# Role of vasodilator mediators in endothelial dysfunction

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## 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.
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## Abstracts of Congress Presentations Related to the Subjects of the Thesis

1. **Csanyi G.**, Mezei Zs., Flesch T., Telegdy Gy., Szabo Gy., Lepran I. Vascular dysfunction in streptozotocin-induced diabetic rats (Vaszkuláris diszfunkció lehetséges mechanizmusa cukorbeteg patkányokban). A Magyar Élettani Társaság LXX. Vándorgyűlése, 7-9 June 2006, Szeged, Hungary.

2. **Csanyi G.**, Franczyk M., Jawien J., Gajda M., Pisulewski P., Chlopicki S. Progression of endothelial dysfunction in APOE/LDLR – double knockout mice; relationship with the development of atherosclerosis. International Society for Heart Research, 26$^{th}$ European Section Meeting, 14-17 June 2006, Manchester, United Kingdom.

3. Lomnicka M., Bartuś M., **Csanyi G.**, Bauer M., Dietl W., Poddeser BK., Chlopicki S. Effects of diabetes, hypertriglyceridaemia and coronary ligation on endothelial function in mesenteric small arteries as compared to aorta. France-New EU members 7$^{th}$ meeting, New Frontiers in Basic Cardiovascular Research, 5-8 October 2006, Debrecen, Hungary.

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5. **Csanyi G.**, Bauer M., Dietl W., Drelicharz L., Skorka T., Lomnicka M., Stepuro T., Podesser BK., Chlopicki S. NO, $PGI_2$ and EDHF in endothelial function in animal models of ischemic and non-ischemic heart failure. The Physiological Society; International Workshop on Endothelium: The Determinant of Cardiovascular Health and Disease, 9-12 May 2007, Krakow, Poland.

6. Mezei Zs., **Csanyi G.**, Flesch T., Telegdy Gy., Szabo Gy., Lepran I. The role of vasoactive mediators in the endothelial dysfunction (Vazoaktív mediatorok szerepe a diabétesz mellituszt kísérő endoteliális diszfunkcióban). A Magyar Élettani Társaság LXXI. Vándorgyűlése, 6-8 June 2007, Pécs, Hungary.

# Abbreviations

| | |
|---|---|
| 6-keto-$PGF_{1\alpha}$ | stable metabolite of $PGI_2$ |
| 12-HHT | 12-L-hydroxy-5,8,10-heptadecatrienoic acid |
| AA | arachidonic acid |
| Ach | acetylcholine |
| ADP | adenosine diphosphate |
| Ang II | angiotensin II |
| APC | activated protein C |
| ApoE | apolipoprotein E |
| AT-III | antithrombin III |
| $BH_4$ | tetrahydrobiopterin |
| BK | bradykinin |
| $BK_{Ca}$ | large conductance $Ca^{2+}$-activated $K^+$ channels |
| cAMP | cyclic adenosine monophosphate |
| cGMP | cyclic guanosine monophosphate |
| COX | cyclooxygenase |
| CYP | cytochrom P450 |
| dpm | disintegrations per minute |
| EDHF | endothelium-derived hyperpolarizing factor |
| EDRF | endothelium-derived relaxing factor |
| EETs | epoxy-eicosatrienoic acids |
| eNOS | endothelial nitric oxide synthase |
| ET-1 | endothelin-1 |
| FAD | flavin adenine dinucleotide |
| FMD | flow-mediated vasodilation |
| FMN | flavin mononucleotide |
| $H_2O_2$ | hydrogen peroxide |
| ICAM-1 | intercellular cell adhesion molecule-1 |
| $IK_{Ca}$ | intermediate conductance $Ca^{2+}$-activated $K^+$ channels |
| iNOS | inducible nitric oxide synthase |
| $K^+$ | potassium ion |
| KCl | potassium chloride |
| KIR | inwardly rectifying $K^+$ channel |
| LDLR | low density lipoprotein receptor |
| L-NAME | $N_\omega$-nitro-L-arginine methyl ester |
| LOX | lipoxygenase |
| LV | left ventricule |
| MICO | miconazole |
| MMP | matrix metalloproteinase |
| NaNP | sodium nitroprusside |
| NF-$\kappa$B | nuclear factor-$\kappa$B |
| nNOS | neuronal nitric oxide synthase |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| $O_2^-$ | superoxide anion |
| PBS | phosphate buffered saline |
| $PGD_2$ | prostaglandin $D_2$ |
| $PGE_2$ | prostaglandin $E_2$ |
| $PGF_{2\alpha}$ | prostaglandin $F_{2\alpha}$ |
| $PGI_2$ | prostacyclin |
| PGIS | prostacyclin synthase |
| Phe | phenylephrine |
| PL | phospholipase |
| sGC | soluble guanylate cyclase |
| $SK_{Ca}$ | small conductance $Ca^{2+}$-activated $K^+$ channels |
| SMC | smooth muscle cell |
| SNAP | *S*-nitroso-*N*-acetylpenicillamine |
| STZ | streptozotocin |
| TEA | tetraethylammonium chloride |
| TFPI | tissue factor pathway inhibitor |
| $TxA_2$ | thromboxane $A_2$ |
| $TxB_2$ | thromboxane $B_2$ (stable metabolite of $TxA_2$) |
| VCAM-1 | vascular cell adhesion molecule-1 |

## Contents

## 1. Introduction

The endothelium, that lines the lumina of the entire surface of the vascular tree is often considered as the body's largest "organ", which weighs about 1 kg, representing 1% of body mass (1). In 1980 Furchgott and Zawadzki observed that acetylcholine caused vasodilation or vasoconstriction in the presence or absence of endothelial integrity and 4 years earlier prostacyclin (initially known as prostaglandin X) was discovered (2-4). Since that time the role of endothelium in the regulation of vascular homeostasis has attracted considerable interest. The last two decades of research have established unambiguously that the vascular 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. The endothelium exerts effects on both the circulating blood elements and the surrounding vascular wall that lead to the following: vasodilation or vasoconstriction, synthesis of growth-inhibiting or growth-promoting substances, prothrombotic or antithrombotic and proinflammatory or anti-inflammatory changes. Physiologically, the endothelium maintains a balance between these opposing mechanisms leading to a proper blood supply to tissues, decreased adhesion of platelets and leukocytes to the vessel wall and attenuated smooth muscle cell growth and migration.

The shift in this balance reported in cardiovascular diseases may lead to arterial endothelial dysfunction that plays an important role in the pathomechanism of atherosclerosis, heart failure, hypertension and diabetes mellitus (5,6). Numerous studies have demonstrated that in humans endothelial dysfunction can be detected in the presence of cardiovascular risk factors prior to any morphological changes in the vascular wall (7,8). Importantly, correction of such risk factors may reverse the impairment of endothelium-dependent relaxation (9). Thus, the measurement of endothelium-dependent relaxation may represent a specific barometer of cardiovascular risk (10). Accordingly, the analysis of the phenotype of endothelial dysfunction may have preventive, diagnostic, therapeutic and prognostic significance (11-13).

## 1.1. Normal endothelial cell function

### 1.1.1. Endothelium in the regulation of vascular tone

Endothelial cells contribute to the regulation of blood flow and blood pressure by releasing vasoactive mediators (Table 1). Indeed, the endothelium synthesizes and releases vasodilators, such as nitric oxide (NO), prostacyclin ($PGI_2$) and endothelium-derived hyperpolarizing factor (EDHF) as well as vasoconstrictors, including endothelin-1 (ET-1) and thromboxane $A_2$ ($TxA_2$). In addition, the endothelium contributes to the formation of vasoconstrictor angiotensin II (Ang II) and degradation of vasodilator bradykinin (BK) by expressing the angiotensin converting enzyme (ACE). Importantly, the net effect on the regulation of vascular tone is determined by the complex interaction between these vasodilator and vasoconstrictor substances.

The relative contribution of vasodilator mediators to endothelium-dependent vasodilation is inversely related to vessel diameter, such that NO- and $PGI_2$- mediated responses predominate in conduit vessels, whereas EDHF is more prominent in resistance arteries (14).

**Table 1. Vasoregulatory substances synthesized by the endothelium**

| *Substance* | *Properties* | *Secretion* | *Compound chemistry* | *Precursor* |
|---|---|---|---|---|
| NO | Vasodilator, exerts antiplatelet action, inhibits leukocyte adhesion, inhibits SMC migration and proliferation | Constitutive and inducible expression. Production is increased by Ach, thrombin, shear stress and ADP | Heterodiatomic free radical | *L*-arginine |
| $PGI_2$ | Vasodilator, exerts antiplatelet action | Constitutive and inducible expression. Synthesis is increased by proinflammatory agents | eicosanoid | AA |
| EDHF | Vasodilator, exerts anti-inflammatory action | Induced by Ach, BK and shear stress | EET, anandamide, potassium, $H_2O_2$, current spread or others | various |
| $TxA_2$ | Vasoconstrictor, induces platelet aggregation and proinflammatory alteration | Induced by Ach, hypoxia and shear stress | eicosanoid | AA |
| ET-1 | Vasoconstrictor, induces SMC proliferation | Induced by hypoxia, shear stress and ischemia | 21-amino acid peptide | Preproendothelin-1 |

NO, nitric oxide; $PGI_2$, prostacyclin; EDHF, endothelium-derived hyperpolarizing factor; $TxA_2$, thromboxane $A_2$; ET-1, endothelin-1; SMC, smooth muscle cells; Ach, acetylcholine; ADP, adenosine diphosphate; BK, bradykinin; EET, epoxy-eicosatrienoic acids; $H_2O_2$, hydrogen peroxide; AA, arachidonic acid.

*Nitric oxide*

In the early 1980s, Furchgott and Zawadzki postulated the existence of an endothelium-derived relaxing factor (EDRF), after observing the obligatory vasodilation in response to factors like acetylcholine (Ach) only in the presence of intact endothelium (4). It was subsequently discovered by two independent research groups that EDRF is identical to NO, an odorless gas previously known as an atmospheric pollutant (15-17)

Now it is well known that NO arises from the conversion of *L*-arginine to *L*-citrulline catalyzed by NO synthase (NOS), which exists as three isoforms. Endothelial NOS (eNOS or NOS III) and neuronal NOS (nNOS or NOS I) are termed constitutive NOS and responsible for the continuous synthesis of small amounts of NO.

These two isoform of NOS require a number of cofactors and prosthetic groups for their activity, including $Ca^{2+}$-calmodulin with NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin ($BH_4$) (18-20). While eNOS is essential for the control of vascular tone, the nNOS is important in neurotransmission, the central control of vascular hemostasis, learning and memory (21,22). The third form of NOS is $Ca^{2+}$-calmodulin-independent enzyme and is termed inducible NOS (iNOS or NOS II). This isoform of NOS produces large amounts of NO in response to cytokines, such as interleukin-1 and tumor necrosis factor α (23). Large amounts of NO, made by iNOS is responsible for the development of hypotension and vasoplegia in endotoxic shock (24).

NO has a biological half-life of about 3-5 sec (25), when formed by the endothelium, NO diffuses into the underlying tissues and permeates the smooth muscle cell (SMC) membrane. In the vascular SMCs, NO activates soluble guanylate cyclase (sGC) and increases levels of cyclic guanosine monophosphate (cGMP), which leads to relaxation of vascular smooth muscle via inhibition of calcium entry into the cell, activation of membrane-bound calcium ATPase and activation of cGMP-dependent protein kinase to phosphorylate myosin light chain kinase (26,27).

The basal NO release from endothelial cells produces a constant, active vasodilator tone that antagonizes a variety of vasoconstrictor substances also released from the endothelium (ET-1 and $TxA_2$). The release of NO can be stimulated by various physical stimuli, such as shear stress (28), cyclic circumferential stretch (29) and physical exercise (30). Moreover, endothelial NO production rises in response to hormones (estrogen) (31,32), a variety of circulating agents (Ach and histamine)

(15,33), and platelet-derived (serotonin and ADP) as well as coagulation factors (thrombin) (34).

*Prostacyclin and other vasoactive prostanoids*

Although NO plays a prominent role in the regulation of vascular tone, other endothelium-derived vasoactive substances should be considered. Indeed, the endothelial cells are able to release other vasoactive substances, such as prostaglandins (PGs), thromboxanes, leukotrienes and epoxyeicosatrienoic acids (EETs), commonly known as eicosanoids (35).

The arachidonic acid (AA), a polyunsaturated 20-carbon fatty acid, is the most prominent precursor of eicosanoids. The AA is released from membrane phospholipids through the action of several phospholipases ($PLA_2$, PLC and PLD) and then becomes available for the eicosanoid synthesis.

Free AA serves as a substrate for cyclooxygenases (COX-1, COX-2 and COX-3), lypoxygenases (LOX-5, LOX-12 and LOX-15) or cytochrome P450 (36).

$PGH_2$, the direct product of COXs, has a half-life of about 5 min and causes vasoconstriction. Most tissues express COX-1 constitutively, in contrast COX-2 expression rises upon cell activation, such as in inflammation (36). Then, $PGH_2$ may undergo further enzymatic conversion into vasodilator ($PGI_2$, $PGD_2$, $PGE_2$ and 12-HHT [12-L-hydroxy-5,8,10-heptadecatrienoic acid]) as well as vasoconstrictor ($PGF_{2\alpha}$ and $TxA_2$) prostanoids, depending on the local tissue distribution of specific PG and Tx synthases. Thus, the synthesis of different prostanoids varies among tissues or cells. For example, the endothelial cells primarily contain prostacyclin synthase (PGIS), which catalyze the conversion of $PGH_2$ to $PGI_2$. Once released, $PGI_2$ activates IP receptor, a seven-membrane spanning G-protein-coupled receptor, which leads to the activation of adenylyl cyclase and concomitant increase in intracellular levels of cyclic adenosine monophosphate (cAMP). Prostacyclin decreases vasomotor tone and inhibits platelet aggregation (37). Furthermore, endothelium in some circumstances can synthesize $TxA_2$, a potent vasoconstrictor and platelet aggregator, as proven by the cloning of an endothelial Tx synthase (38). In addition, endothelial cells can restore the production of $TxA_2$ in aspirin-treated platelets via transcellular metabolism of $PGH_2$ (39).

*Endothelium-derived hyperpolarizing factor*

Next to NO and $PGI_2$ as best defined endothelial vasodilating autacoids, numerous studies undoubtedly revealed the existence of a third vasodilating substance. Indeed, endothelium-dependent relaxation was not blocked completely after the pharmacological inhibition or genetic inactivation of NOS and inhibition of $PGI_2$ production, especially in small resistance arteries (40-44). The observation that this NO- and $PGI_2$-independent relaxation involves hyperpolarization of the target SMC, without increasing the intracellular levels of cyclic nucleotides, led to the concept of an "endothelium-derived hyperpolarizing factor" (EDHF) (44).

