From the Department of Clinical Science, Intervention & Technology, Division of Obstetrics & Gynecology, Karolinska University Hospital-Huddinge, Karolinska Institutet, Stockholm, Sweden

NEW INSIGHTS INTO THE CONTROL OF SMALL ARTERY FUNCTION IN HUMAN PREGNANCY AND ESTROGEN RECEPTOR BETA KNOCKOUT MICE

Leanid Luksha

Karolinska Institutet

Stockholm 2007

Picture on the cover page: subcutaneous fat biopsy with small blood vessels.

Published by Karolinska Institutet.

ISBN 978-91-7357-138-8

Published and printed by
REPROPRINT AB
Stockholm 2007
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

"…In work, do what you enjoy.
In family life, be completely present…"

Tao Te Ching

To my Family

ABSTRACT

Background: Available data clearly indicates functional and morphological differences between small and large arteries, and observations from studies on large arteries may not be applicable to understand the physiology of small arteries (~200-300μm) that actively participate in the regulation of peripheral vascular resistance, blood pressure and flow to target organs. These events confer cardiovascular adaptation to normal pregnancy (NP), however they are disturbed in preeclampsia (PE) and in estrogen receptor β knockout (ERβKO) mice at a certain age.

Aims: (1) To assess endothelial function and morphology with focus on the role and mechanisms of endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation in small subcutaneous arteries isolated from pregnant women with and without PE; **(2)** To estimate the predisposition for sex difference in blood pressure of ERβKO mice at the level of small arteries' function with a focus on endothelium-dependent dilatation (EDHF) and adrenergic vasoconstriction.

Methodology: Small subcutaneous arteries obtained from pregnant women and femoral arteries obtained from age-matched (14-22 weeks old) female and male wild type (WT, ERβ +/+) and ERβKO mice were used in a wire-myography set-up for functional studies. Immunohistochemistry for connexins (Cx) and/or ER subtypes (as appropriate), as well as scanning and transmission electron microscopy techniques were also utilized for evaluation of small arteries' morphology with particular focus on prerequisite for gap junction communications.

Results and conclusions: (1) The overall endothelium-dependent response in arteries from pregnant women with and without PE was similar. However, EDHF-mediated relaxation was reduced in PE. The results demonstrated heterogeneity in the relative contribution of endothelium-derived factors and in the mechanisms responsible for the EDHF-mediated relaxation in PE. Gap junctions and/or H_2O_2 and/or cytochrome P450 epoxygenase metabolites of arachidonic acid appeared to be involved in the EDHF-mediated response in PE. In NP women, communication via gap junctions via Cx 43 represented a common pathway responsible for EDHF action. The link between morphological alterations within the vascular wall, and changes in the contribution of gap junctions to EDHF-mediated relaxation of small arteries isolated from women with PE was suggested.

(2) Endothelium-dependent relaxation in arteries (<200μm of internal diameter) was greater in WT females *vs.* males, and this was attributed to a greater EDHF component in the relaxation. This difference was absent in ERβKO mice. The data suggests that in WT male mice ERβ reduces EDHF-mediated relaxation. The pharmacological evidence and morphological prerequisite for involvement of gap junctions in EDHF-mediated responses was indicated in male arteries. However, the absence of ERβ had no influence on expression of the main Cx subtypes within the vascular wall or on the ultrastructure and morphology of the endothelium. The increased EDHF contribution to endothelium-dependent dilatation in ERβKO male mice vs. WT could not explain the hypertension observed in ERβKO animals.

(3) Femoral arteries from ERβKO male mice demonstrated an enhancement of the contractile response to α_1-adrenoceptor agonist (phenylephrine) that was accompanied by elevated basal tension attributable to endothelial factors. Contractile responses to the mixed adrenoceptor agonist, norepinephrine, were similar in ERβKO and WT mice; however the addition of β-adrenoceptor inhibitor unmasked the enhancement of the underlying α_1-adrenoceptor responsiveness pertinent to males. β-Adrenoceptor-mediated dilatation was also enhanced in ERβKO *vs*. WT males. We suggest that ERβ modifies the adrenergic control of small artery tone in males, but not in females. The alterations in the adrenergic modulation of small artery tone might commence the hypertension in ERβKO males.

Significance: Heterogeneity in manifestation of functional and morphological signs of endothelial dysfunction at the level of small arteries in PE indicates a complexity and multifactor genesis of this pregnancy-related disorder. The relative importance of ERβ for the control of small artery function found in males in the rodent model substantiates a gender-related approach for prevention and treatment of cardiovascular disease.

LIST OF PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals

I. **Luksha L**, Nisell H, Kublickiene KR.
The mechanisms of EDHF-mediated responses in subcutaneous small arteries from healthy pregnant women.
Am J Physiol Regul Integr Comp Physiol. 2004 Jun;286(6):R1102-9.

II. *Lang NN, ***Luksha L**, Newby DE, Kublickiene K.
Connexin 43 mediates endothelium-derived hyperpolarising factor induced vasodilatation in subcutaneous resistance arteries from healthy pregnant women.
Am J Physiol Heart Circ Physiol. 2007 Feb;292(2):H1026-32.
*authors equally contributed

III. **Luksha L**, Nisell H, Luksha N, Kublickas M, Hultenby K, Kublickiene K.
Endothelium-derived hyperpolarizing factor in preeclampsia: heterogeneous mechanisms and morphological prerequisites.
J Physiol. (London) submitted.

IV. **Luksha L**, Poston L, Gustafsson JÅ, Hultenby K, Kublickiene K.
The Estrogen Receptor β contributes to gender related differences in endothelial function of murine small arteries via EDHF.
J Physiol. 2006 Dec 15; 577(Pt 3):945-55.

V. **Luksha L**, Poston L, Gustafsson JÅ, Aghajanova L, Kublickiene K.
Gender specific abnormalities in adrenergic responses of small arteries from estrogen receptor-beta knockout mice.
Hypertension. 2005 Nov;46(5):1163-8.

CONTENTS

LIST OF ABBREVIATIONS

17β-E_2	17beta-estradiol
18α-GA	18-α-Glycyrrhetinic Acid
A23187	Calcium Ionophore
AA	Arachidonic Acid
ACh	Acetylcholine
BK	Bradikynin
BK_{Ca}	Large conductance calcium-activated potassium channels
Ca^{2+}	Calcium ions
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic adenosine monophosphate
$[Ca^{2+}]_i$	Intracellular calcium concentration
CMPs	Connexin Mimetic Peptides
CNP	C-type natriuretic peptide
COX	Cyclooxygenase
Cx(s)	Connexin(s)
CYP450	Cytochrome P450
CVD	Cardiovascular diseases
DFA	Distal femoral artery
EC(s)	Endothelial Cell(s)
EDHF	Endothelium-Derived Hyperpolarizing Factor
EETs	Epoxyeicosatrienoic acids
E_m	Membrane potential
ERs	Estrogen Receptors
ERα, ERβ	Estrogen Receptor alpha or beta
ERαKO	Estrogen Receptor alpha knockout
ERβKO	Estrogen Receptor beta knockout
ET-1	Endothelin-1
GA	Glycyrrhhetinic Acid
Gap26	CMP correspond to amino acid sequence on the first extracellular loop of connexins
Gap27	CMP correspond to amino acid sequence on the second extracellular loop of connexins
H_2O_2	Hydrogen peroxide
ID	Internal Diameter
IEL	Internal Elastic Lamina
IK_{Ca}	Intermediate conductance calcium-activated potassium channels
Indo	Indomethacin
IP_3	Inositol triphosphate
ISO	Isoproterenol
K^+_{ATP}	ATP-sensitive potassium channels
K_{IR}	Inward rectify potassium channels
K_{Ca}	Calcium-dependent potassium channels
K^+-channel	Potassium channel
K^+	Potassium ions

