-2 inhibitors (indomethacin 1-5 µM or rofecoxib 1-5 µM, respectively) were used to test the involvement of COX in Ach-induced vasodilation.

*EDHF-dependent relaxation*

EDHF component of Ach-induced relaxation was assessed after combined incubation with L-NAME and indomethacin. The mechanism of the remaining COX- and NOS-independent vasodilation was analyzed by pre-treatment with the $K^+$ channel blocker tetraethylammonium chloride (TEA, 10 mM, 10 min) or epoxygenase synthesis inhibitor miconazole (MICO, 10 µM, 10 min).

### 3.5. Statistical analysis

Vasodilator responses are expressed as a percentage of Phe-induced preconstriction. All results are expressed as mean ± SEM. The significance of differences between two groups was established by Student's *t*-test. Comparison of means between more than two groups was assessed by ANOVA followed by Scheffe test or Kruskal-Wallis test, for normally and non-normally distributed data, respectively.

Significant differences between the vasodilator and vasoconstrictor COX metabolites was established by modified Student *t* test. $p<0.05$ was considered statistically significant.

## 4. Results

### 4.1. Atherosclerosis

*Progression of atherosclerosis in the aorta of apoE/LDLR$^{-/-}$ mice*

The total cholesterol (21.72±0.61 and 1.76±0.06 mmol/l for 2-month-old apoE/LDLR$^{-/-}$ and control mice, respectively), LDL-cholesterol (15.66±1.03 and 0.65±0.3 mmol/l for 2-month-old apoE/LDLR$^{-/-}$ and control mice, respectively) and HDL-cholesterol (3.37±0.18

and 1.24±0.4 mmol/l for 2-month-old apoE/LDLR$^{-/-}$ and control mice, respectively) levels were already elevated in 2-month-old apoE/LDLR$^{-/-}$ mice and remained approximately at the same level in 4-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice.

Atherosclerotic plaques were nearly absent in 2-month-old apoE/LDLR$^{-/-}$ mice irrespective if we measured in the whole aorta (en-face) or in aortic root (cross-section) (0.64±0.1 % and 0.13±0.07 $10^5$ $\mu m^2$ for en-face and cross section analysis, respectively). However, in 4-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice time-course of progression of atherosclerosis was found by en-face analysis (6.96±1.3, 15.1±1.29 and 23.73±0.95 % of aorta, respectively) and cross-section analysis (3.0±0.14, 5.99±0.42 and 8.03±1.39 $10^5$ $\mu m^2$, respectively).

*Vascular function in the aorta in apoE/LDLR$^{-/-}$ mice*

Endothelium-dependent relaxation was already impaired in the aorta in 2-month-old apoE/LDLR$^{-/-}$ mice and remained impaired in older animals (Ach $10^{-6}$ M: 82.37±3.2%, 89.2±3.64%, 77.83±3.33%, 72.2±3.1% for 2-, 4-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice, respectively) as compared to age-matched controls (Ach $10^{-6}$ M: 101.03±6.09%, 96.13±2.36% and 108.87±3.97% for 2-, 4- and 6-month-old C57BL/6J mice, respectively). The SNAP-induced endothelium-independent relaxations were identical in 2-6-month-old apoE/LDLR$^{-/-}$ mice and maximal relaxations ($10^{-6}$ M) reached approximately 100 % (90.55±3.66%, 97.32±2.94% and 105.8±2.77% for 2-, 4- and 6-month-old apoE/LDLR$^{-/-}$ mice, respectively). In contrast, the endothelium-independent relaxation was decreased in 8-month-old apoE/LDLR$^{-/-}$ mice (SNAP $10^{-6}$ M: 82.03±2.79%). Basal NO production, determined as the magnitude of L-NAME-induced vasoconstriction was diminished in 2- and 4-months-old apoE/LDLR$^{-/-}$ mice (16.66±5.3% and 17.63±8.62%, respectively) as compared to age-matched wild-type animals (31.98±3.05 and 33.32±8.17%, respectively), whereas in 6- and 8-month-old apoE/LDLR$^{-/-}$ mice it was restored (33.79±4.13% and 37.8±4.12%, respectively).