Since all features of EDHF phenomenon could not be explained by a single molecule or pathway the existence of several distinct EDHFs has been assumed:

*1.)* EETs, metabolites of AA through the cytochrome P450 monooxygenase, in human coronary and subcutaneous arteries and rat aorta (43,45,46)

*2.)* the endogenous cannabinoid anandamide, N-arachidonoyl-ethanolamine in rat mesenteric artery (47,48)

*3.)* potassium ions ($K^+$) in human interlobar renal arteries and rat resistance arteries (49,50)

*4.)* hydrogen peroxide ($H_2O_2$) in mouse and human mesenteric arteries (51,52)

*5.)* myo-endothelial gap junctions in submucosal arterioles of guinea-pigs (53)

*ad 1.)* Fisslthaler *et al.* identified three CYP epoxygenases in porcine coronary endothelium and suggested that only the 2C family, specifically 2C8, met the requirements for an EDHF synthase that produced 11,12-EET (54). It seems, however, different CYPs might be expressed in different tissues and species, resulting in a different profile of EETs. For example, Campbell *et al*. reported that bovine coronary artery endothelial cells generated all four EETs (5,6-, 8,9-, 11,12- and 14,15-EETs, respectively), which were equipotent in relaxing the vessels (55). On the other hand, based on the pharmacological evaluation, 5,6-EET was the most potent vasorelaxing agent in the rat heart and the only EET that could be considered as a candidate for an EDHF (56). Once formed in the endothelial cells, EETs diffuse to the underlying tissues and hyperpolarize the vascular smooth muscle via the opening of large conductance $Ca^{2+}$-activated $K^+$ channels ($BK_{Ca}$, sensitive to iberiotoxin) in the SMC membrane (57). Furthermore, EETs may activate adenylyl cyclase in endothelial cells and lead to

increased cAMP-generation, which may spread to the adjacent SMC via myo-endothelial gap junctions, thus facilitating EDHF-mediated relaxation (58,59)

*ad 2.)* Anandamide can activate at least two different pathways to produce hyperpolarization of the vascular SMCs:

*a,* endothelium-dependent relaxation: mediated by the activation of non-CB1 receptor component that requires pertussis toxin-sensitive G proteins and NO.

*b,* endothelium-independent relaxation: mediated by the activation VR1 vanilloid receptors and possibly by the subsequent release of calcitonin gene-related peptide (60).

*ad 3.)* Numerous studies have provided evidence that $K^+$ may be also a candidate for EDHF (49,50). Endothelial cell stimulation by receptor-dependent (Ach and BK) and receptor-independent ($Ca^{2+}$ ionophore A23187) $Ca^{2+}$-eleveting agonists activates small and intermediate conductance $K_{Ca}$ channels ($SK_{Ca}$ and $IK_{Ca}$ channels, sensitive to apamin and charybdotoxin, respectively) leading to the efflux of $K^+$ from endothelial cells and increased levels of extracellular $K^+$. The accumulation of $K^+$ ions in the sub-endothelial space may activate inwardly rectifying $K^+$ (KIR) channels (sensitive to barium) and/or the $Na^+/K^+$ pumps (sensitive to ouabain) in the SMC membrane, leading to hyperpolarization and relaxation (61-63).

*ad 4.)* The role of $H_2O_2$ as a vasoactive substance is not well understood. Indeed, previous studies reported both contractile (64-66) and relaxant (67-69) response to $H_2O_2$ depending on the species, vascular bed and contractile state. Interestingly, it was also reported that $H_2O_2$ can induce both vasoconstriction and vasodilation on the same vasculature depending on whether KCl or phenylephrine was used to preconstrict the vessel (70). This observation suggests that membrane potential of the vascular smooth muscle and $K^+$ ions may influence the effect of $H_2O_2$ on tone.

*ad 5.)* Gap junctions link the cytoplasmic compartments of neighboring cells, thus allowing a direct exchange of ions and second messengers. Gap junctions not only couple endothelial cells to endothelial cells, and SMCs to SMCs, but also couple SMCs to the endothelium (myo-endothelial gap junctions). Some authors have demonstrated the direct link between the EDHF phenomenon and the spread of an electric current through myo-endothelial gap junctions. For example, after the administration of blockers of gap junctions (18β-glycyrrhetinic acid), Ach elicits an outward current in endothelial cells but no longer in the SMCs, which suggests that the two cell types are connected electrically to each other (71,72).

### 1.1.2. Anticoagulant properties of endothelium

*Endothelial control of platelet function*

Under normal physiologic conditions, circulating platelets do not interact with the endothelial surface, due to the endothelial release of NO and $PGI_2$ and endothelial activity of an ecto-ADPase.

Endothelium-derived NO inhibits platelet activation (adhesion, secretion and aggregation) and clot formation, through a cGMP-dependent mechanism and decreased $Ca^{2+}$-sensitive conformational change in the glycoprotein IIb-IIIa (73,74).

Endothelial $PGI_2$ is the most potent inhibitor of platelet aggregation, which exerts its anti-platelet activity through the activation of platelet IP receptors. $PGI_2$ increases the intracellular level of cAMP, thus maintaining platelets in their inactivated state (75).

The endothelial cells constitutively express ecto-ADPase, a membrane-associated ectonucleotidase, which catalyze the metabolism of ADP, a strong platelet activator to AMP (76). In addition, adenosine synthesized by the vascular endothelium inhibits platelet aggregation (77,78).

*Anticoagulant and fibrinolytic activity*

The endothelial cells synthesize heparan sulfate and related glycosaminoglycans that activate antithrombin III (AT-III). The heparan sulfate-AT III complex inactivates thrombin, factors Xa, IXa and XIa.

Another anticoagulant property of normal endothelium involves the expression of tissue factor pathway inhibitor (TFPI), which binds and inactivates the TF/FVIIa/FXa complex, thus preventing thrombin formation and blood clotting (79).

Endothelial thrombomodulin binds to thrombin, which loses its procoagulant activity and the complex instead becomes an activator of protein C (80). Once formed and dissociated from its receptor (EPCR), the activated protein C (APC) binds to protein S, a molecule synthesized by the liver and endothelium (81). This complex proteolitically inactivates factors Va and VIIIa, controls thrombin production and inactivates the tissue plasminogen activator inhibitor. Finally, the endothelial cells express plasminogen, tissue- and urokinase-type plasminogen activator, thus leading to increased plasmin formation and enhanced fibrinolytic activity (82-84).

### 1.1.3. Endothelium as an anti-inflammatory barrier

The endothelium plays also a key role in the inflammatory processes and participates in host defense mechanisms initiated by injury or infections. Maladaptive inflammatory response of endothelium can promote vascular wall inflammation that is a hallmark of atherosclerosis and other cardiovascular diseases.

NO has been described to decrease the expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1) and E-selectin as well as to protect against leukocyte-endothelium interactions (85,86). It is also known that NO inhibits vascular SMC proliferation and migration (87) and regulate the activity of the transcriptional factor, nuclear factor-$\kappa$B (NF-$\kappa$B) (88). Indeed, NO decreases the activity of NF-$\kappa$B by stabilizing its inhibitor IKB$\alpha$, thus leading to the decreased expression of NF-$\kappa$B-regulated genes encoding for VCAM-1 and ICAM-1 (89).

## 1.2. Endothelial dysfunction

Accumulated data have revealed that major cardiovascular diseases, such as atherosclerosis, heart failure, diabetes mellitus and hypertension are all associated with endothelial dysfunction. Since the actions of endothelium are multiple and involve several systems, one single definition of endothelial dysfunction could not cover the whole array of possible disruption in normal function. Some examples of endothelial dysfunction include decreased basal or receptor-stimulated vasodilatory capacity (43), increased production of vasoconstrictor substances (90) as well as proinflammatory and prothrombotic activation of endothelium (91). In clinical practice, the endothelial dysfunction is most frequently diagnosed on the basis of impaired NO-dependent relaxation (92). Obviously, the decrease in NO-mediated vasodilation does not fully account for the development of impaired vasodilatory capacity, but the reduced $PGI_2$, EDHF and BK activity and the increased synthesis of vasoconstrictors (*e.g.*, $TxA_2$, $PGF_{2\alpha}$, ET-1 and Ang II) may be also involved. Nevertheless assessment of NO-dependent vasodilation is increasingly used in clinical practice to investigate endothelial function, and was shown to predict the development of cardiovascular events (7,93,94).

### 1.2.1. Endothelial dysfunction in atherosclerosis

The impairment of endothelium-dependent relaxation in atherosclerosis is considered as primary endothelial dysfunction since the endothelium is a primary target

for risk factors of atherosclerosis (95). Numerous studies demonstrated a defective NO-dependent relaxation in response to Ach or to increased flow (flow-mediated dilatation, FMD) in patients with cardiac risk factors prior to the development of atherosclerotic plaques (7,8,96,97,98). Shechter *et al.* reported that endothelial dysfunction precedes the development of atherosclerosis in humans and the measurement of brachial artery FMD has a prognostic significance in patients with cardiovascular disease (12). Importantly, the correction of cardiovascular risk factors may reverse the impairment of NO-dependent relaxation (9,98,99). Similarly, endothelial dysfunction was repeteadly demonstrated in different animal models of atherosclerosis, such as hypercholesterolemic rabbits (100,101), pigs (102) and non-human primates (103,104). In contrast, in gene-targeted atherosclerotic mice models it is not yet clear whether the endothelial dysfunction is a hallmark of atherogenesis. The impairment of endothelium-dependent relaxation was demonstrated in apoE$^{-/-}$ mice fed Western diet (105,106), in LDLR$^{-/-}$ mice fed Western diet (107) and apoE/LDLR double knockout mice on both chow (108) and Western diet (109). On the other hand, it was reported that in apoE$^{-/-}$ mice fed chow diet the endothelial dysfunction is absent despite the morphological evidence of atherosclerosis (108,110-112), and occurs only at more pronounced stage induced by Western type diet (105,112). Moreover, Crauwels *et al*. demonstrated that endothelial dysfunction is present only in the atherosclerotic but not in the adjacent plaque-free segments and suggested that endothelial dysfunction does not develop in intact arteries despite severe hypercholesterolemia (113).

The results showing that endothelial dysfunction is not detected in apoE$^{-/-}$ mice unless atherogenesis is accelerated by combination with LDL receptor deficiency or Western type diet question the primary role of endothelial dysfunction in gene-targeted mice models of atherosclerosis. Indeed, it is still not known whether the impairment of NO-dependent relaxation precedes the development of atherosclerosis in murine aorta. Furthermore, majority of previous studies investigated only the NO-dependent relaxation in gene-targeted mice models of atherosclerosis and there is no report on the simultaneous alterations of NO, $PGI_2$ and EDHF pathways along the development of atherosclerotic plaques.

On the other hand, there are accumulating data suggesting that activated platelets are important players in the initiation and progression of atherosclerosis (114,115). Gawaz *et al*. recently reviewed the interactions among platelets, leukocytes and endothelium that may trigger autocrine and paracrine activation processes leading to

endothelial inflammation and accelerated atherogenesis (116). Surprisingly, our knowledge about the anti-atherosclerotic effects of antiplatelet drugs, such as aspirin and thienopyridines, is limited and sometimes contradictory (117,118). Cyrus *et al.* reported that aspirin at a dose that selectively inhibits COX-1 in platelets blunt platelet activation, vascular inflammation and progression of atherosclerosis in $LDLR^{-/-}$ mice (119). In contrast, Cayatte *et al.* demonstrated that aspirin did not decrease aortic root lesions and serum ICAM-1 levels in $apoE^{-/-}$ mice (117). Furthermore, it is still not known whether the thienopyridine ticlopidine, an antagonist of the $P2Y_{12}$ subtype of the ADP receptor in platelets (120), yield protection against the progression of atherosclerosis in gene-targeted mice model of this disease.

### 1.2.2. Endothelial dysfunction in heart failure

Heart failure is accompanied by a generalized increase in peripheral vascular resistance. Both the impairment of endothelium-dependent relaxation and increased vasoconstrictor capacity may contribute to the increased peripheral resistance in heart failure. In contrast to atherosclerosis, the impairment of endothelium-dependent relaxation in heart failure is considered as secondary endothelial dysfunction since any alteration in the peripheral endothelial function is the consequence of altered cardiac function and neurohormonal activation (121,122).

However, the rat coronary ligation model has been widely used to investigate the endothelial function in ischemic heart failure, it is still not known whether the endothelium-dependent relaxation is impaired or not in this model. Indeed, some authors have reported impaired (123,124), while others preserved (125) or augmented (126) endothelium-dependent relaxation after coronary artery ligation. These discrepancies between previous studies may be explained by using different animals (race and strain) or by assessing the vascular function in different types of vessels (aorta, mesenteric and renal artery) at different time points (early or late) after the coronary artery ligation. Interestingly, the assessment of endothelial function did not yield similar results even if comparable experimental conditions were used (rat aorta, 10-week after coronary ligation), which suggest the involvement of additional factors, such as infarct-size (small vs. large) or the gender of the animals (125,127). Furthermore, to our knowledge, there is no report about the comprehensive analysis of NO-, $PGI_2$ and EDHF-dependent vasodilation in the thoracic aorta from female and male rats early and late after the coronary artery ligation.

### 1.2.3. Endothelial dysfunction in diabetes mellitus

Whereas some of the earlier reports showed normal (128) or even enhanced (129,130) endothelium-dependent vasodilation, the impairment of endothelium-dependent relaxation has been repeatedly demonstrated in various vascular beds of chemically-induced or genetic models of diabetes mellitus (131-133) as well as in patients with type I or type II diabetes (134,135). Despite numerous studies, the exact mechanisms that lead to the development of endothelial dysfunction in diabetes are still not known. The impairment of NO-dependent vasodilation in diabetes may arise from several mechanisms, such as reduced NOS activity, decreased substrate and co-factor availability (L-arginine and tetrahydrobiopterin) for NOS, enhanced inactivation of NO (increased oxidative stress) and decreased sGC sensitivity to NO (136-141). At the same time, some of the previous studies failed to confirm the above mechanisms (136-144). Furthermore, the decrease in NO-mediated vasodilation in diabetes does not fully explain the impairment of endothelium-dependent vasodilation. Indeed, the reduced $PGI_2$ and EDHF activity (145,146), as well as the increased synthesis of $TxA_2$, $PGF_{2\alpha}$ and ET-1 may be also involved (147-149). Most of the previous studies, however, have investigated only the changes in the relative contribution of NO to endothelial function (150,151) and there are only a few reports concerning the role of prostanoids (145) and EDHF (146) in diabetes.

## 1.3. The aims of the present work

Taking into consideration the gaps in the knowledge, 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 antiplatelet therapy (ticlopidine) on the progression of endothelial dysfunction 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.

## 2. Materials and methods

### 2.1. Materials

The materials used in the experiments were of the following origins: sodium thiopental (Biochemie GmbH, Kundl–Rakusko, Austria); pentobarbitone (Ceva-Phylaxia, Budapest, Hungary); fraxiparine (Sanofi-Synthelabo, Santea, France); 12-L-hydroxy-5,8,10-heptadecatrienoic acid and 12-hydroxy-5,8,10,14-eicosatetraenoic acid unlabeled standards, arachidonic acid (grade I), Cy3-conjugated anti-smooth muscle α-actin, indomethacin, Medium 199 tissue culture, Meyer's hematoxylin, miconazole, $N_\omega$-nitro-L-arginine methyl ester hydrochloride, oil red-O, phenylephrine hydrochloride, rofecoxib, streptozotocin, Sudan IV, tetraethylammonium chloride, ticlopidine, (Sigma-Aldrich, St. Louis, Mo, USA); acetylcholine chloride, sodium nitroprusside (Fluka, Seelze, Germany); calcium chloride, glucose, magnesium sulphate, potassium chloride, potassium phosphate monobasic, sodium chloride, sodium hydrogen carbonate, (Molar Chemicals KFT, Budapest, Hungary); optimal cutting temperature compound (CellPath, Oxford, UK); rat anti-mouse CD68 (Serotec, Oxford UK); rabbit anti-CD3 (Calbiochem, Darmstadt, Germany); goat anti-rat IgG biotinylated antibody, goat anti-rabbit Cy2-conjugated antiserum (Jackson IR, West Grove, PA USA); AMCA-conjugated streptavidin (Vector Burlingame, CA, USA); silica gel thin-layer plates (0.25 mm) (Merck AG, Darmstadt, Germany); 1-[$^{14}$C]-arachidonic acid (spec. act. 2035 MBq/mM) (Amersham, UK); prostaglandin $E_2$, prostaglandin $D_2$, thromboxane $B_2$, prostaglandin $F_{2\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$ (Upjohn Co, Kalamazoo, USA); chloroform, methanol (Reanal, Hungary); pyruvic acid (Serva, Heidelberg); glucose, ethylenediaminetetraacetic acid, (Polskie Odczynniki Chemiczne, Polish Chemical Compounds, Gliwice, Poland).