KPSS	High potassium physiological salt solution
L-NAME	N^{ω}-nitro-L-arginine-methyl ester
L-NNA	N^{w}-nitro-L-arginine
MEGJ(s)	Myoendothelial Gap Junction(s)
Na^{+}–K^{+}-ATPase	Sodium-potassium pump
NE	Norepinephrine
NO	Nitric Oxide
NOS	Nitric Oxide synthase
eNOS	Endothelial Nitric Oxide Synthase
iNOS	Inducible Nitric Oxide Synthase
NP	Normal pregnancy
O_2^{-}	Superoxide anion
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, selective guanylate cyclase inhibitor
OVX	Ovariectomized mice or rats
PE	Preeclampsia
pEC_{50}	Negative log concentration (in mol/l) required to achieve 50% of the maximum response to the agonist
PFA	Proximal femoral artery
PhE	Phenylephrine
PKA	cAMP-dependent protein kinase A
PLA_2	Phospholipase A_2
PGI_2	Prostacyclin
PSS	Physiological Salt Solution
SEM	Standard Error of the Mean
sFlt1	Soluble fms-like tyrosine kinase 1
SK_{Ca}	Small conductance potassium channels
SHR	Spontaneously Hypertensive Rats
SOD	Superoxide dismutase
SMC(s)	Smooth Muscle Cell(s)
SNP	Sodium nitroprusside
TEM	Transmission Electron Microscopy
U46619	Thromboxan A_2 mimetic
vCa^{2+}	Voltage-sensitive calcium channels
vs	versus
VSMC(s)	Vascular Smooth Muscle Cell(s)
WT	Wild Type

"To know that we know what we know, and to know that we do not know what we do not know, that is true knowledge."

Nicolaus Copernicus (1473-1543)

1 INTRODUCTION

Blood vessels proximal to the arterioles with a lumen diameter of 100-300 μm are defined as small arteries. These arteries contribute substantially and participate actively in the regulation of peripheral vascular resistance, blood pressure and flow to the target organs [69]. For many years, the knowledge about their structure and function was mainly based on the investigation of large vessels. Until the 1970s, the information about their properties was gathered from the visual examination of vessels in readily accessible vascular beds or from *in vivo* perfusion studies in combination with histological examinations [265]. In 1976 Professors *M.J. Mulvany* and *W.Halpern* described a new technique for *ex-vivo* investigation of blood vessels with internal diameter (ID) as small as 100μm. Since then the improvement of initial and development of novel techniques substantiated the information specific to small arteries function. Current knowledge implicates the morphological and functional differences between small and large arteries and their physiological role for cardiovascular maintenance in health and disease.

The overall aim of this thesis is to gain further understanding about the functional and morphological features of small arteries by focusing particularly on endothelium-dependent control of vascular reactivity in human pregnancy and in estrogen receptor β knockout mice.

1.1 EDHF AS THE MAIN MECHANISM FOR ENDOTHELIUM-DEPENDENT RELAXATION IN SMALL ARTERIES

More than a quarter of a century has passed since discovery of the vital importance of endothelium for vascular control [134]. Prostacyclin (PGI_2) – a cyclooxygenase-dependent metabolite of arachidonic acid (AA) [108] and nitric oxide (NO) formed through L-arginine and NO synthase (NOS) pathway [280] were identified as major endothelium-derived vasodilators. However, the fact that NO and PGI_2 could not fully account for the agonist-induced relaxation in certain circulations, suggested the existence of an additional vasodilative mechanism defined as endothelium-dependent but NO and PGI_2 independent [179, 203, 204]. Since the residual endothelium-dependent relaxation was concurrent with vascular smooth muscle cell (VSMC) hyperpolarization [60] and abolished by potassium channel (K^+-channel) blockers or by depolarizing concentration of potassium (an increase in extracellular K^+ from basal 4.7 to 25-30 mM) [1], the mediator responsible for this occurrence was termed as *endothelium-derived hyperpolarizing factor* (EDHF) [359]. Thus, by definition, EDHF seems to be a substance and/or electrical signal that is generated or synthesized in and released from the endothelium that hyperpolarizes VSMC followed relaxation [127].

Although the nature and mechanism of EDHF action is wrapped in a shroud of mystery, the importance of EDHF is confirmed by its predominant contribution to

endothelium-dependent modulation of VSMC tone in resistance-sized arteries [117, 252]. Indeed, EDHF-mediated contribution to endothelium-dependent dilatation increases as the vessel size decreases [367, 373]. If NO and PGI_2 inhibitors almost fully prevent endothelium-dependent relaxation in conduit arteries (i.e. aorta) [186, 398], the dilative capacity is equally divided between EDHF and NO in vessels with diameter above 300μm [130]. In smaller vessels the contribution of EDHF increases significantly and the role of NO is minimal [118, 282] (Figure 1; please, note that it is generalized scheme showing the overall tendency; however, the variability between species and vascular beds persists. For example, different contribution of EDHF *vs* NO has been shown between small arteries of hamster isolated from coronary, mesenteric and skeletal muscle vascular beds [71]). The functional evidence is supported by electrophysiological experiments, in which endothelium-dependent changes in membrane potential are more pronounced in smaller *vs* large arteries [367]. An inverse relationship between endothelial NOS (eNOS) expression and vessel size in the aorta and proximal *vs* distal mesenteric arteries has also been reported [332].

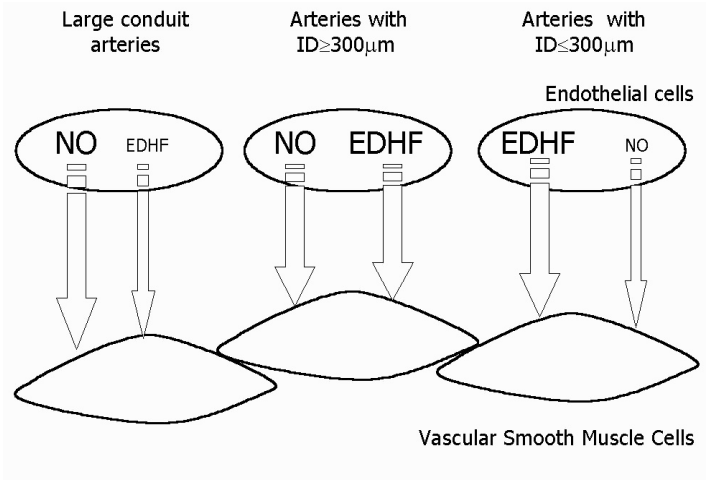

Figure 1. Generalized schematic representation of the balance between agonist-induced NO and EDHF release in arteries depending on the internal diameter (ID).

Given the fact that EDHF-mediated responses are most prominent after NOS inhibition, it seems logical to suggest that the continuous production of NO by endothelial cells (EC) could damp out the generation of EDHF and/or the activity of the proposed EDHF synthase. Therefore, hypothetically EDHF may act as a back-up endothelium-derived vasodilator when NO production is compromised. Indeed, in small mesenteric arteries from mice deficient in eNOS, an up-regulation of EDHF contribution occurs [387]. Exogenously applied NO at concentrations compatible to those achieved after stimulation with endothelium-dependent agonist attenuated EDHF-mediated dilatations in rabbit carotid and porcine coronary arteries, and effect was likely due to interference with synthesis and/or release of EDHF rather than its action *per se* [21]. A negative feedback inhibition of EDHF production by NO involving changes in Ca^{2+} signaling in EC has also been reported. The inhibition of NOS has been shown to enhance intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to the agonists [333] and a higher

endothelial $[Ca^{2+}]_i$ threshold requirement exists for EDHF- *vs* NO-mediated relaxation [239].

Since EDHF appears to be most important in small arteries, it is obvious that changes in the synthesis and/or release of EDHF are of critical importance for the regulation of organ blood flow, peripheral vascular resistance and blood pressure, and, above all, when compromised production of NO is evident. Depending on the scope of cardiovascular disorders, the altered EDHF responses may therefore contribute to [246, 382], or compensate for [51, 189] endothelial abnormalities associated with pathogenesis of several disorders. Consequently, identification of vessel-specific nature of EDHF, and selective activators or inhibitors of its biological activity, might have a significant impact on our understanding of vascular pathophysiology and provide the basis for novel therapeutic strategies [55].