The preincubation with indomethacin (5 µM) did not modify the magnitude of Ach-induced relaxation in either control or apoE/LDLR$^{-/-}$ mice. However, the basal $PGI_2$ production of aorta was gradually increased along to the progression of atherosclerosis (448.1±160.1, 1437.1±439.9, 1938.9±307.3 and 1866.7±404.8 for 1-, 2-, 4- and 6-month-old apoE/LDLR$^{-/-}$ mice, respectively).

In apoE/LDLR$^{-/-}$ mice the EDHF-dependent relaxation, as assessed by Ach in the presence of indomethacin and L-NAME was gradually up-regulated in the aorta starting from



the 2-month-old mice (Ach $10^{-6}$ M: 16.87±4.55%, 40.61±11.84%, 33.03±4.74% and 32.92±2.72% for 2-, 4-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice, respectively) as compared to control animals (Ach $10^{-6}$ M: 5.79±3.71%, 6.57±4.18% and 4.74±2.45% for 2-, 4- and 6-month-old wild-type mice, respectively).

The EDHF-component of Ach-induced vasodilation was abrogated (<3% in each experimental group) in apoE/LDLR$^{-/-}$ mice if KCl (30 mM) was used to preconstrict the vessels instead of phenylephrine or by the preincubation with tetraethylammonium chloride (TEA, 10 mM), an antagonist of $Ca^{2+}$-activated $K^+$ channels or miconazole (MICO, 10 µM), a selective inhibitor of epoxyeicosatrienoic acid (EET) synthesis.

*Effects of ticlopidine on the progression of atherosclerosis and endothelial function*

The total cholesterol and triglycerides in 6-month-old apoE/LDLR$^{-/-}$ mice treated with ticlopidine (90 mg/kg/day) for 4 months were similar to those in non-treated counterparts (total cholesterol: 28.9±1.1 mmol/l (n=5) vs. 26.8±1.3 mmol/l (n=5); triglycerides: 1.8±0.1 mmol/l vs. 2.01±0.1 mmol/l in the non-treated and the ticlopidine-treated group, respectively).

In ticlopidine-treated apoE/LDLR$^{-/-}$ mice the atherosclerotic area in the aortic root (cross section analysis) was markedly diminished as compared with their respective controls (5.65±0.39 $10^5$ $\mu m^2$ vs. 3.08±0.33 $10^5$ $\mu m^2$ in the non-treated and the ticlopidine-treated group, respectively, $p<0.05$).

Ticlopidine-treatment improved the Ach-induced endothelium-dependent vasodilation in the thoracic aorta from 6-month-old apoE/LDLR$^{-/-}$ mice (Ach $10^{-5}$ M: 93.2±2.1%) as compared to non-treated group (Ach $10^{-5}$ M: 78.86±2.6%). Ticlopidine did not modify endothelium-independent relaxation and basal NO production, but increased the EDHF-component of Ach-induced relaxation (Ach $10^{-6}$ M: 28.9±6.52% and 5.7±4.8% for ticlopidine treated and non-treated apoE/LDLR$^{-/-}$ mice, respectively).