Phenylephrine, acetylcholine, sodium nitroprusside and potassium chloride were prepared as stock solutions in distilled water. Indomethacin and $N_\omega$-Nitro-L-arginine methyl ester were dissolved freshly in $NaHCO_3$ (0.6 M) and Krebs-Heinseleit solution, respectively.

### 2.2. Animals

The experiments were conducted according to the Guidelines for Animal Care and Treatment of the European Community and were approved by the local animal ethics commities (Medical Univerities of Szeged, Krakow and Vienna). The investigation

conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### 2.2.1. Animal model of atherosclerosis

Female apoE/LDLR$^{-/-}$ mice of a C57BL/6J x 129/SvJ background (n=33, weighing 22±0.5 g) and wild type C57BL/6J mice (n=20, weighing 23±0.6 g) were obtained from Taconic (Ejby, Denmark). The mice were maintained on 12-h dark/12-h light cycles in air-conditioned rooms with acces to standard chow diet and water *ad libitum*. At 2, 4, 6 and 8 months of age apoE/LDLR$^{-/-}$ mice and age-matched C57BL/6J mice were used to investigate the relationship between the progression of endothelial dysfunction and development of atherosclerotic plaques in the thoracic aorta. Moreover, we characterized the changes in the relative contribution of NO-, $PGI_2$- and EDHF-pathways to endothelium-dependent relaxation in relation to the progression of atherosclerosis.

We used female apoE/LDLR$^{-/-}$ mice to investigate the effects of ticlopidine-treatment on vascular function and progression of atherosclerosis in thoracic aorta. At the age of 8 weeks apoE/LDLR$^{-/-}$ mice were put on a Western diet (consisting of 21% fat by weight, 0.15% cholesterol by weight and no cholic acid; Sniff, Soest, Germany) 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. Six-month-old mice were used for vascular experiments.

### 2.2.2. Animal model of heart failure

Male (n=25, weighing 300±20 g) and female (n=25, weighing 200±20 g) Sprague–Dawley rats (Core Unit of Biomedical Research Himberg of the Medical University of Vienna) were used to analyze the alterations in NO-, $PGI_2$- and EDHF-dependent endothelial function in the thoracic aorta 7 and 42 days after coronary ligation. The rats were housed in an isolated room at controlled temperature with free acces to food and drinking water. Coronary artery ligation was performed according to Pfeffer *et al.* (152). Sham operated rats underwent the same surgical procedure, except coronary ligation. The surviving rats were randomly assigned to be sacrificed at day 7 (4 female and 4 male) or 42 days after MI (4 female and 5 male), while 15 rats (7 female and 8 male) served as sham controls.

### 2.2.3. Animal model of diabetes mellitus

Male Sprague-Dawley rats (weighing 190±10 g) were randomized to receive a single iv injection of streptozotocin (STZ, 65 mg/kg body weight, dissolved in 1 ml of citrate buffer) (diabetic group, n=16) or vehicle (1 ml of citrate buffer) (control group, n=16). We analyzed diabetes-induced alterations in NO-, $PGI_2$- and EDHF-dependent relaxation in the aorta 4 and 8 weeks after the injection.

## 2.3. Model quantifications

### 2.3.1. Atherosclerosis

*Quantification of atherosclerosis in aortic roots (cross-section analysis)*

Fifteen minutes before anaesthesia (thiopental; 100 mg/kg body weight, ip) 1000 IU of fraxiparine was injected into the peritoneum, then mice were sacrificed by cervical dislocation. The heart and whole aorta were perfused by phosphate buffered saline (PBS, pH 7.4) at a constant pressure of approximately 100 mm Hg. Next, the excised heart and the ascending aorta were embedded in optimal cutting temperature (OCT) compound and snap-frozen. Ten micrometer-thick cryosections were cut from the aortic root (153) and after fixation (4% paraformaldehyde; pH 7.0) Meyer's hematoxylin and oil red-O (ORO) were used to stain the sections. Total area of the lesion was measured semiautomatically in each slide using LSM Image Browser 3 software (Zeiss, Jena, Germany). For each animal eight sections were used to calculate a mean lesion area, reflecting the cross section area covered by atherosclerotic lesions (154).

*Quantification of atherosclerosis in descending aorta (en face analysis)*

After removal of connective tissue, the whole descending aorta (from arch to bifurcation) was isolated and fixed in 4% formaldehyde. The longitudinally opened aorta was pinned onto brown wax plates and stained with Sudan IV. The Aphelion software was used to calculate the aortic lesion area and total aortic area.

*CD68 immunostaining*

Sections were cut from the proximal 1 mm of the aortic root. Acetone-fixed aortic sections were incubated overnight with primary antisera: Cy3-conjugated anti-smooth muscle α-actin, rat anti-mouse CD68 and rabbit anti-CD3. Next, goat anti-rat IgG biotinylated antibodies, goat anti-rabbit Cy2-conjugated antiserum and

AMCA-conjugated streptavidin were used. Epifluorescence Olympus BX50 microscope (with appropriate filter cubes) was used to show Cy3 (red), Cy2 (green) and AMCA (blue) fluorescence for smooth muscle α-actin, CD3 and CD68 respectively. In each section, the total area occupied by CD68-immunopositive macrophages was measured manually using LSM Image Browser softwer.

*Analysis of total cholesterol and triglyceride levels*

For plasma lipoprotein measurements, blood was collected after fasting for 8 hours from the right ventricule. Plasma was separated by centrifugation at 1000 g at 4 °C for 10 min. and were immediately frozen and stored at -80 °C. The lipid profile was analyzed using commercially available kits for HDL-, LDL-, (Olympus Diagnostica GmbH, Hamburg, Germany) total-cholesterol and triglycerides (CORMAY, Lublin, Poland).

### 2.3.2. Heart failure

*In vivo haemodynamic measurements*

Before the coronary artery ligation and 42 days post-MI, in vivo haemodynamic measurements were determined by transthoracic echocardiography. Imaging was performed under light anaesthesia and spontaneous respiration (xylazin 10 mg/kg and ketamin 100 mg/kg bodyweight, respectively) with a 7.5 MHz standard paediatric transducer connected to an echocardiographic computer console (VINGMEDSound, CFM 800, software version 1.0) (155).

The interrogation depth was set at 4 cm. A parasternal long axis view was followed by a parasternal short axis view. Level of the left ventricle in 2-dimensional echocardiography had been obtained M-mode was added for measurement of left ventricular dimensions at end-systole and end-diastole. Fractional shortening of left ventricule diameters was calculated.

*Determination of infarct-size*

Seven or 42 days after coronary artery ligation, the anaesthetized (thiopental; 120 mg/kg body weight, ip) rats were sacrificed by cervical dislocation. After opening the thorax, the heart was quickly removed, blotted to dry and weighed to determine the total heart weight. Ventricles were separated from the atria, the right ventricule was removed from the left ventricule (LV), and each part was weighed. The infarcted area was isolated from the intact LV, then the infarct-size was expressed as the ratio of infarcted

region to total LV mass. Importantly, animals with an infarct size more than 30% of LV were used for the experiments.

### 2.3.3. Diabetes mellitus

*Serum glucose and $HbA_{1c}$ analysis*

Four or eight weeks after diabetes induction, fasted blood samples were taken from the aortic bifurcation of anaesthetized rats (pentobarbitone, 60 mg/kg body weight, ip). Plasma glucose (Human GmbH, Wiesbaden, Germany) and $HbA_{1c}$ levels (BIO-RAD Laboratories GmbH, Germany) were determined according to the manufacturer's instructions. Furthermore, the development of diabetic state was validated by body weight measurement and 24-hour urine collection.

## 2.4. Analysis of eicosanoids in aortic rings

### 2.4.1. Determination of basal prostacyclin production by enzyme immunoassay

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

### 2.4.2. 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. The cells are not activated by such a low concentration of arachidonic acid. Thirty minutes later, the enzyme reaction was stopped by bringing the pH of the incubation mixture to 3 with formic acid. 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. The plates were developed to a distance of 15 cm in the organic phase of ethyl acetate : acetic acid : 2,2,4-trimethylpentane : water (110:20:30:100) by means of overpressure thin-layer

chromatography (Chrompres 25, Labor MIM, Hungary) (156). Each 3-mm band of the chromatograms was then scraped off and the radioactivity was determined by liquid scintillation analyzer (TRI-CARB 2100TR, Canberra Packard, USA), using 5 ml toluene containing 0.44% (w/v) of 2,5-diphenyloxazole, 0.02% (w/v) of 1,4-di-[2-(5-phenyl)oxazoyl]benzene and 10% (v/v) of ethanol. 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 (157). Prostacyclin and $TxA_2$ were determined as their stable metabolites (6-keto-$PGF_{1\alpha}$ and $TxB_2$, respectively). Assuming that the exogenously administered 1-[$^{14}$C]arachidonic acid, as a tracer is converted in the same way as the endogenous source, our method allows to measure the relative amount of various prostanoids.

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

At sacrifice, the descending thoracic aorta was quickly removed, and after washing with ice-cold saline it was placed in cold, freshly prepared Krebs-Heinseleit solution 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); pH 7.4. 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 that was continuously bubbled with 95% $O_2$/5% $CO_2$ mixture. Rings were mounted between 2 hooks attached to an isometric force transducer (Szeged: UTC 2, Gould Statham USA and Krakow: Biegastab K30 type 351; Hugo Sachs March-Fr, Germany) connected to a recorder (Szeged: Data Acquisition System, Powerlab 8SP, AD Instruments Great Britain and Krakow: Graphtec WR3320, UK) for continuous recording of tension. After 1-h equilibration under a resting tension of 7.35 mN (mouse aorta) or 39.2 mN (rat aorta), viability of the vessels was verified as the magnitude of vasoconstriction induced by potassium chloride (KCl, 30 or 60 mmol/l for mice and rat aortic rings, respectively).

### 2.5.1. 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).

### 2.5.2. 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.

### 2.5.3. 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).

## 2.6. 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 were established by modified Student $\underline{t}$ test (158). $p<0.05$ was considered statistically significant.

# 3. Results

## 3.1. Atherosclerosis

### 3.1.1. Progression of atherosclerosis in the thoracic aorta of apoE/LDLR$^{-/-}$ mice

*Development of hypercholesterolemia*

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 elevated already in 2-month-old apoE/LDLR$^{-/-}$ mice and remained approximately at the same level in 4-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice. In contrast, triglyceride level remained unchanged irrespectively of the age of the animals (1.75±0.16, 0.9±0.11, 1.51±0.11 and 1.46±0.06 mmol/l for 2-, 4-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice, respectively). Neither cholesterol nor triglyceride levels were altered in age-matched C57BL/6J mice. For comparison lipid profile of 6-month-old C57BL/6J mice is also shown (1.85±0.33, 0.59±0.2 and 1.21±0.2 and 0.89±0.25 mmol/l for total, LDL- and HDL-cholesterol and triglyceride levels, respectively) (Figure 1).

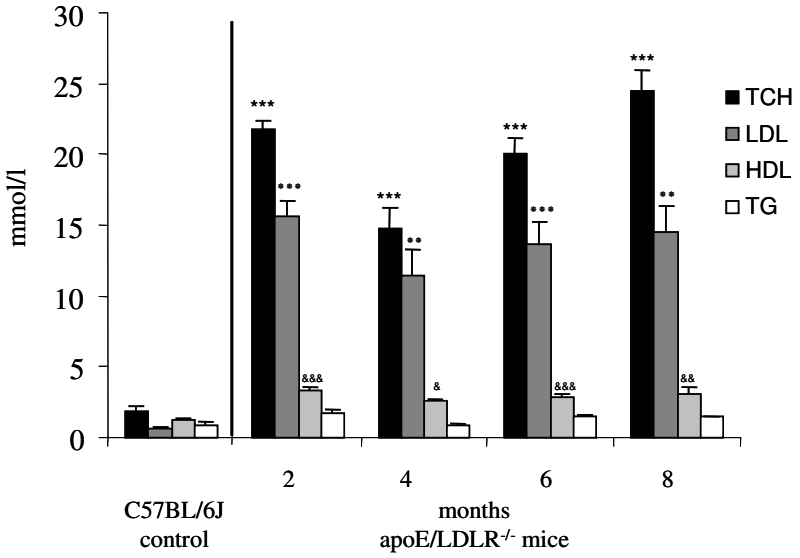



**Figure 1. Elevation of cholesterol level in apoE/LDLR$^{-/-}$ mice**

Plasma liporotein concentrations in 6-month-old C57BL/6J (n=12) and 2-, 4-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice (n=11, 7, 12 and 8, respectively) fed a normal diet. Data represent mean±SEM. *** indicates a significant difference ($p<0.001$) in total cholesterol levels (TCH) when apoE/LDLR$^{-/-}$ mice of different age was compared with C57BL/6J mice. ** and *** indicate a significant difference ($p<0.01$ and $p<0.001$) in LDL levels when apoE/LDLR$^{-/-}$ mice of different age was compared with C57BL/6J mice. $^{\&}$, $^{\&\&}$ and $^{\&\&\&}$ indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$) in HDL levels when apoE/LDLR$^{-/-}$ mice of different age was compared with C57BL/6J mice.