Considering the importance of small arteries in the maintenance of blood supply requirements to the target organs, the prevalence of EDHF-mediated responses in these arteries plays a vital biological role. The accessibility of EDHF-mediated mechanism in addition to NO- and PGI_2-mediated dilatation may provide a "factor of safety" to preserve vasodilative capacity of the endothelium in circulation where endothelium-dependent relaxation appears to be of vital importance. The diverse nature of EDHF, as demonstrated in the same arteries by different research groups, may also reflect a flexibility of the mechanisms responsible for EDHF-mediated relaxation, which depends on the physiological or diseased state of the organism.

1.2 MECHANISMS OF EDHF RELEASE AND ACTION

The fact that acetylcholine (ACh) causes hyperpolarization of VSMCs was reported prior to the detection of the importance of the endothelium in dilatation [209]. The final recognition that agonist-induced hyperpolarization occurs through a release of an endothelial factor named EDHF was reached a few years later [33] after the classical study describing endothelium-dependent dilatation was published [134].

The overall picture of EDHF release and/or generation of a hyperpolarizing signal within EC and the response of VSMC are shown in Figure 2. The basic mechanism of EDHF-mediated response can be separated into two stages based on the place where the events occur. An increase in $[Ca^{2+}]_i$, activation of Ca^{2+}-dependent K^+-channels (K_{Ca}) and K^+ efflux followed by hyperpolarization, synthesis of substance or generation of signals capable of diffusing through membranes or myoendothelial gap junctions (MEGJ) to VSMC confer endothelial stage of EDHF-mediated response. The following stage reflects the mechanism by which endothelial hyperpolarization is transferred to VSMC. At the level of VSMC, EDHF activates K^+-channels and causes endothelium-dependent hyperpolarization (EDH) accompanied by closure of voltage-sensitive Ca^{2+}-channels (vCa^{2+}) that results in relaxation [43, 252].

The elevation of $[Ca^{2+}]_i$ in EC is a critical event for the synthesis or release of EDHF [61], however the relative role of either transmembrane Ca^{2+} influx or Ca^{2+} release from intracellular sources remains in dispute. It has been suggested that emptying of the intracellular stores of Ca^{2+} serves as a triggering pathway to initiate EDHF production,

while transmembrane Ca^{2+} influx through nonselective cation channels is an important step for sustained EDHF-mediated response [132, 368].

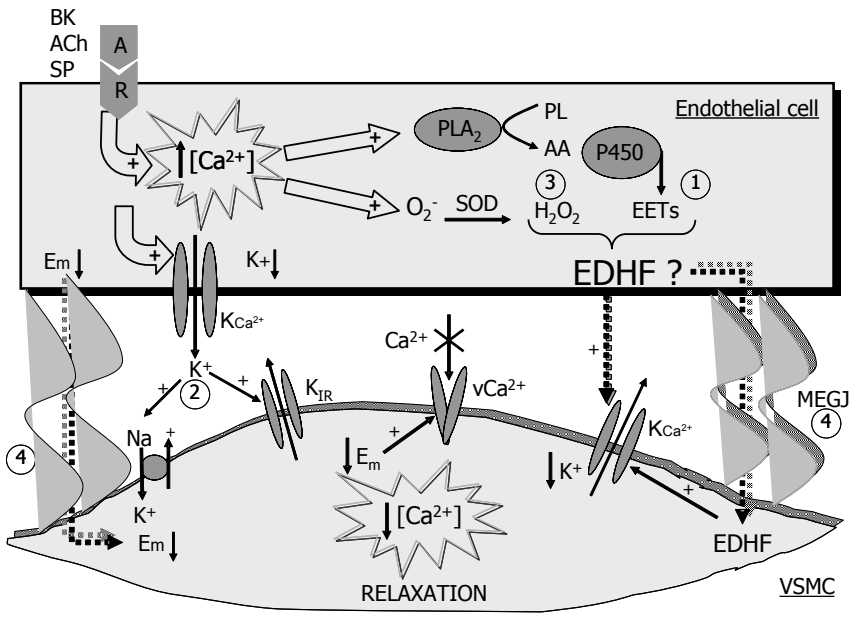

Figure 2. Summary of current view involving potential pathways in endothelium-dependent hyperpolarization.

Endothelium-dependent agonists (A) activate endothelial cell (EC) receptors (R) leading to the entry of extracellular and the release of intracellular Ca^{2+} and synthesis of EDHF. Along with the synthesis of EDHF, the hyperpolarization of ECs occurs, since Ca^{2+} activates Ca^{2+}-dependent K^+-channels (K_{Ca}^{2+}) and induces K^+ efflux. EDHF diffuses to the vascular smooth muscle cells (VSMCs), activates K_{Ca}^{2+}-channels and causes endothelium-dependent hyperpolarization. VSMCs contain voltage-sensitive Ca^{2+}-channels (vCa^{2+}) and a drop in membrane potential closes vCa^{2+}-channels and induces relaxation. Three main candidates for EDHF have been proposed: (1) Cytochrome P450 (CYP450) products, (2) potassium ions (K^+) and (3) H_2O_2. (1) CYP450 products: An increase in Ca^{2+} in EC activates phospholipase A_2 (PLA_2) known as the rate-limiting enzyme for the release of arachidonic acid (AA) from phospholipids (PL). AA is a substrate for superfamily of CYP450 enzymes (P450). Epoxygenase products of AA, epoxyeicosatrienoic acids (EETs), directly activate K_{Ca}^{2+}-channels in VSMCs and induce relaxation. (2) K^+ *per se*: the opening of EC K_{Ca}^{2+}-channels could result in an increase of extracellular K^+ that could hyperpolarizate VSMCs via the activation of the ouabain-sensitive electrogenic Na^+–K^+-ATPase and inward rectify K^+-channels (K_{IR}). (3) H_2O_2: an increase in Ca^{2+} in EC activates enzymes that produce superoxide anions (O_2^-) as a by-product. Superoxide dismutase (SOD) accelerates the dismutation of O_2^- into H_2O_2 and molecular oxygen. H_2O_2 activates K_{Ca}^{2+}-channels and causes hyperpolarization followed by relaxation. (4) Myoendothelial gap junctions (MEGJ) provide the means by which hyperpolarization of ECs is transferred to VSMCs. MEGJ facilitate the EDHF diffusion from the ECs to VSMCs or may serve as a channel for electrical signal transduction.

At the level of SMC, changes in $[Ca^{2+}]_I$ determine the constriction-relaxation process. It is important to note that in small arteries sarcoplasmic reticulum, an intracellular store of Ca^{2+}, is poorly developed [335]. This contrasts to large conductance vasculature, and therefore small arteries are exceptionally dependent on extracellular Ca^{2+} and its influx through vCa^{2+}.

The nature and distribution of K^+-channels involved in EDHF-mediated response within EC and VSMC has yet to be finalized. Initially, three channels have been considered, as only the combination of two toxins, i.e. charybdotoxin that blocks both large (BK_{Ca}) and intermediate conductance calcium-activated K^+-channels (IK_{Ca}), and apamin that inhibits small conductance K^+-channels (SK_{Ca}), as a rule, abolished EDHF-mediated responses [81]. Later, however, a combination of either charybdotoxin or apamin with selective inhibitors of BK_{Ca} or SK_{Ca} channels, has indicated an essential role for IK_{Ca} and SK_{Ca}, but not BK_{Ca}, channels [413, 414]. IK_{Ca} and SK_{Ca} are localized in EC and are not expressed in the VSMCs [112, 390], suggesting an importance of EC hyperpolarization in the EDHF-mediated response. Also, it has been shown that ECs do not express the BK_{Ca} channels whereas, SMCs densely express BK_{Ca} channels [137]. Finally, it can not be excluded that the synthesis and action of EDHF involves novel K^+-channels either on EC and/or on VSMC [413].

The mechanisms responsible for changes in K^+ dynamics at the level of VSMC during EDHF-induced hyperpolarization appear also to be heterogeneous and involve BK_{Ca}, inwardly rectifying K^+-channels (K_{IR}) or K^+_{ATP} and Na^+–K^+-ATPase [211, 386]. Therefore, the selective inhibitor of BK_{Ca} iberiotoxin does not serve as a universal blocker of EDH at the level of SMC. The variety of mechanisms involved in EDH at the level of VSMC seems to depend on different pathways and currently it is anticipated that EDHF may not act as a single factor. It is more likely that differences on the nature and cellular targets of EDHF exist depending on species, tissue type, and whether or not it is in a healthy or diseased state.