**4.2. Heart failure**

*Development of heart failure after coronary ligation*

There was no significant difference in infarct-size between female and male rats either 7 (32.8±1.4% and 35.8±2.1% for female and male rats, respectively) or 42 days (42.5±2.9% and 40.7±3.2% for female and male rats, respectively) after coronary ligation. However, 42 days (3.6±0.3 and 2.8±0.1 mg/g for female and male rats, respectively) but not 7 days (2.8±0.2 and 2.5±0.1 mg/g for female and male rats, respectively) after the coronary artery ligation the LV/body weight ratio was significantly increased as compared to sham animals (2.8±0.1,

2.5±0.1, 2.7±0.1 and 2.4±0.1 mg/g for 7 days female, 7 days male, 42 days female and 42 days male sham rats, respectively). Forty two days after coronary ligation the fractional shortening was reduced to an equal extent in female and male MI hearts (17.2±1.7% and 16.8±6.1% for female and male MI rats, respectively) as compared to sham animals (35.3±6.9% and 42.6±3.6% for female and male MI rats, respectively).

*Vascular function in the aorta after coronary artery ligation*

The magnitude of Ach-induced endothelium-dependent relaxation was not different between MI rats and sham operated animals, irrespectively of the gender of the animals and the time period after MI. Seven days after coronary ligation maximal Ach-induced relaxation ($10^{-5}$ M) in the aorta was: 94.07±2.25%, 89.14±2.32%, 98.27±1.47% and 96.81±2.88% for female-MI, female-sham, male-MI and male-sham respectively. After 42 days the maximal Ach-induced relaxation ($10^{-5}$ M) was: 96.01±3.22%, 92.01±2.23%, 99.03±3.36% and 98.28±3.9% for female-MI, female-sham, male-MI and male-sham, respectively.

Moreover, the endothelium-independent relaxation induced by NaNP was nearly identical in MI and sham operated rats, irrespective of gender. The magnitude of endothelium-independent relaxation induced by NaNP ($10^{-6}$ M) was not different between female and male MI groups and sham operated rats (42 days after coronary ligation: 101.55±2.71%, 101.44±2.87%, 100.97±4.46% and 105.1±1.8% for female-MI, female-sham, male-MI and male-sham, respectively).

Basal NO production was significantly decreased in both female and male MI rats 7 days after coronary ligation (21.76± 2.89%, 54.32±5.11%, 37.61±4.81% and 51.98±5.05% for female-MI, female-sham, male-MI and male-sham, respectively). Forty two days after coronary ligation the attenuation of basal NO production in MI group vs. sham group was present in male (21.42±2.6% vs. 33.85±4.1%, respectively) but not in female animals (24.49±2.78% vs. 27.01±2.56%, respectively).

The incubation of aortic rings with indomethacin (1 μM) did not modify the magnitude of Ach-induced relaxation. Moreover, the basal $PGI_2$ synthesis of aortic rings did not differ significantly between MI and sham rats at either 7 or 42 days after coronary ligation (in pg/mg: 66.28± 13.2, 101.79±41.9, 85.43±34.96 and 81.5±24.74 for female-MI, female-sham, male-MI and male-sham 42 days after MI, respectively).

At the early phase of post-MI period (7 days) the magnitude of EDHF-dependent relaxation (as assessed by Ach in the presence of indomethacin and L-NAME) was substantially up-regulated in both female (36.24±5.62%) and male MI rats (41.8±5.9%) as



compared to respective sham animals (8.37±2.43% and 14.91±4.79% for female and male rats, respectively). However, at the late phase (42 days) the up-regulation of EDHF-mediated relaxation was seen only in female MI (25.99±4.02%) but not male MI rats (3.79±1.09%).

If KCl (60 mM) was used to preconstrict the vessels instead of phenylephrine, the EDHF-mediated relaxation was abrogated in all experimental groups. Furthermore, the up-regulation of EDHF-pathway was completely blocked by pretreatment with TEA (10 mM) or MICO (10 μM).