Figure 2/A

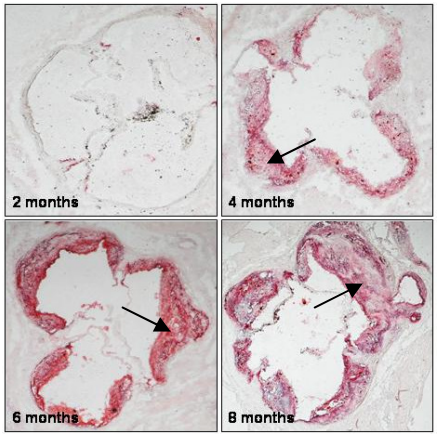



Figure 2/B

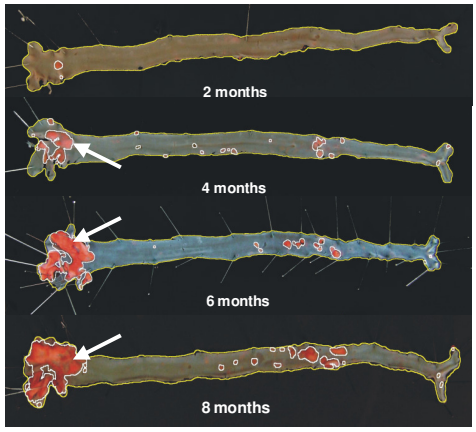
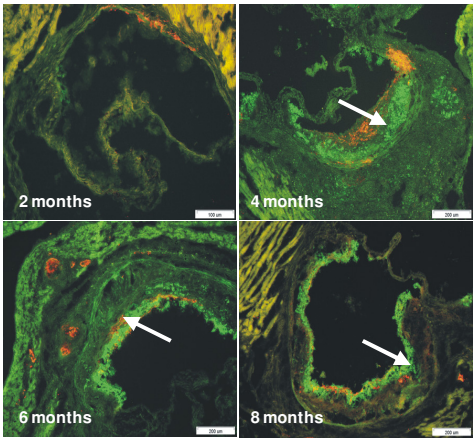



**Figure 2. Progression of atherosclerosis in the thoracic aorta in apoE/LDLR$^{-/-}$ mice**

2/A. Representative photomicrographs showing oil red-O-stained lesions (arrows) in aortic roots in 2-, 4-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice

2/B. Sudan IV-stained lesions (arrows) in whole aorta from 2-, 4-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice

Figure 2/C




2/C. Representative images showing progressive macrophage accumulation (arrows) in the subendothelial space of aortic roots in 2-, 4-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice. (magnification 4x)

Figure 2/A, 2/B and 2/C are not published results. Department of Experimental Pharmacology, Chair of Pharmacology, Jagiellonian University Medical College and Chair of Histology, Jagiellonian University Medical College, Krakow, Poland. With permisson.

*Atherosclerotic plaque development*

Macrophages were absent and atherosclerotic lesions were not visible in the aorta in C57BL66J mice. Atherosclerotic plaques were nearly absent in 2-month-old apoE/LDLR$^{-/-}$ mice, irrespective if we measured in the aortic root (cross-section) (Figure 2/A) or in the whole aorta (en-face) (Figure 2/B). However, in 4-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice the progression of atherosclerosis was visible by cross-section (Figure 2/A) and en-face analysis (Figure 2/B). Furthermore, CD68-immunopositive macrophages were scarce in the subendothelial space of aortic roots isolated from 2-month-old apoE/LDLR$^{-/-}$ mice, while the stained macrophages were abundant at later stage of plaque development (Figure 2/C).

### 3.1.2. Vascular function in the aorta from apoE/LDLR$^{-/-}$ mice

*NO-dependent function*

Endothelium-dependent relaxation was similar in control C57BL/6J mice, irrespectively of the age of the animals (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). Moreover, endothelium-independent relaxation induced by SNAP was also similar and maximal relaxation ($10^{-6}$ M) amounted to approximately 100 % (103,96±3,99% for 6-month-old C57BL/6J mice, respectively). In contrast, in apoE/LDLR$^{-/-}$ mice as early as at the age of 2 months the Ach-induced endothelium-dependent relaxation was impaired as compared to 6-month-old C57BL/6J mice (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 (Figure 3). 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%).

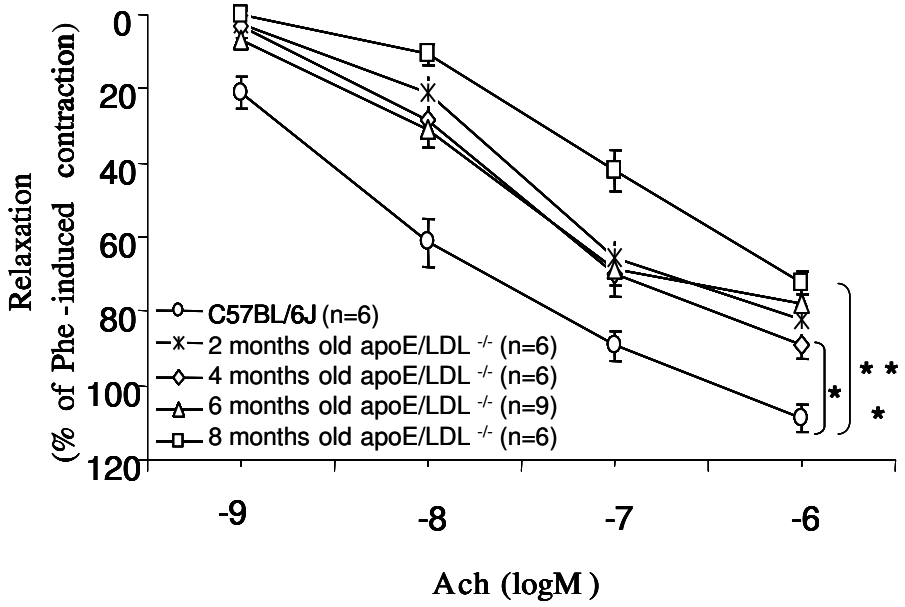



**Figure 3. Impairment of endothelium-dependent relaxation in apoE/LDLR$^{-/-}$ mice**

Concentration-response curves for acetylcholine-induced relaxation in aortic rings from 6-month-old C57BL/6J and 2-, 4-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice. Data represent mean±SEM. *p<0.05 indicates a significant difference in Ach-induced ($10^{-6}$ M) relaxation between C57BL/6J and 4-month-old apoE/LDLR$^{-/-}$ mice. ***p<0.001 indicates a significant difference in Ach-induced ($10^{-6}$ M) relaxation between C57BL/6J and 2-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice.

Basal NO production, determined as the magnitude of L-NAME-induced vasoconstriction (300 µM) was diminished in 2- and 4-month-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).

*COX-dependent endothelial function*

The preincubation with indomethacin, a non-selective COX inhibitor, did not modify the magnitude of Ach-induced relaxation in either control or apoE/LDLR$^{-/-}$ mice. The basal $PGI_2$ production of aorta was slightly increased with age in control mice (in pg/mg: 145.8±52.4, 160.9±71.3 and 202.7±46.3 for 2-, 4- and 6-month-old C57BL/6J mice, respectively). The age-dependent up-regulation of $PGI_2$ synthesis was more pronounced in apoE/LDLR$^{-/-}$ mice (in pg/mg: 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). The up-regulation of basal $PGI_2$ synthesis was blocked by indomethacin (in pg/mg: 26.4±9.1, 43.4±9.38, 40.1±17,5 and 49.2±10.8 for 1-, 2-, 4- and 6-month-old apoE/LDLR$^{-/-}$ mice, respectively). The COX-2 inhibitor rofecoxib partially blocked the up-regulation of $PGI_2$ synthesis in aorta in apoE/LDLR$^{-/-}$ mice (in pg/mg: 151.9±72.2, 135.4±69.1, 505±339.5 and 783.4±370.8 for 1- and 2-, 4- and 6-month-old apoE/LDLR$^{-/-}$ mice, respectively) (figure 4).

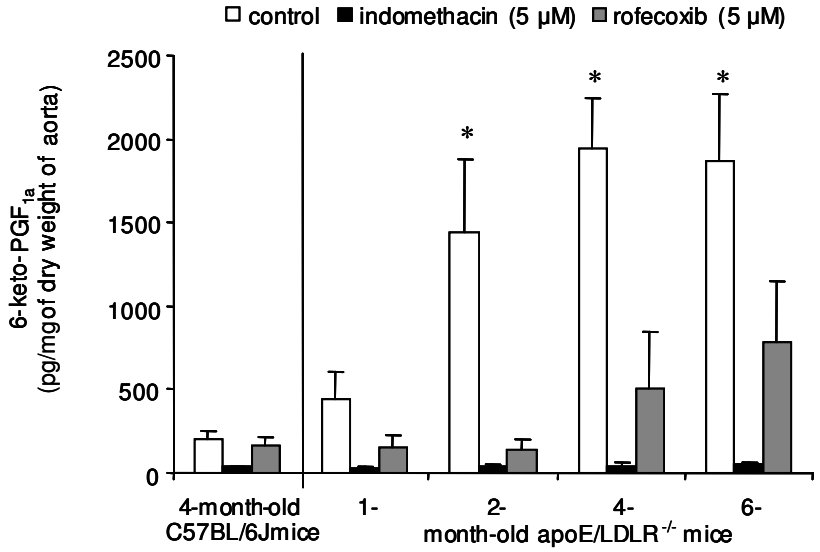



**Figure 4. Age-dependent up-regulation of basal $PGI_2$ synthesis in apoE/LDLR$^{-/-}$ mice**

Basal $PGI_2$ (determined as its stable metabolite 6-keto-$PGF_{1\alpha}$) production in aortic rings in 4-month-old C57BL/6J and 1-, 2-, 4- and 6-month-old apoE/LDLR$^{-/-}$ mice. The enzymatic source of $PGI_2$ was analyzed by non-selective COX or selective COX-2 inhibitors such as indomethacin (5 µM) or rofecoxib (5 µM), respectively. Data represent mean±SEM for n=5. *$p<0.05$ indicates a significant difference between C57BL/6J and 2-, 4- and 6--month-old apoE/LDLR$^{-/-}$ mice.

*EDHF-dependent relaxation*

The EDHF-mediated vasodilation in the aorta was assessed on the basis of Ach response elicited in the presence of NOS inhibitor L-NAME (300 µM, 15 min) and indomethacin (5 µM, 30 min). In control C57BL/6J mice, the Ach-induced vasodilation was nearly completely blocked after the combined preincubation with L-NAME and indomethacin. In 2-, 4- and 6-month-old C57BL/6J mice the EDHF-component of Ach-induced vasodilation ($10^{-6}$ M) amounted to 5.79±3.71%, 6.57±4.18% and 4.74±2.45%, respectively.

In contrast, in apoE/LDLR$^{-/-}$ mice the EDHF-dependent relaxation was gradually up-regulated starting from 2-month-old mice. The magnitude of Ach-induced response ($10^{-6}$ M) in the presence of L-NAME and indomethacin amounted to 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 (figure 5).

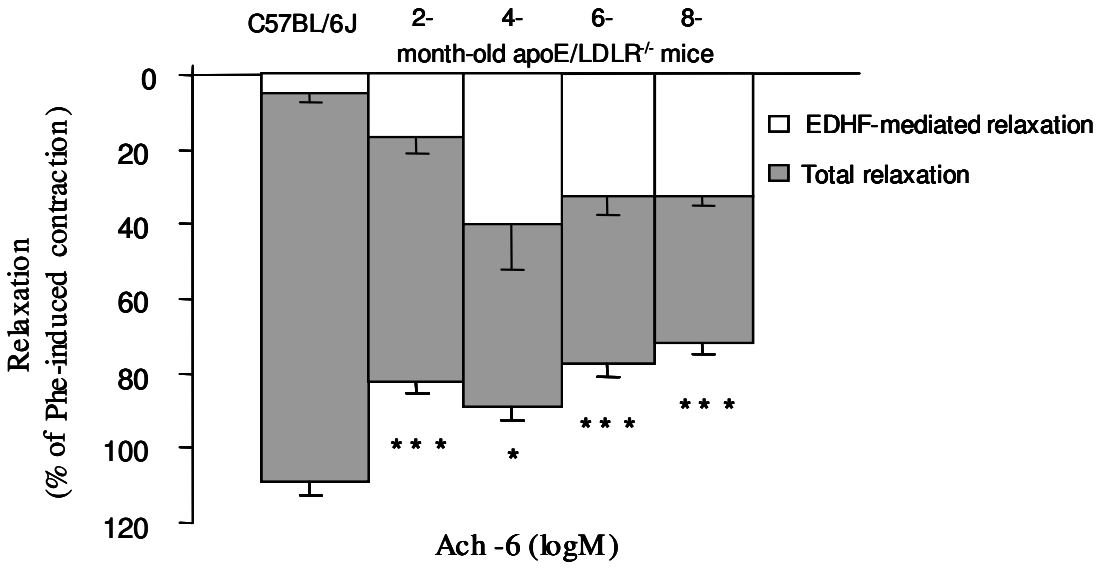



**Figure 5. Up-regulation of EDHF-mediated relaxation in apoE/LDLR$^{-/-}$ mice**

Grey bars show the total relaxation induced by Ach ($10^{-6}$ M) in aortic rings from 6-month-old C57BL/6J and 2-, 4-, 6- and 8-month old apoE/LDLR$^{-/-}$ mice. Data represent mean±SEM for n=6-9. *p<0.05 indicates a significant difference in Ach-induced ($10^{-6}$ M) relaxation between C57BL/6J and 4-month-old apoE/LDLR$^{-/-}$ mice. ***p<0.001 indicates a significant difference in Ach-induced ($10^{-6}$ M) relaxation between C57BL/6J and 2-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice. White bars show the up-regulation of EDHF-mediated relaxation (Ach in the presence of indomethacin and L-NAME) along the progression of atherosclerosis. *p<0.05 indicates a significant difference between C57BL/6J and 4-, 6- and 8-month-old apoE/LDLR$^{-/-}$ mice (n=6-9).

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. In addition, 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 also blocked the EDHF-dependent vasodilation in the thoracic aorta (figure 6).

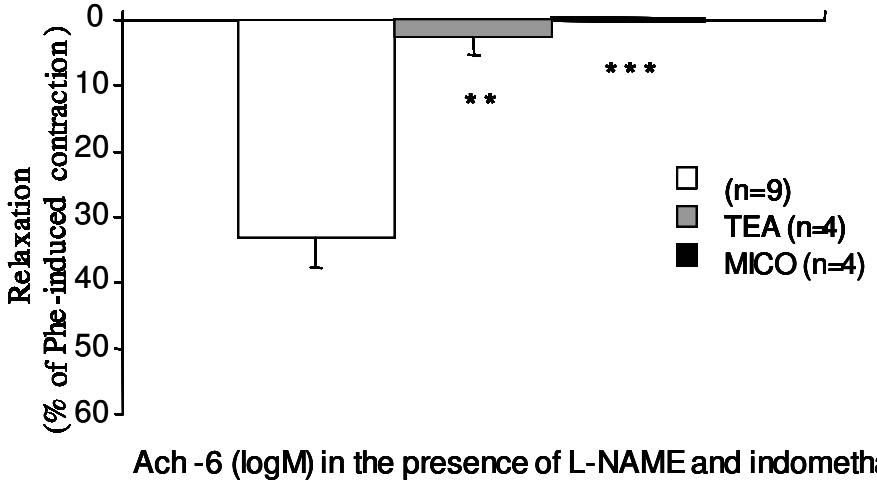



**Figure 6. The role of cytochrome P450 metabolites and potassium channels in the mechanism of EDHF-mediated relaxation in apoE/LDLR$^{-/-}$ mice**

The remaining EDHF-mediated (white bar) relaxation [Ach $10^{-6}$ M after L-NAME (300 μM) and indomethacin (5 μM)] was abolished after incubation with the antagonist of $Ca^{2+}$-activated $K^+$ channels tetraethylammonium chloride (TEA, 10mM) (grey bar) or the inhibitor of epoxyeicosatrienoic acid synthesis miconazole (MICO, 10 μM) (black bar) in aortic rings from 6-month-old apoE/LDLR$^{-/-}$ mice. Data represent mean±SEM**.** **p<0.01 and ***p<0.001 indicate significant differences between treatments with and without TEA and MICO, respectively.

### 3.1.3. Effects of ticlopidine on the progression of atherosclerosis

*Plasma lipids in ticlopidine-treated apoE/LDLR$^{-/-}$ mice fed on Western diet*

The total cholesterol and triglycerides in 6-month-old apoE/LDLR$^{-/-}$ mice treated with ticlopidine for 4 months (90 mg/kg/day) 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].