To date the cytochrome P450 (CYP450) products of AA [307], potassium ions [110], hydrogen peroxide [244] and C-type natriuretic peptide (CNP) [54] have been introduced as the potential candidates for EDHF. EC hyperpolarization could be also transmitted to SMC through myoendothelial gap junctions [58] that are clusters of intercellular channels formed by connexin (Cx) proteins. The potential candidates for EDHF are introduced below and above in Figure 2. For more comprehensive details please see recent reviews [43, 128, 151, 252, 329].

1.2.1 EDHF or NO is EDHF?

Classically, EDHF mediated response is a hyperpolarization with subsequent relaxation maintained after inhibition of NO and prostaglandins, namely PGI_2, synthesis. Since both NO and PGI_2 in certain circumstances and in some types of arteries and/or species may also hyperpolarize the SMC, theoretically, they could be considered as EDHF. Because all available cyclooxygenase (COX) inhibitors completely abolish the prostaglandins production in vasculature, any endothelium-dependent hyperpolarization observed in the presence of one inhibitor is unlikely to be mediated by PGI_2 [119]. In contrast, inhibitors of NOS do not entirely block the production of NO [76]. Furthermore, some vessels (e.g. human coronary artery) are able to generate NO from a

non-L-arginine source, and NOS inhibitor-insensitive production may take a place [193]. NO can also be stored and under circumstances of restricted NO production, could be released independently from NOS [119]. Recently Stankevicius et al (2006) reported that activation of apamin and charybdotoxin-sensitive K^+-channels, which are unique characteristics of EDHF-mediated relaxation, induce hyperpolarization of ECs and NO release in the rat mesenteric artery [343], although the NO release after incubation with apamin and charybdotoxin could be up-regulated by abolishment of EDHF-mediated mechanism.

Considering the fact that NO-dependent responses, involving the hyperpolarization, as a mechanism of relaxation, may occur due to incomplete inhibition of NOS pathway, NO scavengers or inhibitors of guanylate cyclase are encouraged for use to rule out a residual contribution of NO in EDHF-mediated response. Although, it is important to note that the hyperpolarization mediated by NO requires a 40-fold higher concentration than that necessary to mediate relaxation through guanylate cyclase pathway [157]. Nevertheless, when all approaches have been covered, a third pathway of endothelium-dependent relaxation (i.e. EDHF) has been demonstrated in a variety of blood vessels [376].

"The best way to get a good idea is to get a lot of ideas"

Linus Pauling (1901-1994)
The Nobel Prize in Chemistry 1954,
The Nobel Peace Prize 1962

1.3 THE PATHWAYS TO EXPLAIN EDHF-MEDIATED RELAXATION

Several criteria must be considered while identifying the mediator for EDHF [297]. First, EDHF-mediated relaxation should be basically abolished by interventions that affect synthesis, release or effect of the candidate *per se*. Second, the proposed substance should mimic EDHF-mediated relaxation. Third, the candidate has to be derived from the endothelium in an amount sufficient to initiate EDHF-mediated response. Finally, the agents that are known to increase the synthesis, release or effect of the candidate, have to be able to potentate EDHF-mediated relaxation.

Several candidates exist that fulfill the criteria listed above, and two general pathways that explain EDH (i.e. diffusible factors and contact-mediated mechanisms) are suggested [314]. Diffusible factors are endothelium-derived substances that are able to pass through internal elastic lamina (IEL), reach underlying SMC at a concentration sufficient to activate ion channels, and initiate smooth muscle hyperpolarization and relaxation. Contact-mediated mechanisms confer to endothelial hyperpolarization that passively spreads to the smooth muscle through intercellular coupling and therefore EDH considered as a solely electrical event.

1.3.1 Diffusible factors

1.3.1.1 Epoxyeicosatrienoic acids

Historically, the metabolites of AA were the first candidates suggested for the role of EDHF [308], although currently there is strong supporting evidence for it mostly in

coronary circulation as demonstrated in several species including humans [40]. A number of enzymes metabolize AA into numerous compounds that influence the tone of blood vessels. In a variety of arteries EDHF-mediated responses are inhibited by inhibitors of phospholipase A_2 (PLA_2), the enzyme responsible for the liberation of AA from membrane phospholipids [2, 407].

Despite a variety of prostaglandins derived from AA through the COX pathway, the products of AA generated by another metabolizing enzyme were proposed to be responsible for the synthesis of EDHF. Epoxygenase CYP-450 products of AA, notably 5,6-; 8,9-; 11,12-; or 14,15- epoxyeicosatrienoic acid (EETs) have been suggested to serve as EDHF, at least, in some vascular beds.

The idea that EETs are EDHF is based on the following observations. 1) Endothelium-dependent agonists [47, 109], pulsative stretch [126] and shear stress [177], all stimulate the EETs release. 2) The CYP450 epoxygenases are expressed in ECs of coronary arteries [34, 125]. 3) Pharmacological inhibition of CYP-450 epoxygenases by sulfaphenazole and so-called "EET antagonists" nearly abolishes the EDHF-mediated responses, at least in some arteries [136]. 4) Incubation of porcine coronary arteries with antisense oligonucleotides against the coding region of DNA for CYP-450 epoxygenase markedly reduced its mRNA and protein, and attenuated EDHF-mediated hyperpolarization and relaxation without compromising responsiveness to endogenous or exogenous NO [34, 125]. 5) EETs induce relaxation in endothelium-denuded arteries and activate BK_{Ca}-channels as well as Na^+–K^+-ATPase in native and cultured SMCs [296]. 6) The vasodilator potency of EETs increases with the reduction in vessel size [297], and conduit vessels such as the aorta do not normally synthesize EETs [287]. 7) NO antagonizes heme-containing proteins such as CYP450 [259] and may theoretically explain the inhibitor influence of NO on EDHF-mediated responses. 8) Finally, an inducer of CYP450 β-naphthoflavone enhances the formation of EETs, as well as EDHF-mediated hyperpolarization and relaxation in native ECs, at least, in porcine coronary arteries [125].

In humans, the CYP450-dependent, EDHF-mediated responses have been observed in coronary [261] and mammary [12] arteries, in forearm [159] and in skeletal circulation [169], although EDHF-mediated relaxation in mesenteric [242], myometrial [195], and renal arteries [44], appeared to be CYP450 independent. A clear diversity exists in respect to subcutaneous circulation [74], and currently the role of EETs acting as EDHF is questioned for several reasons. First, some of CYP450 inhibitors act non-specifically on K-channels which are directly involved in EDHF-mediated relaxation [252]. Secondly, in only few cases (i.e. coronary artery) iberiotoxin alone inhibits EDHF-mediated relaxation [293], while only a combination of apamin and charybdotoxin effectively inhibits the EDHF-mediated response. Finally, since EETs are lipophilic, their diffusion from the endothelium to the VSMC down a concentration gradient will be too slow to confer a rapid EDHF-mediated response, and molecules of EETs are not able to pass through MEGJ.

Recently, Fleming et al (2004) suggested that EETs may serve as key messengers or modulators of EDHF-mediated response rather than EDHF *per se* (i.e. a factor that diffuses from the endothelium to the VSMC) [128]. Indeed, metabolites of CYP450 epoxygenase may regulate Ca^{2+} entry into EC [311], activate endothelial $K_{Ca}{}^{2+}$ channels

[19, 220] and facilitate gap junctional communication via protein kinase C-dependent processes [292].

1.3.1.2 Hydrogen peroxide

The hypothesis that H_2O_2 could serve as a possible candidate for EDHF was proposed in 1991 [25] due to the evidence that H_2O_2 is produced by EC, and could relax and hyperpolarize SMC. However, at that time, there was no supporting evidence, a few years later, H_2O_2 was reconsidered as a candidate but only in conditions associated with limited availability of L-arginine [83]. At that time, links between H_2O_2 and EDHF were considered either weak or nonexistent [117, 377].