**4.3. Diabetes mellitus**

*Development of diabetes after streptozotocin injection*

Four weeks after STZ injection, the plasma glucose (216±16.4 mg/dl) and $HbA_{1c}$ levels (2.2±0.4 %) were significantly elevated as compared to age-matched controls (102.1±13.1 mg/dl and 1.6±0.3 %). Both plasma glucose (298.8±22.6 mg/dl) and $HbA_{1c}$ (3.0±0.2 %) were further increased 8 weeks after the STZ injection. Moreover, 4 and 8 weeks after the STZ injection the volume of 24-hour urine collection was increased significantly in diabetic rats (27.3±6.0 ml and 45.6±8.0 ml for 4 and 8 weeks diabetes, respectively), while the body weight was significantly decreased (221.9±51.2 g and 226.3±41.6 g for 4 and 8 weeks diabetes, respectively) as compared to those of the control rats (8.5±3.1 ml and 10.0±3.6 ml, 319.2±27.3 g and 395.7±19.7 g for 24-hour urine collection and body weight 4 and 8 weeks after vehicle injection, respectively).

*Vascular function in the aorta in diabetic rats*

Relaxation curves induced by Ach were identical in 4 and 8 weeks control animals (Ach $10^{-6}$ M: 71.2±6.1% and 73.1±7.4% respectively). In contrast, there was an impairment of Ach-induced endothelium-dependent relaxation 8 weeks after STZ-injection (Ach $10^{-6}$ M: 56.7±7.9%).

Endothelium-independent relaxation was significantly decreased at a lower concentration range of NaNP 8 weeks after the STZ-injection (NaNP $10^{-8}$ M: 35.9±9.3% and 76.4±9.2% for diabetic and control rats, respectively). However, the maximal response induced by NaNP was similar in control (NaNP $10^{-6}$ M: 104.7±2.7% and 104.4±5.5% for 4 and 8 weeks control rats, respectively) and in diabetic rats (NaNP $10^{-6}$ M: 104.7±4.2% and 103.4±4.8% for 4 and 8 weeks diabetic rats, respectively) irrespective of the age of the animals.

Basal NO production was not different between control (30.4±5.3% and 29.2±5.7% for 4 and 8 weeks control rats, respectively) and diabetic rats (31.4±7% and 31.1±10.7% for 4 and 8 weeks diabetic rats, respectively).

Indomethacin (2 μM) alone did not modify the magnitude of endothelium-dependent relaxation induced by Ach in either control or diabetic rats. The basal production of the total amount of vasodilator COX metabolites (6-keto-$PGF_{1\alpha}$, $PGE_2$, $PGD_2$ and 12-HHT) was significantly higher in diabetic aorta (9.52±1.5 × $10^3$ dpm), as compared with controls (5.51±0.5 × $10^3$ dpm). Indeed, the activity of 6-keto-$PGF_{1\alpha}$ (4.64±0.9 × $10^3$ dpm) and 12-HHT (2.34±0.4 × $10^3$ dpm) were significantly increased 8 weeks after the STZ-injection in the aorta when compared to the corresponding controls (2.23±0.4 × $10^3$ dpm and 1.33±0.2 × $10^3$ dpm for 6-keto-$PGF_{1\alpha}$ and 12-HHT, respectively). Furthermore, the synthesis of vasoconstrictor and platelet aggregator $TxA_2$ was significantly increased in diabetic aorta (1.33±0.1× $10^3$ dpm and 0.87±0.05 × $10^3$ dpm for diabetic and control rats, respectively).

The combined preincubation of aortic rings with L-NAME and indomethacin almost completely blocked the Ach-induced vasodilation in all experimental groups (< 3 % both in diabetic and control rats, respectively).