*Development of atherosclerosis in ticlopidine-treated apoE/LDLR$^{-/-}$ mice*

The effect of ticlopidine treatment on the progression of atherosclerosis in the whole aorta of apoE/LDLR$^{-/-}$ mice (en face analysis) was not evident (25.15±2.9% and 21.5±5.71% of aorta in the non-treated and the ticlopidine-treated group, respectively, p=0.0617). In contrast, 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 $10^5$ μm$^2$ vs. 3.08±0.33 $10^5$ μm$^2$ in the non-treated and the ticlopidine-treated group, respectively, p<0.05).

### 3.1.4. Effects of ticlopidine on endothelial function in thoracic aorta from apoE/LDLR$^{-/-}$ mice

*NO-dependent function*

Acetylcholine-induced endothelium-dependent vasodilation in the thoracic aorta from 6-month-old apoE/LDLR$^{-/-}$ mice was significantly impaired (Ach $10^{-5}$ M: 78.86±2.6%). Ticlopidine-treatment markedly improved the Ach-induced relaxation in 6-month-old apoE/LDLR$^{-/-}$ mice (Ach $10^{-5}$ M: 93.2±2.1%) (Figure 7).

The magnitude of endothelium-independent vasodilation induced by SNAP was similar between ticlopidine-treated and non-treated apoE/LDLR$^{-/-}$ mice (SNAP $10^{-5}$ M: 115.16±11.2% and 103.32±1.5% for non-treated and ticlopidine-treated group, respectively). Basal NO production, as measured by the magnitude of L-NAME-induced (300 μM) contraction was similar in ticlopidine-treated and non-treated apoE/LDLR$^{-/-}$ mice (26.37±5.9% and 23.09±5.6% for non-treated and the ticlopidine-treated group, respectively).

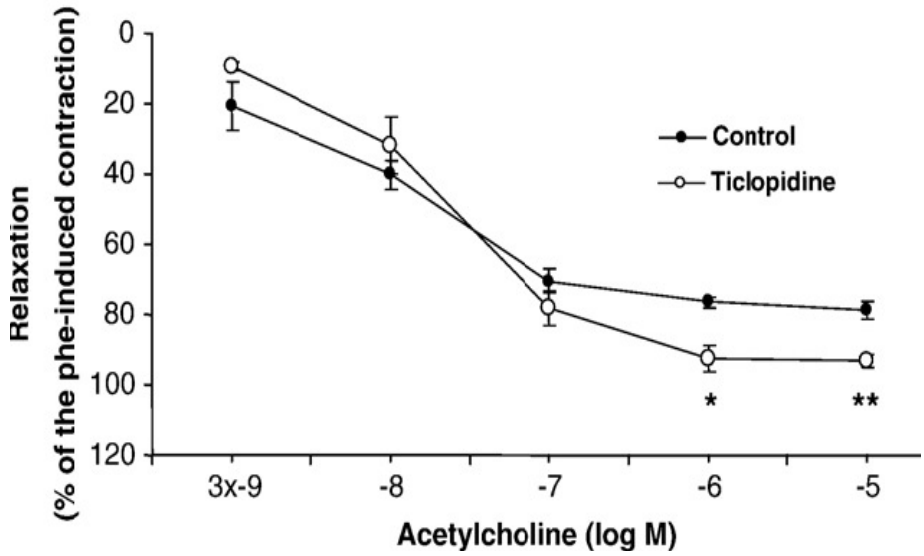



**Figure 7. Ticlopidine improved endothelium-dependent relaxation in aorta in apoE/LDLR$^{-/-}$ mice**

Concentration-response curves for acetylcholine-induced relaxation in aortic rings from ticlopidine-treated (90 mg/kg/day for 4 months) and non-treated apoE/LDLR$^{-/-}$ mice. Data represent mean±SEM from n=5 mice in each group. *p<0.05 indicates a significant difference between non-treated and ticlopidine-treated apoE/LDLR$^{-/-}$ mice. **p<0.01 indicates a significant difference between non-treated and ticlopidine-treated apoE/LDLR$^{-/-}$ mice.

*COX- and EDHF-dependent endothelial function*

Acetylcholine-induced response in the aortic rings from non-treated apoE/LDLR$^{-/-}$ mice was completely blocked in the presence of L-NAME, while in

aortic rings from ticlopidine-treated apoE/LDLR$^{-/-}$ mice the effect of L-NAME was less pronounced (Figure 8). The NO-independent component of the Ach-induced response, that was up-regulated in ticlopidine-treated animals, was not modified by indomethacin (5 μM), but was absent when rings were preconstricted with KCl (60 mM) instead of phenylephrine.

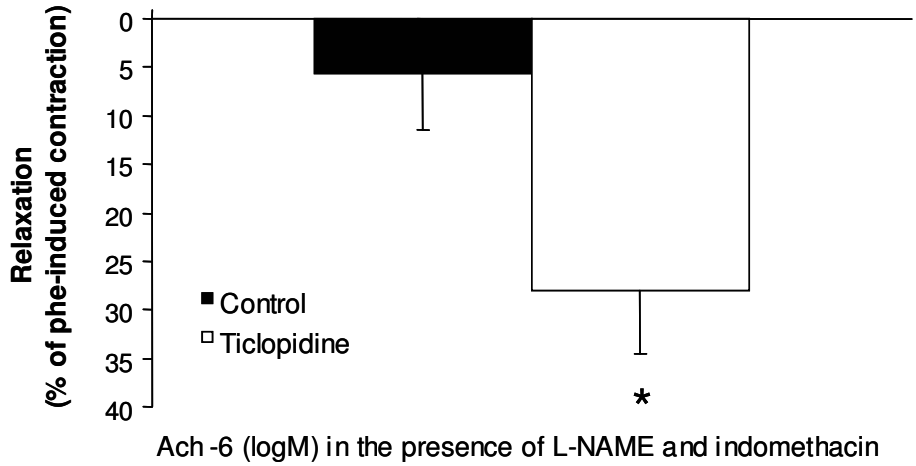



**Figure 8. Up-regulation of EDHF-mediated relaxation in ticlopidine-treated apoE/LDLR$^{-/-}$ mice**

Acetylcholine-induced vasodilation in the presence of L-NAME (300 μM) and indomethacin (5 μM) in aortic rings taken from ticlopidine-treated and non-treated groups. Data represent mean±SEM from n=5 mice in each group. *p<0.05 indicates a significant difference between non-treated and ticlopidine-treated apoE/LDLR$^{-/-}$ mice.

## 3.2. Heart failure

### 3.2.1. Post-MI remodeling and development of heart failure after coronary ligation

*Animal characteristics and morphometric results*

The body weight of male MI and sham rats was greater than of respective female rats. There was no significant difference in infarct-size between female and male rats either 7 or 42 days after coronary ligation (Tables 2/A and 2/B). However, 42 days but not 7 days after the coronary artery ligation the LV/body weight ratio was significantly increased as compared to sham animals. This increase was more pronounced in female than male rats with MI (3.6±0.3 vs. 2.8±0.1; p< 0.05).

Table 2/A. 7 days after myocardial infarction

| Gender | Female | | Male | |
|---|---|---|---|---|
| Parameter | SHAM (n=6) | MI (n=6) | SHAM (n=6) | MI (n=6) |
| Infarct-size (% LV) | 0±0 | 35.4±2.8 | 0±0 | 38.8±3.4 |
| Body weight (g) | 208.6±13.4 & | 214.4±10.7 | 305.5±8.5 | 318.2±9.8 ¤ |
| Heart weight (mg) | 830±61 & | 1005±63 | 1210±67 | 1295±60 ¤ |
| LV weight (mg) | 540±13 & | 587±35 | 773±35 | 798±45 ¤ |
| RV weight (mg) | 189±12 | 205±10 | 212±27 | 226±23 |
| LV/body (mg/g) | 2.6±0.1 | 2.7±0.13 | 2.5±0.1 | 2.5±0.1 |

Table 2/B. 42 days after myocardial infarction

| Gender | Female | | Male | |
|---|---|---|---|---|
| Parameter | SHAM (n=6) | MI (n=6) | SHAM (n=6) | MI (n=6) |
| Infarct-size (% LV) | 0±0 | 43.42±2.4 | 0±0 | 42.4±3.1 |
| Body weight (g) | 262.2±6.4& | 283.8±5.6 | 427.2±13.4 | 421.7±5.7¤ |
| Heart weight (mg) | 1045±76 & | 1563±125* | 1506±73 | 1685±120 |
| LV weight (mg) | 713±32 & | 1083±72* | 1048±43 | 1210±53 |
| RV weight (mg) | 158±16 & | 198±32 | 226±22 | 265±63 |
| LV/body (mg/g) | 2.7±0.1 | 3.7±0.3* | 2.4±0.1 | 2.7±0.1# ¤ |
| Fractional shortening | 35.3±6.9 | 17.2±1.7* | 42.6±3.6 | 16.8±6.1# |

* P<0.05 between female MI and SHAM

# P<0.05 between male MI and SHAM

¤P<0.05 between female MI and male MI

& P<0.05 between female SHAM and male SHAM

**Table 2. Animal characteristics and and morphometric results 7 (2/A) and 42 days (2/B) after post-MI**

Fractional shortening was measured in Ludwig Boltzmann Cluster for Cardiovascular Research, c/o Institute for Biomedical Research, Allgemeines Krankenhaus Wien, Vienna, Austria. With permission.

*In vivo heart function analysis*

Forty two days after coronary ligation both systolic and diastolic LV diameters were significantly increased, irrespective the gender of animals. Consequently, the fractional shortening was reduced to an equal extent in female and male MI hearts compared to sham (Table 2/B).

### 3.2.2. Vascular function in the aorta after coronary artery ligation

*NO-dependent function*

Maximal relaxation induced by Ach ($10^{-5}$ M) (Figure 9) and EC50 values were similar in all experimental groups. Seven days after coronary ligation EC50 values (logM) in the aorta were: −7.25±0.1, −6.98±0.1, −7.54±0.04 and −7.26±0.1 for female-MI, female-sham, male-MI and male-sham, respectively. Forty two days after myocardial infarction EC50 values (logM) were: −6.97±0.1, −7.13±0.1, −6.91±0.1 and −7.21±0.1 for female-MI, female-sham, male-MI and male-sham, respectively. It is however worth noting that response to acetylcholine at a concentration of $10^{-7}$ to $10^{-6}$ was augmented in female and male MI vs. sham operated rats 7 days after the coronary ligation, though only in males this difference reached statistical significance. This augmentation of Ach-response was not seen 42 days after the coronary ligation.

Moreover, the relaxation curves induced by NaNP were 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 the 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). EC50 values for relaxation to NaNP were −8.43±0.02, −8.38±0.04, −8.31±0.03 and −8.24±0.05 for female-MI, female-sham, male-MI and male-sham, respectively.

Figure 9/A. 7 days after myocardial infarction

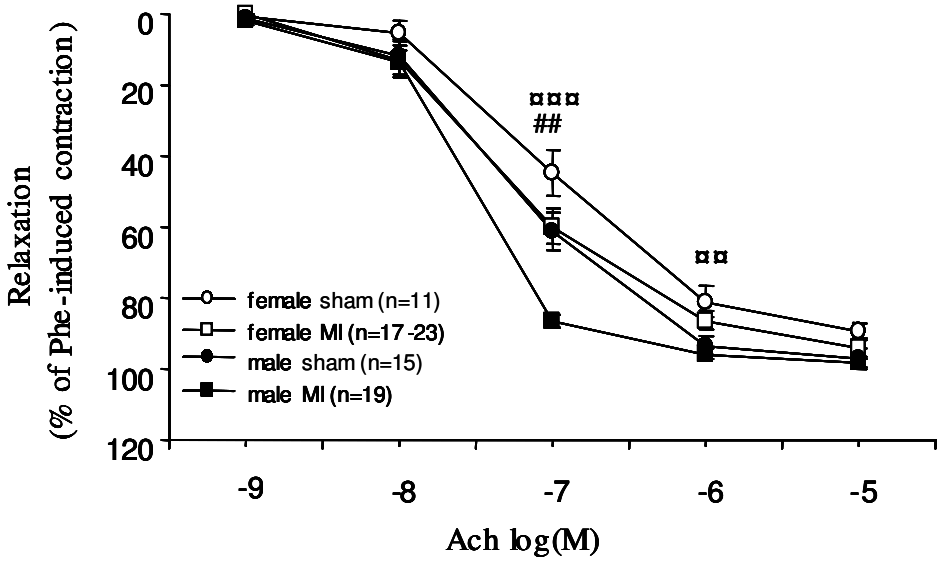



Figure 9/B. 42 days after myocardial infarction

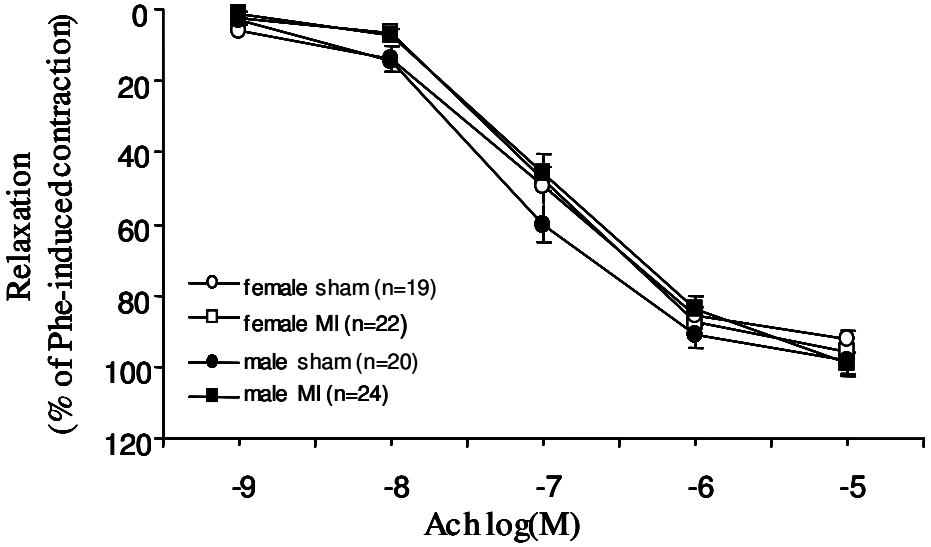



**Figure 9. Lack of impairment of endothelium-dependent response in the aorta in the early (A) and late (B) phases of the post-MI period in female and male rats**

**9/A.:** Concentration–response curves for acetylcholine-induced relaxation in aortic rings taken from female (□), male (■) MI rats and female (○), male (●) sham operated rats 7 days after coronary ligation. Data represent mean±SEM for aortic rings, *n*=11-23. ## indicates p<0.01 when male-MI was compared with male-sham, ¤¤ indicates p<0.01 when female-MI was compared with male-MI, ¤¤¤ indicates p<0.001 when female-MI was compared with male-MI.

**9/B.:** Concentration–response curves for acetylcholine-induced relaxation in aortic rings taken from female (□), male (■) MI rats and female (○), male (●) sham operated rats 42 days after coronary ligation. Data represent mean±SEM for aortic rings, *n*=19-24.

Basal NO production, determined as the magnitude of L-NAME-induced vasoconstriction 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).

*COX-dependent endothelial function*

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).