However, Matoba et al (2000) has argued based on experimental evidence in mesenteric arteries from mice [244], human [242], porcine [243] and canine [401] coronary arteries that H_2O_2 fulfils the criteria for EDHF, since catalase, a specific inhibitor of H_2O_2, abolished EDHF-mediated hyperpolarization and relaxation [330]. Afterward, it has also been reported that endothelium-derived H_2O_2 is an EDHF in human coronary [260] and piglet pial microvessels [212].

The capacity of ECs to produce superoxide anions (O_2^-) from several intracellular sources, including eNOS, lipoxygenases, COX, CYP450 epoxygenases and NAD(P)H oxidases, is well known [331]. They are converted by superoxide dismutase (SOD) to H_2O_2, which may stimulate ion channels on the VSMCs by increasing K^+ conductance and causing hyperpolarization followed by relaxation. However, the mechanism of H_2O_2-induced hyperpolarization appears to be complex and different types of K^+ channel might be involved. It is also possible that H_2O_2 non-selectively targets various K^+ channels in VSMC and response of these channels (activation or inhibition) may vary between vascular preparations studied [115].

Since two products of NOS, NO and O_2^- react spontaneously, they would be expected to suppress each other's effects. In order to form H_2O_2, O_2^- has to escape interacting with NO and, under normal conditions, only a small amount of O_2^- will survive long enough to act with SOD and generate H_2O_2. Normally NO overcomes H_2O_2 production and serves as a main endothelium-derived vasodilator. In contrast, in diseased conditions, the production of O_2^- (largely from sources other than eNOS) will overwhelm the production of NO [379]. Thus, the relative contribution of NO *vs* H_2O_2 to control the vascular tone will be inversely proportional to each other and the appearance of one is likely to compensate for the absence of the other. In the presence of oxidative stress when deactivation of NO occurs, it is possible that H_2O_2 production will compensate the impairment of endothelium-dependent relaxation. If this hypothesis is correct, there should be then an increased contribution of H_2O_2 in the diseased state [370]. Some studies report that in tetrahydrobiopterin-deficient mice uncoupled eNOS can serve as a source for H_2O_2 and an increased contribution of H_2O_2 to endothelium-dependent relaxations has been demonstrated as a compensatory response [82, 83, 214]. Recently, a compensatory cardioprotective role of endogenous H_2O_2 has been implicated in coronary ischemia-reperfusion injury against loss of NO contribution [400].

However, several studies failed to show that catalase inhibits L-NAME and indomethacin-resistant relaxations [133, 160, 233, 291]. In contrast to Matoba et al (2000) [244], extended work by Ellis et al (2003) [114] have failed to confirm the existence of a catalase-sensitive pathway, and therefore H_2O_2-mediated, endothelium-dependent relaxations in small mesenteric arteries from the same strain of mice (C57BL/6).

Moreover, H_2O_2 candidacy is questioned by the facts that it has an inhibitory action on K^+ channels, at least in some vascular beds [115]. H_2O_2 has been shown to alter the activity of pathways controlling intracellular calcium homeostasis, including Ca^{2+}-ATPase pump, Na^+–K^+-ATPase and some Ca^{2+}-channels that may lead to an increase in intracellular Ca^{2+} levels followed by SMCs contraction [115, 405,349].

It is more likely that H_2O_2 may indirectly affect the other pathways responsible for relaxation. For example, the production of AA can be induced by H_2O_2 [115] and is followed by the production of different metabolites that, in turn, could influence EDHF-mediated responses or serve as EDHF *per se*. Recent studies reported that H_2O_2 up-regulates eNOS expression *in vitro* and *in vivo* [105] and H_2O_2 acutely stimulates NO production by eNOS [361].

It can not be excluded that H_2O_2 is endothelium-derived vasodilator, but it is not an EDHF. It was demonstrated that in isolated rat femoral arteries H_2O_2 derived from eNOS, since catalase reduced ACh-induced relaxations in physiological salt solution (PSS), but had no effect on residual relaxation after L-NAME [216]. The finding is in accordance with a previous study in which the production of H_2O_2 was impaired in eNOS-knockout mice [244]. Also, in rabbit iliac arteries NO/prostanoid-independent relaxations evoked by A23187 were mediated by H_2O_2, but could not be regarded as EDHF, as the catalase-sensitive component was not associated with smooth muscle hyperpolarization [57]. Recently, Drouin et al (2007) reported that eNOS-derived H_2O_2 is an endothelium-derived vasodilator in pressurized cerebral arteries from mice and H_2O_2 shares a similar dilatory pathway with NO since H_2O_2-induced dilation was prevented by ODQ, the soluble guanylate cyclase inhibitor [104].

1.3.1.3 Potassium ions

The activation of endothelial K_{Ca}^+ channels causes an efflux of K^+ from ECs towards the extracellular space. Theoretically, this could increase the concentration of extracellular K^+ in the space between the endothelium and media. An increase of extracellular K^+ within the range of 1 to 10 mmol/l has been shown to activate the ouabain-sensitive electrogenic Na^+–K^+-ATPase and K_{IR}-channels and followed by hyperpolarization and SMC relaxation. Using a K^+-selective microelectrode in rat hepatic arteries, Edwards et al. (1998) [110] reported that the ACh-mediated increase in K^+ from 4.6 to 11.6 mmol/l occurs in the exstracellular space between ECs and SMCs. This has been further supported by studies in other vascular beds, including humans [110, 254, 271, 369]. However it was later opposed by the facts that combination of Ba^{2+} and ouabain failed to influence EDHF-mediated relaxation and an increase in extracellular K^+ does not mimic EDHF dilatation in arteries from both humans and animals [9, 74, 77, 415]. The involvement of K^+ ions into EDHF-mediated

relaxation through gap junctions is discussed below (please see Figure 5) and this event does not necessarily involve the activation Na^+–K^+-ATPase and inward rectifying K^+-channels. It's more likely that K^+ ions and gap junctions can be involved in EDHF-mediated relaxation simultaneously or sequentially and may also act synergistically [40, 119].

1.3.1.4 C-Type Natriuretic Peptide

C-type natriuretic peptide (CNP), a member of the natriuretic peptide family, has been shown to exert a variety of cardiovascular effects including vasodilatation and hyperpolarization of arteries through the opening of K_{Ca}^+-channels [54, 350]. CNP is widely distributed in the cardiovascular system and it has been found at high concentrations particularly in ECs [62]. Therefore, endothelium-derived CNP has been proposed as a putative hyperpolarizing factor [55, 393], and its action has been suggested to be associated with specific C subtype of natriuretic peptide receptor (NPR-C) followed by G_i-dependent activation of G protein-gated K_{IR}-channels in the vascular smooth muscle that brings hyperpolarization and, thereby, relaxation [55]. However, the expression and activity of G protein-gated K_{IR}-channels at the VSMCs level is far from clear. Moreover, there is no direct evidence to suggest that CNP can activate G protein-gated K_{IR}-channels [119]. In addition, CNP induces weak relaxation and hyperpolarization of coronary arteries, and it is unlikely that CNP acts as a mediator for endothelium-dependent hyperpolarization, at least, in those arteries [20]. On the other hand, endothelium-derived CNP could reduce ischemia–reperfusion injury [171] and participate in anti-inflammatory and anti-atherogenic processes within the vascular wall [5].

1.3.2 Contact-mediated mechanism

A second suggestion to explain the mechanism for EDHF-mediated response is that hyperpolarization generated in EC could spread electronically to the underlying SMC through direct cell-cell coupling. Indeed, analysis of transmission electron microscopy (TEM) pictures indicates that cells within the vascular wall are coupled through both homocellular and myoendothelial gap junctions (MEGJs) and pharmacological influence on these channels has crucial impact on vasodilatory and vasoconstrictory responses [99, 309].