**5. Discussion**

Taking the results of the present work altogether, the comprehensive analysis of endothelial dysfunction encompassing NO, $PGI_2$ and EDHF revealed important differences in the phenotype of endothelial dysfunction in atherosclerosis, heart failure and diabetes mellitus. In all of these three diseases there was a decrease in NO-mediated vasodilation in aorta, however the phenotype of decreased NO-dependent function was quiet different. Indeed, the impairment of endothelium-dependent and -independent relaxation was demonstrated in atherosclerosis and diabetes mellitus but not in heart failure, while the decrease in basal NO production was shown in atherosclerosis and heart failure but not in diabetes mellitus. Surprisingly, the accompanying changes in basal $PGI_2$ production and EDHF-mediated relaxation were also not similar. In apoE/$LDLR^{-/-}$ mice there was a striking up-regulation of $PGI_2$ and EDHF along the progression of atherosclerosis. In diabetes mellitus only a slight compensatory up-regulation of $PGI_2$ was visible with no up-regulation of EDHF. In heart failure there was no up-regulation of basal $PGI_2$ production, while an up-regulation of EDHF was demonstrated that was transient in males and long-lasting in females. On the basis of our pharmacological analysis we suggest that a cytochrome P-450 metabolite of arachidonic acid, most likely an EET is up-regulated in atherosclerosis and heart failure.



The results of the present study indicate that up-regulation of EDHF-mediated relaxation and basal $PGI_2$ production in aorta may compensate for the decreased NO-dependent relaxation in female apoE/$LDLR^{-/-}$ mice. Our results furthermore suggest that increased EDHF activity is not sufficient to preserve normal endothelium-dependent relaxation in atherosclerosis, therefore it is likely that compensatory up-regulation of EDHF operates in conjunction with NO rather than in stead. After coronary ligation the long-lasting up-regulation of EDHF in females may play an important role in maintaining endothelial function and may serve as a possible explanation for the survival advantage of female patients with heart failure as compared to males reported in clinical trials. Furthermore, the lack of significant up-regulation of EDHF may explain why diabetes is associated with much higher cardiovascular event rate than other diseases with endothelial dysfunction.

Data presented in the present work also suggest that pharmacology of endothelium in atherosclerosis, heart failure and diabetes mellitus could perhaps be tailored differently. Augmentation or potentiation of EDHF-dependent mechanisms for example by ticlopidine could be efficient in restoring vascular homeostasis in atherosclerosis but not in diabetes. In turn, the stimulation of $PGI_2$-dependent mechanism could be an efficient way to reverse endothelial dysfunction as well as to inhibit thrombotic and inflammatory processes in the cardiovascular system in atherosclerosis, but not in heart failure. In the present work it was shown that anti-platelet ticlopidine stimulated EDHF-mediated vasodilation and restored endothelium-dependent relaxation. However, it remains to be established how important is the EDHF-mediated mechanism to the anti-atherosclerotic effect of ticlopidine.

## 6. Publications Related to the Thesis

I. **Csanyi G.**, Bauer M., Dietl W., Lomnicka M., Stepuro T., Podesser BK., Chlopicki S.: Functional alterations in NO, $PGI_2$ and EDHF pathways in the aortic endothelium after myocardial infarction in rats.
*European Journal of Heart Failure* 2006, 8:769-776.

II. Jawien J., **Csanyi G.**, Gajda M., Mateuszuk L., Lomnicka M., Korbut R., Chlopicki S.: Ticlopidine attenuates progression of atherosclerosis in apolipoprotein E and low density lipoprotein receptor double knockout mice.
*European Journal of Pharmacology* 2007, 556:129-135.

III. **Csanyi G.**, Lepran I., Flesch T., Telegdy Gy., Szabo Gy., Mezei Zs.: Lack of endothelium-derived hyperpolarizing factor (EDHF) up-regulation in endothelial dysfunction in aorta in diabetic rats.
*Pharmacological Reports* 2007, 59:447-455.

IV. **Csanyi G.**, Franczyk-Zarow M., Gajda M., Mateuszuk L., Mezei Zs., Jawien J., Gwozdź P., Lomnicka M., Walski M., Wojnar L., Pisulewski PM., Stefan Chlopicki.: Progression of endothelial dysfunction in apoE/LDLR – double knockout mice; relationship with the development of atherosclerotic plaques.
*Atherosclerosis* 2008, Submitted.



## 7. Acknowledgements

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