*EDHF-dependent relaxation*

At the early phase of post-MI period (7 days) the magnitude of EDHF-dependent relaxation (as assessed by Ach from $10^{-8}$ to $10^{-5}$ M in the presence of indomethacin and L-NAME) was substantially up-regulated in both female and male MI rats as compared to respective sham animals (Figure 10/A). However, at the late phase (42 days) the up-regulation of EDHF-mediated relaxation was seen only in female MI rats (Figure 10/B).

The magnitude of the EDHF-component of Ach-induced ($10^{-6}$ M) relaxation in relation to gender in the early (36.24±5.62% and 41.8±5.9% in female and male-MI, respectively) and in the late phase (25.99±4.02% and 3.79±1.09% in female and male-MI, respectively) of the post-MI period is shown in Figure 11.

If KCl (60 mM) was used to preconstrict the vessels instead of phenylephrine, the EDHF-mediated relaxation was abrogated in all experimental groups (<1% in female-MI, female-sham, male-MI and male-sham animals both 7 and 42 days after coronary ligation). Furthermore, the up-regulation of EDHF-pathway was completely blocked by pretreatment with the antagonist of $Ca^{2+}$-activated $K^+$ channels tetraethylammonium chloride (10 mM) or the EET synthesis inhibitor miconazole (10 μM) (Figure 12).

Figure 10/A. 7 days after myocardial infarction

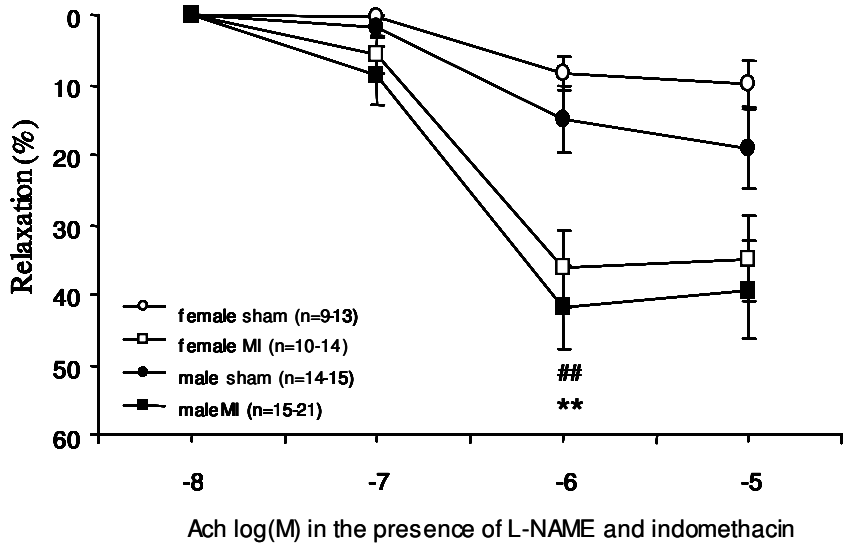



Figure 10/B. 42 days after myocardial infarction

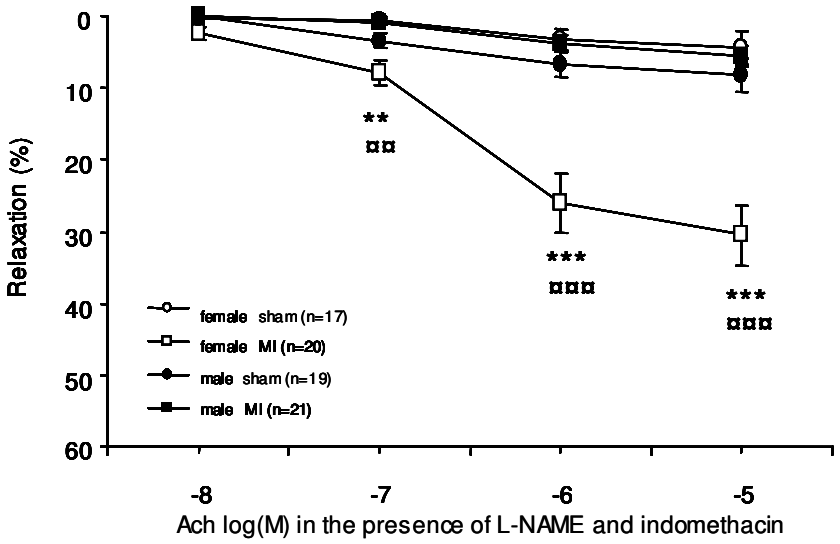



**Figure 10. Up-regulation of the EDHF-component of acetylcholine-induced vasodilation**

**10/A.:** Acetylcholine-induced vasodilation in the presence of L-NAME (300 μM) and indomethacin (1 μM) in aortic rings taken from female (□), male (■) MI rats and female (○), male (●) sham operated rats 7 days after coronary ligation. Vessels were preconstricted with phenilephrine and L-NAME. Data represent mean±SEM for aortic rings, *n*=9-21. ** indicates p<0.01 when female-MI was compared with female-sham, ## indicates p<0.01 when male-MI was compared with male-sham.

**10/B.:** Acetylcholine-induced vasodilation in the presence of L-NAME (300 μM) and indomethacin (1 μM) in aortic rings taken from female (□), male (■) MI rats and female (○), male (●) sham operated rats 42 days after coronary ligation. Vessels were preconstricted with phenilephrine and L-NAME. Data represent mean±SEM for aortic rings, *n*=17-21. ** indicates p<0.01 when female-MI was compared with female-sham, *** indicates p<0.001 when female-MI was compared with female-sham, ¤¤ indicates p<0.01 when female-MI was compared with male-MI, ¤¤¤ indicates p<0.001 when female-MI was compared with male-MI.

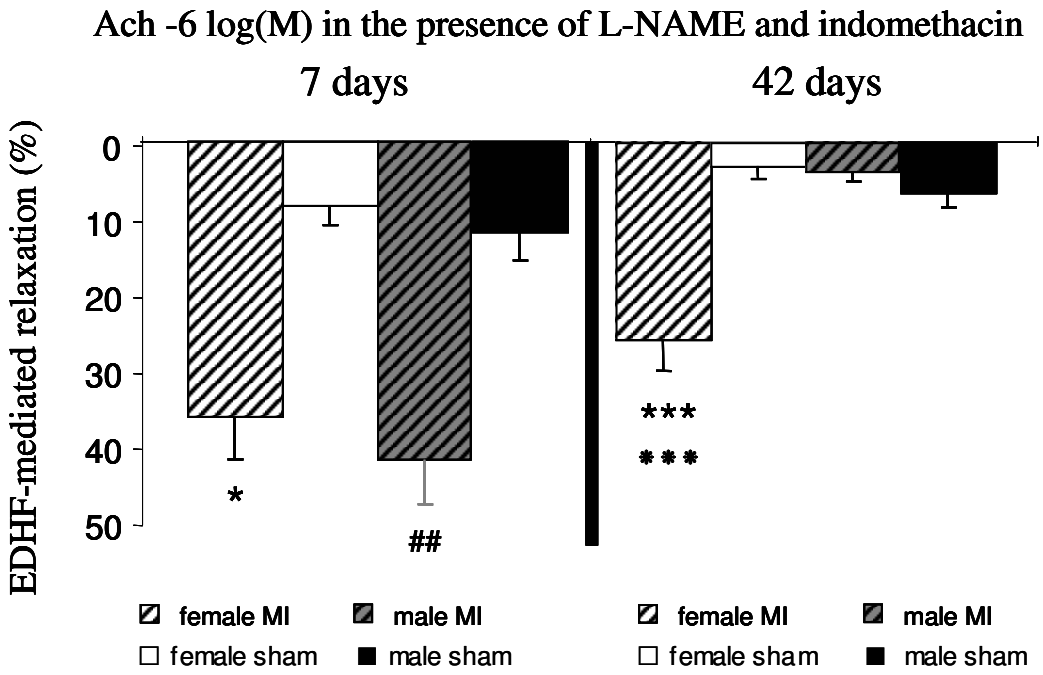



**Figure 11. Comparison of the magnitude of the EDHF-component of vasodilation induced by $10^{-6}$ M acetylcholine in the early and late phases of the post-MI period**

Acetylcholine-induced ($10^{-6}$ M) vasodilation in the presence of L-NAME (300 μM) and indomethacin (1 μM) in aortic rings from female and male rats. Data represent mean±SEM for aortic rings, *n*=9-21. * indicates p<0.05 when female-MI was compared with female-sham (7 days), *** indicates p<0.001 when female-MI was compared with female-sham (42 days), ## indicates p<0.01 when male-MI was compared with male-sham, *** indicates p<0.001 when female-MI was compared with male-MI.

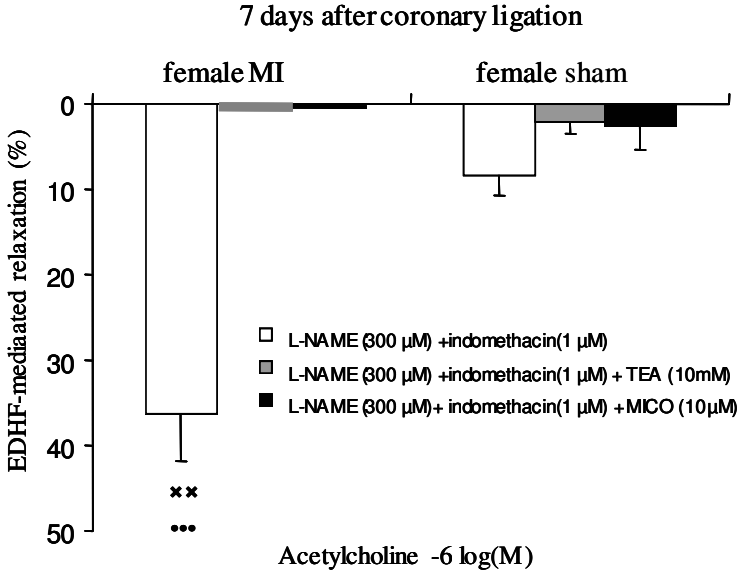



**Figure 12. The mechanism of the EDHF-component of acetylcholine-induced vasodilation in the post-MI period in aorta from female rats**

Aortic rings from female rats 7 days after coronary ligation were treated with L-NAME and indomethacin (white bars), L-NAME, indomethacin and TEA (grey bars) or L-NAME, indomethacin and MICO (black bars). Data represent mean±SEM for aortic rings, *n*=12. ×× indicates p<0.01 when L-NAME + indomethacin treatment was compared with the combination of L-NAME + indomethacin + TEA, ••• indicates p<0.001 when L-NAME + indomethacin treatment was compared with the combination of L-NAME + indomethacin + MICO.

### 3.3. Diabetes mellitus

#### 3.3.1. Development of diabetes after streptozotocin injection

Four weeks after STZ injection, the plasma glucose and $HbA_{1c}$ levels were significantly elevated as compared to age-matched controls. Both plasma glucose and $HbA_{1c}$ levels 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, while the body weight was significantly decreased as compared to those of the control rats (Table 3).

| | Control | | Diabetic | |
|---|---|---|---|---|
| | 4 weeks n=7 | 8 weeks n=8 | 4 weeks n=7 | 8 weeks n=8 |
| Body weight (g) | 319,2±27,3 | 395,7±19,7[a] | 221,9±51,2[c] | 226,3±41,6[d] |
| Urine (ml/24h) | 8,5±3,1 | 10,0±3,61 | 27,3±6,0[c] | 45,6±8,0[b d] |
| Blood glucose (mg/dl) | 102,1±13,1 | 101,2±13,9 | 216,0±16,4[c] | 298,8±22,6[b d] |
| HbA1c (%) | 1,6±0,3 | 1,6±0,1 | 2,2±0,4[c] | 3,0±0,2[b d] |

Values are means ± SEM.

[a] $P < 0.05$ four weeks control vs. eight weeks control
[b] $P < 0.05$ four weeks diabetic vs. eight weeks diabetic
[c] $P < 0.05$ four weeks control vs. four weeks diabetic
[d] $P < 0.05$ eight weeks control vs. eight weeks diabetic

**Table 3. Body weight, 24-h urine, blood glucose and $HbA_{1c}$ in control and diabetic rats**

#### 3.3.2. Vascular function in the aorta in diabetic rats

*NO-dependent vascular function*

Relaxation curves induced by Ach were identical 4 and 8 weeks after vehicle injection (Ach $10^{-6}$ M: 71.2±6.1% and 73.1±7.4% respectively), therefore control animals were combined for further analysis. 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%) as compared to control rats (Figure 13).

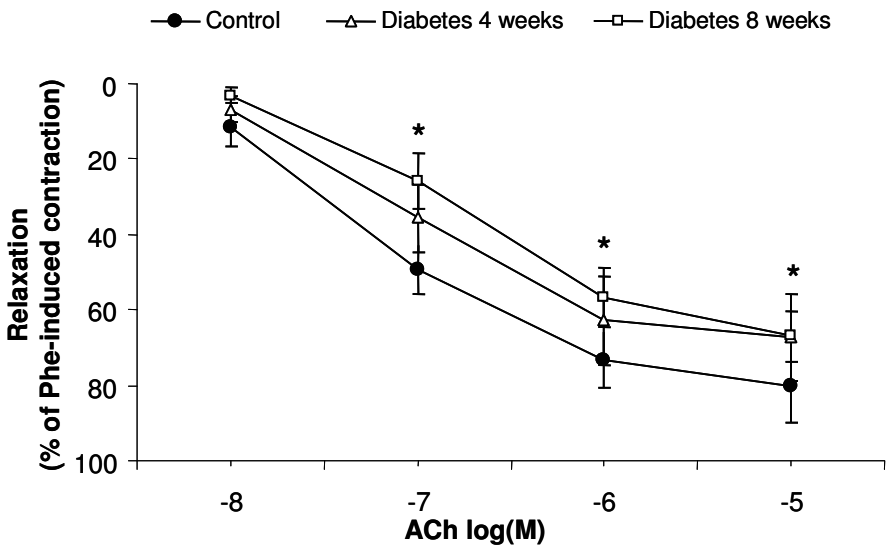



**Figure 13. Impairment of acetylcholine-induced endothelium-dependent relaxation in 8 weeks diabetic aorta**

Endothelium-dependent vasodilation was analyzed by acethylcholine in isolated aortic rings of control (combined data) and diabetic (4 and 8 weeks after STZ-injection) rats. Vessels were preconstricted with phenylephrine ($2x10^{-7}$ M). Data represent mean ± SEM of 7-8 animals. * indicates $p<0.05$ when 8 weeks diabetic rats were compared with control rats.