Gap junctions are clusters of transmembrane channels that cross the intercellular gap and allow the transfer of ions and second messengers (water-soluble molecules less that 1 kDa) between adjacent cells. For example, to form MEGJ, the EC and VSMC must shape extensions that could pass through penetrations in the internal elastic lamina (IEL) and create contacts with each other throughout the structure of aqueous pore. This can only occur when the distance between cells (i.e. thickness of IEL) is not too big. Therefore, the incidence of MEGJ is inversely correlated with arterial diameter, suggesting that MEGJ plays a predominant role in smaller vessels [168].

1.3.2.1 Gap junction structure

Each gap junction is composed of two hemi-channels, termed connexons, each provided by one of the two cells. Although a gap is left between the adjacent cell membranes, two connexons interact to form a tightly sealed, double-membrane intercellular channel [323]. Each connexon is composed by six protein subunits connexins (Cx, Figure 3). More than 20 Cxs have been identified, but only Cx37, Cx40, Cx43 and occasionally Cx45 are detected in endothelium and/or smooth muscle, and the expression of them differs depending on the species and vascular bed studied [168, 411]. Connexons can be assembled from one Cx (homomeric connexon) or more than one Cx (heteromeric connexon). Thus, a gap junction may be composed by two identical homomeric connexons (homotypic junction) or by two connexons of different heteromeric or homomeric composition (heterotypic junction) [341].

All members of the Cx family share a common architecture: four hydrophobic transmembranous domains (1–4), two extracellular loops (EL1 and EL2), and three cytoplasmic domains, including an intracellular loop (IL) and carboxy- and amino-terminal domains (Figure 3). Two extracellular loops, containing between 31 and 34 amino acids are highly conserved and connected by three invariant disulphide bonds, and are crucially dictate the formation of connexons and gap junctions as well.

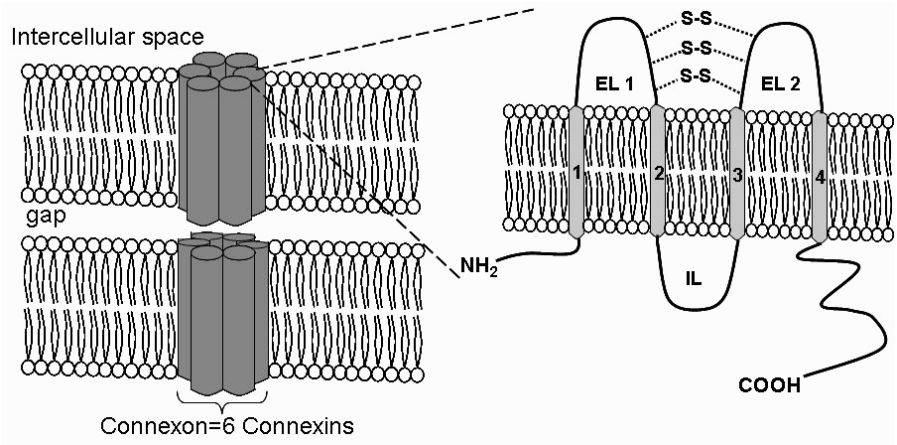

Figure 3. Common architecture of gap junction and connexin.

1.3.2.2 Connexins within the vascular wall

The expression of Cx is not EC and SMC specific, although differences in expression pattern have been found. The presence of Cx40 and Cx37 is mainly attributed to the endothelium [168, 230]. In contrast, expression of Cx43 in the endothelium is controversial and appears to vary with vessel type, size, species and disease conditions [135, 174, 240, 340].

Cx distribution in smooth muscle is less clear and all vascular Cx [374] as well as absence of them [156] has been reported. Cx43 has been generally considered to be predominant in the media [325]. However, the majority of studies have reported the

expression of Cx43 in VSMC of large elastic arteries, such as the aorta [174, 325, 374], whereas, expression of Cx43 in media of muscular arteries is not so obvious [174, 240]. Thus, Cx expression is not identical in all blood vessels although Cx profiles in different parts of the vasculature have not been completely described yet. The differences in Cx expression has also been related to the disease, such as hypertension [187, 309] and atherosclerosis [158, 210], supporting their role in the genesis of vascular disorders.

1.3.2.3 A knockout approach

The development of Cx knockout animals has helped to define the role of Cx subtypes in cardiovascular function. Cx40 knockout animals (Cx40(-/-)) exhibit diminished conduction of dilatation in response to ACh or BK and they are hypertensive [96, 123]. Cx40(-/-) arterioles exhibit spontaneous, irregular vasomotion leading temporarily to complete vessel closure [97]. The results suggested an important role of Cx40 in microcirculation and in the control of blood pressure in mice [158]. Hypertension in Cx40(-/-) is not related to the renin-angiotensin system or changes in action or release of NO or other endothelial factors [97].

Mice lacking Cx37 are viable and have a normal cardiovascular phenotype, although females are infertile [336]. Since Cx37 and Cx40 are co-expressed in ECs and could overlap functionally, the role of junctional communication may only be revealed after elimination of both. Indeed, Cx37-/-Cx40-/- animals display severe vascular abnormalities with pronounced vessel dilatation and congestion and they die perinatally [337].

Cx43 knockout mice also die perinatally probably due to pulmonary and cardiac rather than vascular abnormalities [337]. However, a recent study indicates that dysregulated coronary vasculogenesis plays a key role in cardiac abnormalities in Cx43 knockout mice suggesting an essential role for Cx43 in vasculogenesis and remodeling [389]. To investigate the function of Cx43 in EC, independent from the role that Cx may play in SMC, an endothelial-specific Cx43 deletion was studied in mice. It has been reported that the loss of Cx43 in the endothelium was associated with hypotension and bradycardia [222] and was accompanied by enhanced levels of NO, plasma angiotensin I and angiotensin II [222]. However in another study, lack of Cx43 in ECs had no effect on resting blood pressure [360].

Thus, investigations of vascular function in Cx knockout animals show that Cx are crucial for the function of vasculature, especially at level of microcirculation and contribute to the physiological control of peripheral vascular resistance and as a consequence to the control of blood pressure. However, the knockout approach to study the contribution of specific Cx to the construction of MEGJ needs to be further explored more productively.

1.3.2.4 A pharmacological approach

The verification of MEGJ's contribution to EDHF-mediated responses relies on the use of a diverse range of compounds, which inhibit intercellular communication across gap junctions. Licorice derivates (glycyrrhetinic acid and carbenoxolone), long-chain

alcohols, such as heptanol and, more recently, Cx-mimetic peptides have been utilized, although the majority of them has been questioned due to a variety of non-specific effects, or there have been only few electrophysiological studies about their effects on electrical coupling [78].

The glycyrrhhetinic acid (GA) metabolites (18-α-glycyrrhetinic acid (18α-GA), 18-β-glycyrrhetinic acid and carbenoxolone) have been used to uncouple gap junction channels. The molecular mechanisms of their inhibitory actions are still unknown, although phosphorylation or changes in the aggregation of Cx subunits have been suggested [99]. The 18-α-GA is most popular [92, 167, 233, 241, 245] due to experimental evidence that the α-form is more specific and less toxic than other GA compounds [91]. However, debate continues with regards to if GA compounds could also alter the activity of ion transport processes, including ion channels [357] or if their action is indeed specific on gap junctions [147, 241].

1.3.2.5 Connexin mimetic peptides

Recently a new approach has been introduced and *connexin mimetic peptides* (CMPs) have been proposed to be highly selective and specific. The most frequently used CMPs, Gap26 and Gap27, correspond to an amino acid sequence on the first and second extracellular loops of certain Cx (Figure 4). Gap 26 and Gap 27 peptides appear to act in a Cx-specific manner and have now been widely applied to block gap junctions composed of Cx37, Cx40 and Cx43. Indeed, CMPs do not suppress endothelial hyperpolarization directly and they do not influence relaxation to exogenous nitrovasodilators or K_{ATP} channel openers [151].

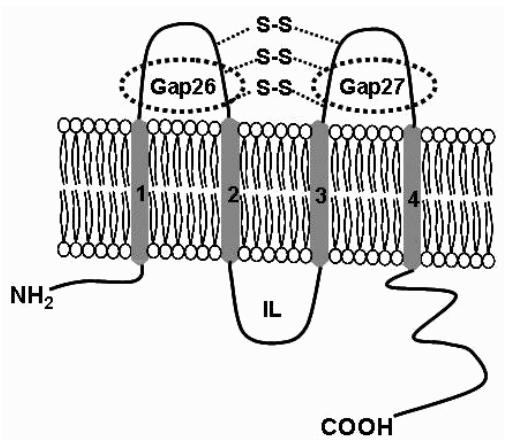

Figure 4. Gap 26 corresponds to amino acid sequences in the first and Gap 27 in the second extracellular loops of certain Cxs and it makes docking of them impossible.

CMPs inhibit intercellular channel formation rather than disrupt existing gap junctions, since they inhibit connexon formation. Therefore, a relatively long incubation time is required to affect the gap junctions [116]. Blockade by CMPs is effected from the outside of the cell membrane and is reversible, with selectivity in targeting specific Cx

subtypes according to the sequence homology [152]. Thus, different combinations of CMPs might deduce a functional importance of certain Cx subtypes. Recently, Chaytor et al, 2005 [56] demonstrated that specific CMPs could be employed to inhibit electronic signaling *via* myoendothelial and homocellular smooth muscle gap junctions in a selective fashion.

Gap27 and Gap 26 have been shown to attenuate the endothelium-dependent hyperpolarization and relaxation without influence on NO- and prostanoids-mediated responses both in isolated arteries and *in vivo* [58, 94, 153]. Paired or triple CMPs combinations were used to target more than one Cx subtype to attenuate EDHF-mediated relaxation [26, 57, 59, 372], suggesting that in general more than one Cx subtype is involved in construction of MEGJs. A triple combination of CMPs has been recommended as the most effective and reliable way to block vascular gap junctions [151].

However, CMPs may not clarify the relative role of MEGJs *vs* gap-junctional coupling within the endothelial or smooth muscle layers (and not between the different layers). Moreover, Edwards et al, 2000 argued that CMPs preferentially block homocellular smooth muscle gap junctions, rather than MEGJs, at least in porcine coronary arteries [111], and CMPs failed to modify muscle hyperpolarization immediately below the IEL, but spread through the media was markedly reduced after 60 min artery pre-exposure to 500μM of Gap 27 [111]. Also, despite a high incidence of MEGJs in murine mesenteric arteries, neither ^{37,40}Gap26 nor ^{37,43}Gap27, nor the two in combination, significantly reduced the EDHF response in these arteries [103].

1.3.2.6 What flows through MEGJ to initiate EDHF-mediated response?

There are at least two possible scenarios. One suggests that MEGJ offer a way for the passage of diffusible factors indicated for EDHF. This will speed up the diffusion of endothelial factors towards SMCs without the dilutional effect of transfer through the extracellular space. However, not every factor could pass through MEGJ due to the size or solubility, as for example, lipophilic compounds such as EETs will fail to pass in contrast to charged water-soluble species [39]. H_2O_2 is one of the most stable reactive oxygen products and it can easily cross cell membranes without passing MEGJ.

The second possibility involves a direct transfer of charge or small signaling molecules between EC and SMC. The transfer of charge represents the current of ions passing from EC to SMC or visa versa, and candidate molecules have to initiate the changes in SMC membrane potential. There is some support for direct exchange through MEGJs of different molecules and ions (e.g.,cAMP, IP_3, Ca^{2+}, K^+). cAMP formed in EC may diffuse via MEGJ to reduce smooth muscle tone via activation of K_{Ca}^{2+} channels, through phosphorylation of cAMP-dependent protein kinase A (PKA) and myosin light chain kinase, or enhanced sequestration of Ca^{2+} within the sarcoplasmic reticulum [154]. It may also enhance the EC hyperpolarization and augment electronic spread of endothelial hyperpolarization through the vascular wall [153, 154] (Figure 5), however then EDHF-mediated response would be similar to that induced by PGI_2 and could be reduced by COX inhibitors, but this is not the case [43].

K^+ may carry the current through MEGJ followed by subsequent hyperpolarization (Figure 5) and the movement of K^+ out of vascular SMC through MEGJ to the endothelium and eventually to the extracellular space and would produce a net hyperpolarization of the media [40]. However, whether a single layer of ECs can drive the hyperpolarization of multiple layers of SMCs is unclear, although the structure of vascular wall contributes to the maximal connection between EC and SMC. Indeed, SMCs are fusiform cells running circularly around blood vessel, whereas, ECs are aligned parallel to the longitudinal axis of the vessel, therefore, one EC crosses about twenty SMCs [24] and the majority of SMCs within the vascular wall of small arteries could be connected with at least one EC.

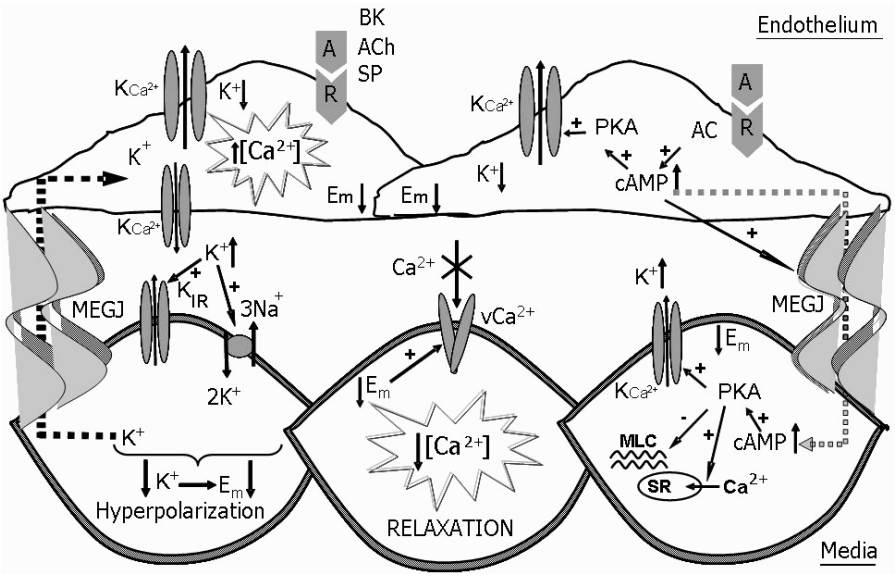

Figure 5. Schematic presentation showing possible mechanisms responsible for the transformation of endothelial hyperpolarization to smooth muscle layer through MEGJ.

Efflux of K^+ from the endothelium results in hyperpolarization of SMCs through the activation of ouabain-sensitive electrogenic Na^+-K^+, ATPase and inward rectify K^+-channels (K_{IR}). Efflux of K^+ in opposite direction is also possible through MEGJ to compensate the deficiency of positive charge in hyperpolarized ECs and, in turn, it results in hyperpolorazation of SMC. Elevation in endothelial cAMP levels could be involved in hyperpolorazation of both ECs and SMCs mainly through protein kinase A (PKA) dependent activation of K^+ channels. cAMP could enhance MEGJ permeability, and, when passing through MEGJ, cAMP may influence contractile apparatus of SMC via phosphorylation of the myosin light chain (MLC) kinase and enhance sequestration of Ca^{2+} within the sarcoplasmic reticulum (SR).

"Truth in science can be defined as the working hypothesis best suited to open the way to the next better one."

Konrad Lorenz (1903-1989)
The Nobel Prize in Physiology and Medicine 1973

1.3.3 Summary

Currently, the term *endothelium-derived hyperpolarising factor* is misleading since EDHF may represent a mechanism rather than a specific factor *per se*. The mechanisms of endothelium-dependent hyperpolarization (i.e. EDHF-mediated relaxation) seem to be heterogeneous depending on several issues (e.g. size and vascular bed), surrounding environment (oxidative stress, hypercholesterolemia) and demand (compensatory). There is also the possibility that different endothelial mediators or pathways involved in EDHF-mediated relaxation could work simultaneously and support or interchange the contribution of each other depending on the situation. Certainly, all known so far diffusible factors suggested for the role of EDHF are involved into the control of vascular tone to a greater or lesser degree although not all of them are fit entirely to the classical definition of EDHF. For example, generation of H_2O_2 and the production of EETs or CNP do not necessarily require the hyperpolarization of ECs. It has been suggested that CYP450 epoxygenase metabolites may serve as messengers or modulators of EDHF and H_2O_2 could be a separate endothelium-derived vasodilator rather than a factor working through the EDHF-mediated pathway. Therefore, it is quite reasonable requirement has been raised by Feletou&Vanhoutte (2006) in recent review paper [119]. Once the involvement of a certain endothelium-derived vasodilator is confirmed in a given vascular bed, it must be referred to by their proper name, i.e., endothelium-derived H_2O_2, EETs, and CNP, and should no longer be termed "EDHF" [119].