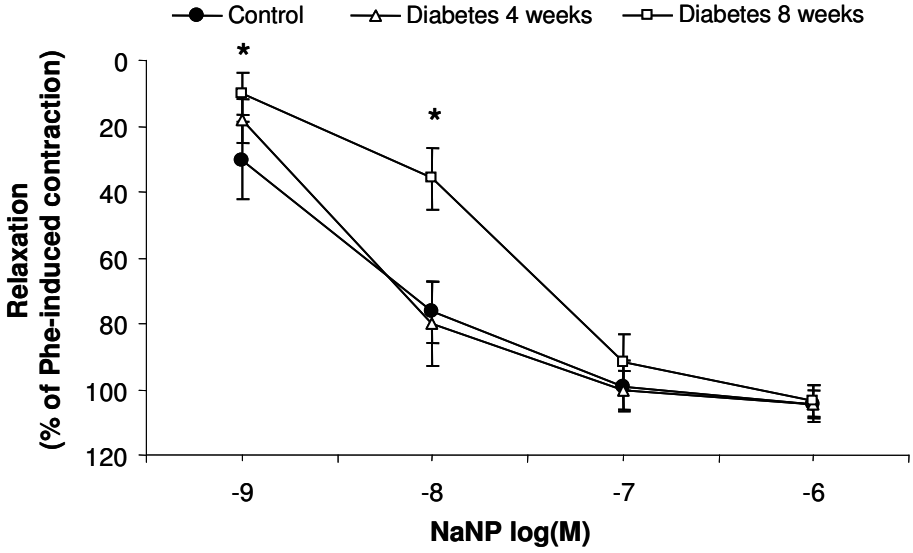



**Figure 14. Impairment of sodium nitroprusside-induced endothelium-independent relaxation in 8 weeks diabetic aorta**

Endothelium-independent vasodilation was assessed by sodium nitroprusside in isolated aortic rings of control (combined data) and diabetic (4 and 8 weeks after STZ-injection) rats. Vessels were preconstricted with phenylephrine ($2x10^{-7}$ M). Data represent mean ± SEM of 7-8 animals. * indicates $p<0.05$ when 8 weeks diabetic rats were compared with control rats.

The response induced by NaNP at a concentration of $10^{-9}$ to $10^{-8}$ was significantly decreased 8 weeks after the STZ-injection. 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 (Figure 14).

Basal NO production, assessed as the magnitude of L-NAME-induced vasoconstriction 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).

*The role of COX-metabolites in diabetic rat aorta*

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) (Figure 15). 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) (Figure 16).

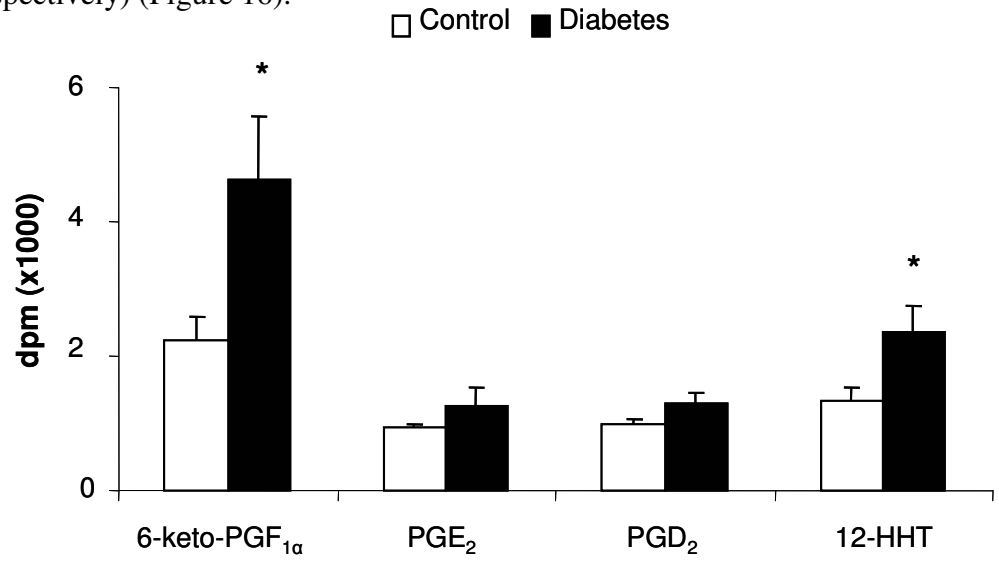



**Figure 15. Increased synthesis of $PGI_2$ (measured as its stable metabolite 6-keto-$PGF_{1\alpha}$) and 12-HHT in abdominal aorta 8 weeks after streptozotocin injection**

Comparison of 6-keto-$PGF_{1\alpha}$ (stable metabolite of $PGI_2$), $PGE_2$, $PGD_2$ and 12-HHT synthesis at 8 weeks in control and diabetic abdominal aorta (15 mg wet weight/in each sample). Data represent mean ± SEM (x $10^3$ dpm) of 6 animals. * indicates $p<0.05$ in diabetic vs. control rats.

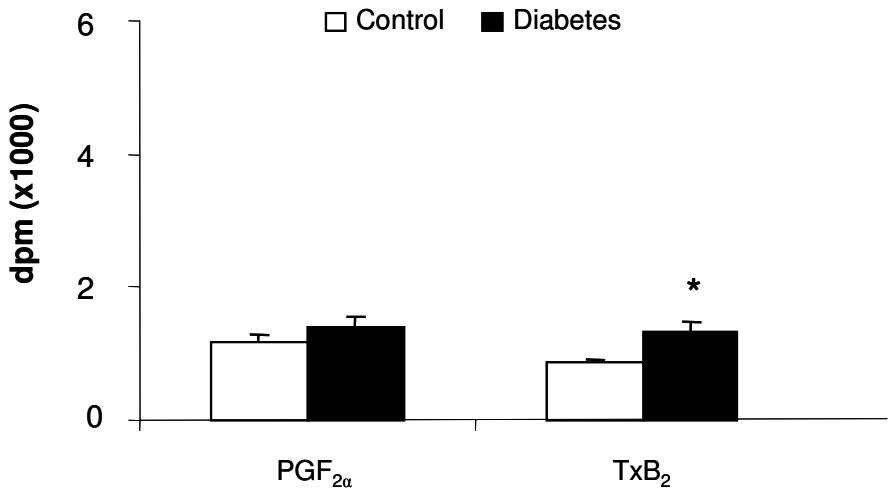



**Figure 16. Increased synthesis of $TxA_2$ (measured as its stable metabolite $TxB_2$) in abdominal aorta 8 weeks after streptozotocin injection**

Comparison of $PGF_{2\alpha}$ and $TxB_2$ (stable metabolite of $TxA_2$) synthesis at 8 weeks in control and diabetic abdominal aorta (15 mg wet weight/in each sample). Data represent mean ± SEM (x $10^3$ dpm) of 6 animals. * indicates $p<0.05$ in diabetic vs. control rats.

*EDHF-dependent relaxation in diabetic aorta*

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).

### 3.4. Summary of results

*Atherosclerosis*

Atherosclerotic lesions were virtually absent in apoE/LDLR$^{-/-}$ mice fed chow diet at the age of 2 months, while they were detected in 4-8-month-old mice both in aortic root tissue (cross-section) and in whole aorta (en-face). Similarly, macrophages (CD68 immunostaining) were scarce in subendothelial space of aortic roots of 2-month-old apoE/LDLR$^{-/-}$ mice, while the stained macrophages were abundant in 4-8-month-old apoE/LDLR$^{-/-}$ mice. Acetylcholine-induced relaxation in aorta was already impaired in 2-month-old apoE/LDLR$^{-/-}$ mice (before the development of atherosclerotic plaques) and remained diminished in older apoE/LDLR$^{-/-}$ mice. Basal NO production of aorta was impaired in 2-month-old apoE/LDLR$^{-/-}$ mice, but it was restored in older animals. Importantly, EDHF-component of Ach-induced vasodilation and COX-2 derived $PGI_2$ production were gradually up-regulated in apoE/LDLR$^{-/-}$ mice along the progression of atherosclerosis. The EDHF-mediated vasodilation involved

cytochrome P450-dependent metabolites, most likely epoxyeicosatrienoic acids and opening of $Ca^{2+}$-dependent $K^+$ channels. Finally, *S*-nitroso-*N*-acetyl-penicillamine-induced endothelium-independent relaxation was preserved in aorta of apoE/LDLR$^{-/-}$ mice until 6 months of age but it was blunted in 8-month-old apoE/LDLR$^{-/-}$ mice.

Ticlopidine treatment (90 mg/kg/day) for 4 months decreased the atherosclerotic area in the aortic root in 6-month-old apoE/LDLR$^{-/-}$ mice fed Western diet. In ticlopidine-treated apoE/LDLR$^{-/-}$ mice the Ach-induced endothelium-dependent vasodilation in the thoracic aorta was improved as compared to their non-treated counterparts. Finally, we demonstrated that ticlopidine did not modify endothelium-independent relaxation and basal NO production, but increased the EDHF-component of Ach-induced relaxation.

*Heart failure*

Myocardial infarction in rats did not modify the magnitude of acetylcholine- and sodium nitroprusside-induced relaxation in the aorta, but induced the impairment of basal NO production and adaptive up-regulation of EDHF-mediated vasodilation. Our pharmacological analysis indicate that EDHF-mediated vasodilation up-regulated by myocardial infarction involves the cytochrome P450-dependent metabolites epoxyeicosatrienoic acids. Basal $PGI_2$ production by the aortic rings did not differ significantly between MI and sham rats either 7 or 42 days after coronary ligation. Interestingly, 7 days after coronary ligation, basal NO production was impaired and the EDHF component of Ach-induced vasodilation was up-regulated in both male and female rats. However, 42 days after coronary ligation, basal NO was only impaired in male rats, while EDHF was only up-regulated in female animals.

*Diabetes mellitus*

Eight weeks after streptozotocin-induced diabetes both acetylcholine-induced endothelium-dependent and sodium nitroprusside-induced endothelium-independent relaxations were impaired in the aorta. In contrast, basal NO production of aorta was preserved either 4 or 8 weeks after diabetes induction. Eight weeks after streptozotocin injection basal production of $PGI_2$, 12-HHT and $TxA_2$ was increased as compared to control animals. Streptozotocin-induced diabetes did not induce the up-regulation of EDHF-mediated vasodilatation in aorta in diabetic rats.

## 4. Discussion

Because of the variety of functions carried out by the endothelium, endothelial dysfunction is likely to involve a number of abnormalities, such as impaired endothelium-dependent vasodilation, prothrombotic and proinflammatory activation of endothelium and increased production of $O_2^-$. Therefore, it is interesting that in clinical practice endothelial dysfunction is diagnosed exclusively on the basis of impaired endothelium-dependent relaxation, which is measured as a decrease in stimulated (by pharmacological agonists or increased blood flow) NO-mediated vasodilation (92,93,159). Endothelial dysfunction described in this way have been associated with a variety of conditions and disease states, including hypercholesterolemia, hyperhomocysteinemia, atherosclerosis, hypertension, heart failure and diabetes mellitus (93,99,160-162). Importantly, the phenotype of endothelial dysfunction in these disorders 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 impairment of endothelium-dependent relaxation. 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 the above mentioned diseases. To our knowledge, there is no report on simultaneous alterations of NO, $PGI_2$ and EDHF activity in animal models of atherosclerosis, ischemic heart failure and diabetes mellitus. Therefore, the aim of the present work was to assess the changes in NO, $PGI_2$ and EDHF pathways in the thoracic aorta along with the development of atherosclerosis (apoE/LDLR$^{-/-}$ mice), heart failure (after coronary ligation in rats) and diabetes mellitus (induced by streptozotocin in rats).

### 4.1. Atherosclerosis

*Nitric oxide*

Numerous studies reported the impairment of NO-mediated relaxation in patients with atherosclerosis (8,95). On the other hand, it is still not clear whether the decrease in NO bioavailability is a hallmark of atherosclerosis in gene-targeted mice developing atherosclerotic plaques.

We demonstrated that basal NO production was decreased and Ach-induced endothelium-dependent vasodilation was impaired in 2-month-old apoE/LDLR$^{-/-}$ mice prior to the development of atherosclerotic plaques. Along with the atherosclerotic plaque progression, in 4-8-month-old apoE/LDLR$^{-/-}$ mice the basal NO production was restored and the Ach-induced endothelium-dependent vasodilation was still impaired. The endothelium-independent relaxation induced by the NO-donor *S*-Nitroso-*N*-acetylpenicillamine remained unchanged in 2-6-month-old apoE/LDLR$^{-/-}$ mice, while at the more pronounced stage of atherosclerosis (8-month-old animals) the sensitivity of SMCs to exogenous NO was decreased.

In the present work it was demonstrated for the first time that impairment of NO-mediated relaxation in the aorta taken from the atherosclerotic apoE/LDLR$^{-/-}$ mice occurs early as a primary alteration prior to any morphological evidence of plaque development. Our results suggest that impairment of NO-mediated relaxation in 2-month-old apoE/LDLR$^{-/-}$ mice is associated with elevated LDL level but not with the presence of atherosclerotic plaques. Previous studies reported that endothelial expression of VCAM-1 is increased in response to hypercholesterolemia that leads to enhanced binding of various classes of leukocytes to endothelium, initiating an inflammatory process (163). In addition, hypercholesterolemia increases endothelial $O_2^-$ production via xanthine oxidase activation (164). The prolonged increase in the generation of $O_2^-$ in the presence of continuous NO results in the formation of highly reactive peroxynitrite ($ONOO^-$), leading to decreased NO bioavailability, oxidative damage of cell membranes and enhanced lipid peroxidation (165,166). Therefore, it is conceivable that impairment of NO-mediated relaxation, seen as an early alteration in 2-month-old apoE/LDLR$^{-/-}$ mice, is associated with hypercholesterolemia-induced $O_2^-$ production. The results of a previous study are in line with our conception for the prerequisite role of oxidant stress and hypercholesterolemia for initiating the development of endothelial dysfunction since they demonstrated that *in vitro* treatment of normal mouse vessels with oxidized LDL but not with native LDL leads to the impairment of endothelium-dependent relaxation (167).

Our results indicate that aortic endothelium from 2-month-old apoE/LDLR$^{-/-}$ mice, due to an impaired NO bioavailability, exhibit a spectrum of actions that promote coagulation, inhibit fibrinolysis and favour atherogenesis. Indeed, the impaired NO mediated vasodilation is accompanied by decreased endothelium-dependent vasodilation, increased adhesion of platelets and leukocytes to the vessel wall and

enhanced smooth muscle cell growth and migration, each of them plays a key role in atherogenesis.

Finally, we demonstrate that SMC sensitivity to nitrovasodilators is fairly preserved at the early stage of atherosclerosis and impairment of endothelium-independent relaxation occurs only at the very advanced stage of plaque development. This observation suggests that reduced activity of soluble guanylate cyclase may further contribute to the impairment of vascular function in the late phase of atherosclerosis.

*Prostacyclin*

During the last three decades many studies have addressed the role of COX enzymes and different prostanoids in atherosclerosis (168,169). Despite numerous studies, there is a considerable debate as to whether COX-2 is pro-atherogenic or anti-atherogenic in nature. Cipollone *et al.* reported that overexpression of functionally coupled COX-2 and matrix metalloproteinase (MMP), an enzyme responsible for fibrous cap digestion occurs simultaneously in atherosclerotic lesions (170), which suggest that increased COX-2 activity may contribute to a destabilization of the atherosclerotic plaque. Moreover, neovessel formation requires the presence of COX-2 (171), suggesting that overexpression of COX-2 within the lesion may contribute to the formation of new blood vessels, thus allowing the plaque to expand. On the other hand, it seems that COX-2 may exert anti-atherosclerotic activity as well (172,173), via catalyzing the synthesis of $PGI_2$.

Furthermore, it is known that macrophages play an important role in atherogenesis and produce an array of prostanoids, including $TxA_2$ and $PGE_2$, considered the more atherogenic prostanoids. Indeed, $TxA_2$ is a vasoconstrictor and platelet aggregator (174), while $PGE_2$ may increase cholesterol synthesis and augment MMP activity (175,176).

We have demonstrated that macrophages are absent in the subendothelial space in aortic roots from wild-type mice. Moreover, basal $PGI_2$ production of aorta was slightly increased with age in C57BL/6J mice and mediated exclusively by COX-1. In contrast, at the early stage (2-month-old apoE/LDLR$^{-/-}$ mice) of atherosclerosis the macrophages were already present in the aorta and COX-2-derived $PGI_2$ production was increased. At later stages of plaque development (4-8-month-old apoE/LDLR$^{-/-}$ mice) macrophages were abundant in the subendothelial area of atherosclerotic lesions and COX-2-derived $PGI_2$ production of aorta remained elevated. This is in line with previous studies that

reported the increased $PGI_2$ biosynthesis in atherosclerosis (177) and the expression of COX-1 and COX-2 within atherosclerotic lesion (178).

Our results suggest the ability of atherosclerotic aorta to compensate for the loss of NO by increased production of COX-2 derived $PGI_2$. Interestingly, previous studies reported that estrogen increases the synthesis of COX-2 derived $PGI_2$ by acting on estrogen receptor α (179). On the other hand, it is also known that activated platelets induce endothelial inflammation, a crucial event in the progression of atherosclerosis (116). Therefore, it is feasible that increased synthesis of COX-2-derived $PGI_2$ in female apoE/LDLR$^{-/-}$ mice may attenuate the atherosclerotic process through the inactivation of platelets and inhibition of their aggregation.

In fact, increased COX-2 activity may lead not only to increased production of anti-atherogenic $PGI_2$, but also to pro-atherogenic prostanoids, such as $TxA_2$ and $PGE_2$. Therefore, further studies are needed to determine whether and how enhanced COX-2 activity affects atherogenesis and the net effect of the interplay between the anti- and pro-atherogenic prostanoids.

*Endothelium-derived hyperpolarizing factor*

Acetylcholine-induced vasodilation was completely blocked by preincubation with L-NAME and indomethacin in C57BL/6J mice of all age groups. In contrast, Ach-induced vasodilation was partially resistant to inhibitors of COXs and NOS in the aorta taken from female apoE/LDLR$^{-/-}$ mice. The EDHF-component of Ach-induced vasodilation was up-regulated starting from the 2-month-old apoE/LDLR$^{-/-}$ mice and remained elevated at later stages of plaque development.

The up-regulation of EDHF-mediated relaxation was abrogated if KCl was used to pre-contract the aortic rings instead of phenylephrine. Moreover, the preincubation with the $K^+$ channel blocker tetraethylammonium chloride or epoxygenase synthesis inhibitor miconazole blocked the EDHF-component of Ach-induced vasodilation. On the basis of our pharmacological analysis we therefore suggest that a cytochrome P-450 metabolite of arachidonic acid, most likely an EET is up-regulated in atherosclerotic apoE/LDLR$^{-/-}$ mice.

Previous studies reported that following endothelial cell stimulation with Ach, the intracellular concentration of $Ca^{2+}$ increases and leads to the activation of PLC, which liberates arachidonic acid from membrane phospholipids (180). The subsequent activation of CYP2C results in the generation of EETs, which increase the efflux of $K^+$

through $K_{Ca}$ channels, enhance the generation of cAMP by adenylyl cyclase and activates gap junctional coupling in the endothelial cells. Following the generation of EETs, the spread of an electric current through myo-endothelial gap junctions to the adjacent SMCs leads to the hyperpolarization of myocytes or the increased $K^+$ efflux through endothelial $SK_{Ca}$ and $IK_{Ca}$ channels elicits the hyperpolarization of the surrounding SMCs by activating $K_{IR}$ channels and/or $Na^+$-$K^+$-ATPase.

Our observations are in line with a recent study showing a compensatory up-regulation of EDHF in female $eNOS^{-/-}$ and $COX\text{-}1^{-/-}$ mice (181). Interestingly, the function of EETs is not limited to its vasodilatory action, but it exhibits a potent anti-inflammatory activity both *in vitro* and *in vivo*. Indeed, Node *et al.* reported that EETs decreased cytokine-induced VCAM-1, ICAM-1 and E-selectin expression, and EETs prevented leukocyte adhesion to the vessel wall by a mechanism involving the inhibition of transcription factor NF-$\kappa$B (182). Since atherosclerosis is a chronic inflammatory disorder (183), it is conceivable that up-regulation of anti-inflammatory EDHF (184) may play a compensatory role in the aorta of apoE/$LDLR^{-/-}$ mice, leading to delayed atherogenesis. Moreover, the up-regulation of EDHF in apoE/$LDLR^{-/-}$ mice contributes to the Ach-induced endothelium-dependent vasodilation to compensate for the decreased NO-dependent relaxation. Our results furthermore suggest that increased EDHF activity that operate to compensate for the lack of NO is not sufficient to preserve normal endothelium-dependent relaxation. It is therefore likely that compensatory up-regulation of EDHF operates in conjunction with NO rather than in stead. In this respect, there is a great need to determine the functional consequence of adaptive up-regulation of EDHF in atherogenesis.

### 4.2. Heart failure

*Nitric oxide*

Despite numerous studies, the phenotype of dysfunctional endothelium in the thoracic aorta in rats with heart failure in particular in relation to gender is not entirely clear. Our results showed that magnitude of Ach-induced endothelium-dependent relaxation was not modified in the thoracic aorta after coronary artery ligation, irrespectively the time-period after MI or the gender of animals. Moreover, there was no impairment of sodium nitroprusside-induced endothelium-independent relaxation in either female or male MI rats. In contrast, we observed a gender-specific alteration in

basal NO production in the late but not in the early phase of post-MI remodeling. Indeed, the basal NO production was impaired in the aorta of both female and male rats 7 days after coronary ligation. However, 42 days after MI the decrease in basal NO acitvity was seen only in male but not in female rats. This finding may explain the survival advantage of female patients with heart failure as compared to males reported in clinical trials. (185). Our findings stay in line with previous reports in which estrogen up-regulated the expression of eNOS (186) and restored the vasodilator role of basal NO in ovariectomized rats with chronic heart failure (187).

Our results also suggest that assessment of Ach-induced endothelium-dependent vasodilation – at least in our experimental settings - is not sensitive enough to detect endothelial dysfunction in post-MI aorta, as compared to the measurment of basal NO production. This observation is in agreement with a previous study (188) and could explain why some authors reported the lack of endothelial dysfunction in the same model if they measured the Ach-induced response alone (189). Interestingly it was previously reported that basal NO synthesis is more sensitive to $O_2^-$-dependent inactivation than NO stimulated by acetylcholine (190), suggesting that impairment of basal NO activity could be due to increased generation of $O_2^-$.

Obviously, the impairment of basal NO synthesis in the early phase of post-MI remodeling is related to the increased sympathetic and neurohumoral activity as well as the enhanced production of $O_2^-$ (191). In addition to these mechanisms, the impaired cardiac function may also contribute to the vascular changes at the late phase of post-MI period. We showed that despite the more pronounced hypertrophy in the female hearts 42 days after MI, the ejection fraction was equally reduced in female and male MI hearts. Therefore, any differences found between female and male animals during the assessment of vascular function is not due to a gender-specific decrease in cardiac function but to heart failure itself.

*Prostacyclin*

Prostacyclin, as a potent systemic and pulmonary vasodilator, has the ability to decrease both left and right ventricular afterload, leading to a pronounced increase in stroke volume and cardiac output. Intravenous administration of $PGI_2$ has been shown to improve right ventricular performance in pulmonary hypertension (192) and after heart transplantation (193). Moreover, $PGI_2$ infusion improves pulmonary hemodynamics and left ventricular function in patients with severe congestive heart

failure (194). In addition to decreased pulmonary and systemic vascular resistance, the beneficial effect of $PGI_2$ in congestive heart failure seems to involve an improved positive inotropic effect (194).

Since it is not known whether the thoracic aorta has the ability for the compensatory synthesis of $PGI_2$ in heart failure we measured the $PGI_2$ component of Ach-induced relaxation and basal $PGI_2$ production of aorta. The incubation of aortic rings with indomethacin, a non-selective COX inhibitor did not modify the magnitude of Ach-induced vasodilation in either MI or sham rats, irrespectively of the age and the gender of animals. Morever, there was no difference in basal $PGI_2$ production between MI and sham rats at either 7 or 42 days after coronary ligation. Consequently, the question emerges why basal $PGI_2$ synthesis of aorta was up-regulated in atherosclerosis but not in heart failure. We suppose that this discrepancy in basal $PGI_2$ production between atherosclerosis and heart failure may be explained by the different pathomechanism and etiology of these disorders. Indeed, it has been reported that endothelial dysfunction is a primary phenomenon in atherosclerosis (95), while it develops as a secondary consequence of altered cardiac function in heart failure (122). Moreover, platelets are well recognized to play an important role in the initiation of atherosclerosis as well as in thrombotic complications of this disease. In contrast, it is not clear whether platelets are the main players in the pathogenesis of heart failure. Therefore, one might speculate that overproduction of platelet inhibitor $PGI_2$ in atherosclerosis represents an important compensatory response to the increased platelet activation, while this does not occur in heart failure.

*Endothelium-derived hyperpolarizing factor*

Another important finding of this work was the gender-specific up-regulation of NOS- and COX-independent vasodilation in the late but not in the early phase of post-MI period. Indeed, EDHF-mediated relaxation was up-regulated both in female and male rats 7 days after coronary ligation, while it remained elevated 42 days after MI only in female rats. Similarly to atherosclerotic aorta, the elevated extracellular $K^+$ concentration, preincubation with tetraethylammonium chloride or miconazole blocked the EDHF-component of Ach-induced vasodilation. The results of our pharmacological analysis indicate the contribution of EETs to the EDHF-mediated vasodilation in the aorta from rats with MI. It is tempting to speculate that long-lasting up-regulation of EDHF in females plays an important role in maintaining endothelial function in heart

failure. In addition, the function of EDHF is not limited to the regulation of vasomotor tone, but it displays a potent anti-inflammatory action (184), which could be important in heart failure.

### 4.3. Diabetes mellitus

*Nitric oxide*

Similarly to atherosclerosis and heart failure, we demonstrated the impairment of NO-mediated relaxation in aorta from diabetic rats. In the present work, we showed that both acetylcholine-induced endothelium-dependent and sodium nitroprusside-induced endothelium-independent relaxation were decreased in the aorta 8 weeks after the STZ-injection. This finding correlates with that of others (195), who also reported an impaired endothelium-dependent and -independent relaxation in diabetes. Taking this into consideration, we may conclude that both decreased endothelial NO-release and reduced SMC sensitivity to NO may contribute to the impairment of endothelium-dependent relaxation in the thoracic aorta of STZ-induced diabetic rats.

In our experiments the magnitude of L-NAME-induced vasoconstriction did not differ between control and diabetic rats. This suggests that, in contrast to atherosclerosis and heart failure, measurement of basal NO activity alone is not suitable to diagnose endothelial dysfunction in this diabetes model.

*Prostacyclin*

We have demonstrated that basal production of the total amount of vasodilator and vasoconstrictor COX metabolic products was significantly higher in aorta from diabetic rats, as compared with control animals. The synthesis of 6-keto-$PGF_{1\alpha}$ (stable metabolite of $PGI_2$), 12-HHT and $TxB_2$ (stable metabolite of $TxA_2$) was significantly increased in aorta 8 weeks after the STZ-injection when compared to the corresponding controls. In contrast, the preincubation of the aortic rings with indomethacin did not modify the sensitivity and the maximal value of Ach-induced relaxation either in control or diabetic rats, suggesting that stimulated release of COX metabolites plays only a minor role in the Ach-induced vasodilatation of aorta.

Previous studies reported that prostanoid production varies according to the time period after the STZ-injection in rats (145). Interestingly, the decreased production of $PGI_2$ or the increased synthesis of $TxA_2$ may reflect the severity of diabetes, especially the progression of vascular complications (196,197). Indeed, Peredo *et al.* (145) have

shown that $PGI_2/TxA_2$ ratio was decreased in the aorta 120 and 180 days but not after 30 days of diabetes. In the present work we demonstrated that diabetes of 8 weeks duration induced an increased synthesis of $PGI_2$, 12-HHT and $TxA_2$ in the aorta. $PGI_2$ is a potent vasodilator and platelet inhibitor, while 12-HHT stimulates $PGI_2$ synthesis (198). In addition, 12-oxoheptadeca-5(Z)-8(E)-10(E)-trienoic acid, the primary metabolite of 12-HHT has a $TxA_2$ receptor antagonistic effect (199). Therefore, our results suggest that overproduction of $PGI_2$ and 12-HHT may have protective effect at the early stage of diabetes, while it is conceivable that these mechanisms do not operate at later stages displaying an increased risk for cardiovascular complications in diabetes.

*Endothelium-derived hyperpolarizing factor*

In this work we demonstrated the up-regulation of EDHF pathway in the aorta of female animals with atherosclerosis and heart failure. In contrast, the combined preincubation with L-NAME and indomethacin completely blocked the Ach-induced vasodilation in the aorta in male diabetic rats. Since the up-regulation of EDHF seems to be important in females (181), we used female diabetic rats (data not shown) where the results were similar to males. Our results therefore suggest that lack of EDHF-pathway up-regulation in the thoracic aorta may contribute to the development of endothelial dysfunction as well as the vascular complications of diabetes.

### 4.4. The effect of ticlopidine on endothelial function in aorta in apoE/LDLR$^{-/-}$ mice

These days the improvement of endothelial function is not an accepted paradigm of treatment of cardiovascular diseases yet. Still many drugs, which are used in medicine today possess pleiotropic effect on endothelial function, such as angiotensin converting enzyme inhibitors (200), HMG CoA reductase inhibitors (201), aldosterone (202) and some $\beta_1$-adrenoreceptor antagonists (203). All of them improve NO-dependent function (202-205), some of them have an effect on $PGI_2$ production (206), while their effect on EDHF is largely unknown.

In the present work we investigated the effect of platelet inhibitor ticlopidine on the progression of endothelial dysfunction and plaque development in apoE/LDLR$^{-/-}$ mice. Our results showed that the area covered by atherosclerotic plaques was decreased in the aortic roots of apoE/LDLR$^{-/-}$ mice after ticlopidine-treatment. We also demonstrated that ticlopidine stimulated EDHF-mediated vasodilation and restored endothelium-dependent relaxation (207). It may well be that up-regulation of EDHF

pathway by ticlopidine plays a role in maintaining proper endothelium-dependent relaxation and contributes to the anti-atherosclerotic effect of ticlopidine. However, the question whether the attenuated plaque development and improved endothelial function after ticlopidine-treatment is due to the anti-platelet effects of ticlopidine, to the up-regulation of EDHF-pathway, or to both of them, remains to be answered.

## 5. Conclusion

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. Perhaps 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 or soluble epoxide hydrolase inhibitors (sEHI) (208) could be efficient in restoring vascular homeostasis in atherosclerosis but not in diabetes. In turn, the stimulation of $PGI_2$-dependent mechanism for example by 1-methylnicotinamide 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 (206,209). In the present work it was shown that anti-platelet ticlopidine

stimulated EDHF-mediated vasodilation and restored endothelium-dependent relaxation. It remains to be established how important is the EDHF-mediated mechanism to the anti-atherosclerotic effect of ticlopidine.

Nevertheless, we believe that analysis of the effects of cardiovascular drugs on NO, $PGI_2$ and EDHF pathways are important to find out what are their optimal indication to treat endothelial dysfunction in cardiovascular diseases. Hopefully, this direction of further research could be continued with the aim to improve endothelial function in patients with cardiovascular diseases that would lead to vascular protection and preservation of vascular homeostasis.

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