1.4 EDHF-MEDIATED RESPONSES IN NORMAL PREGNANCY AND PREECLAMPSIA

1.4.1 Normal pregnancy

Maternal cardiovascular adaptation to pregnancy is associated with decreased peripheral vascular resistance that plays an important role for blood pressure reduction despite an increase in plasma volume and cardiac output. The vascular adaptation to pregnancy mainly depends on enhanced endothelium-dependent dilatation [29, 149].

It is now generally accepted that NO plays an important role as a systemic vasodilator in pregnancy [338]. More recently, a role for EDHF has been introduced [143] and several studies have suggested that EDHF may play an important role in the enhancement of endothelium-dependent relaxation, as demonstrated in vasculature of pregnant rats [89, 138]. However, an equal (i.e. mesenteric artery) or even reduced (i.e. uterine artery) contribution of EDHF has been reported in pregnant mice [80].

The anticipation that human pregnancy is associated with an enhanced contribution of EDHF is less conclusive [199, 233, 286] and an interindividual heterogeneity, vascular

bed specificity, choice of agonist used to characterize the relative contribution of NO and EDHF to endothelium-dependent relaxation may play an important role. Arteries obtained from subcutaneous fat circulation in normal pregnant (NP) women demonstrate a residual bradikynin (BK)-induced relaxation after incubation with inhibitors of NO and PGI_2 production. This endothelium-dependent relaxation was significantly higher, as confirmed by differences in EC_{50} values, compared to that in non-pregnant women [199]. In contrast, Ang et al (2002) failed to show any difference in the contribution of EDHF to carbachol-induced relaxation in the same arteries between pregnant and non-pregnant women [10]. An endothelium-dependent relaxation in response to both ACh and BK was completely accounted for EDHF in small omental arteries, however no difference existed in EDHF-typed contribution between pregnant and non-pregnant women [286]. In myometrial arteries from NP women, the absence of NO has been shown to be compensated by EDHF, the effect was not apparent in arteries from non-pregnant women [194].

The mechanisms responsible for the up-regulation of EDHF-mediated responses in pregnancy remain unclear and could be partly attributed to increased levels of circulating estrogen in the pregnant *vs* non-pregnant state. Moreover, the mechanism of EDHF-mediated response may differ in certain vascular beds in the pregnant *vs* non-pregnant state. Pascoal and Umans (1996) showed that EDHF-mediated response to BK is abolished after incubation with inhibitor of non-selective K-channels in omental arteries from non-pregnant but not in NP women, suggesting different mechanism behind EDHF [286]. Recently, the pathway for EDHF type responses in human pregnancy has been explored in small myometrial arteries [194], in which gap junctions were implicated [195]. In support, the contribution of EDHF to ACh-induced relaxation is amplified in the aorta from pregnant rats due to an increased contribution of gap junction communications [89] and increased expression of mRNA for Cx43 found not only in the thoracic aorta but also in mesenteric and uterine arteries [89].

1.4.2 Preeclampsia

Preeclampsia (PE) is a disorder specific to human pregnancy. It is characterized by hypertension (i.e. blood pressure ≥140/90 mmHg after the 20th week of gestation in previously normotensive women), proteinuria and other systemic disturbances occurring after 20 weeks of gestation and resolving after delivery. Early onset of PE (i.e. <32 weeks) accompanied by multi-organ involvement, haemolysis, elevated liver enzymes and low platelets (HELLP syndrome), renal impairment, pulmonary oedema and/or severe central nervous system symptoms is referred to as “severe” PE that results in a higher rate of growth restricted neonates [334]. Growth restriction will increase the risk of cardiovascular diseases and diabetes in adulthood. Current epidemiological evidence implies that women with a history of PE have an increased risk to develop hypertension, coronary and cerebro-vascular disease [236].

PE occurs in 2-5% of pregnancies and continues to be a major cause of maternal morbidity and mortality with 15-20% of the total maternal mortality in developed countries [334], whereas in developing countries maternal mortality is more common (up to 3 times higher), accounting for 50000 deaths yearly [107]. Despite intensive research the aetiology of PE is elusive, this multisystemic syndrome cannot yet be

prevented and current treatment, as it has been for the last 100 years, is the delivery of the fetus and placenta. There are many risk factors for PE including genetic factors, pregnancy–related factors (e.g. primiparity, multiple pregnancies etc.), and maternal constitution (obesity, diabetes etc.) [106]. However, this does not always, even with a combination of several risk factors, result in the development of PE.

It is generally agreed that PE originates in the placenta where an inadequate trophoblastic invasion in uterine spiral arteries results in poor remodeling of arteries with followed reduction in uteroplacental perfusion. This is called poor placentation and is considered the first stage of PE. The hypoxic and dysfunctional placenta is presumed to release factors into the maternal circulation that cause the second stage of this condition, multisystemic maternal syndrome, that can be ascribed to generalized endothelial dysfunction [302]. Furthermore, poor placentation increases the level of trophoblast debris to the maternal circulation, which elicits an inflammatory response and further endothelial dysfunction [300].

Several factors have been implicated for initiation of maternal endothelial abnormalities, but none of them has yet proved to be causative *in vivo* [299]. The strongest candidate is the soluble receptor for vascular endothelial growth factor (VEGF), also known as sFlt1 (soluble fms-like tyrosine kinase 1) [344]. sFlt1 binds VEGF and placental growth factor (PlGF) and in this way it deprives the systemic endothelium from essential survival factors [299,188]. However, an increase in sFlt1 does not seem to be solely specific to PE, as its increase has been observed in intrauterine growth restriction as well [344]. Moreover, sFlt1 is not increased in all women with PE, including some with even severe syndrome [218]. An additional molecule, i.e. soluble endoglin seems to cooperate with sFlt to induce endothelial malfunction and induce clinical signs of severe PE, including development of the HELLP syndrome and restriction of fetal growth [383].

PE seems to be extremely heterogeneous in pathogenesis since reduced placental perfusion must interact with maternal factors to result in clinical manifestation of PE. Poor placentation does not always lead to PE [302]. Poor placentation is also present in intrauterine growth restriction and in about one third of the cases of spontaneous preterm birth [302]. There is a point of view that pregnancy *per se* is a metabolic and vascular stress test [319], which reveals a woman's health in later life and which is consistent with the higher incidence of ischemic heart disease, stroke, and hypertension that becomes evident many years after an episode of PE [196, 299].

1.4.3 Endothelial dysfunction in preeclampsia: role of EDHF

Generalized maternal endothelial dysfunction is conferring clinical signs such as hypertension, proteinuria and edema. Endothelial dysfunction in PE is confirmed by alterations in endothelium-dependent relaxation [150, 381]. This in line with hemodynamic changes, increased vascular responsiveness to vasoconstrictors, reduced placental perfusion, platelet activation and activation of hemostatic system, which are all characteristic of maternal syndrome PE [381]. Vascular cellular adhesion molecule-1, intercellular adhesion molecule-1, E-selectin, endothelin-1 and cellular fibronectin,

all soluble markers of endothelial dysfunction are raised in PE. Some are evident before the clinical features of the disease [381].

Several reports have described a relative contribution of EDHF *vs* NO in arteries from women with PE. Pascoal et al (1998) have demonstrated differences in endothelium-dependent relaxation in omental arteries from women with PE. The response to ACh, but not to BK, was entirely abolished in PE, suggesting a defect of muscarinic signal transduction rather than changes in endothelium-derived relaxing factor *per se* [285]. It should be stressed that in this study endothelium-dependent relaxation to BK and ACh was NO/prostanoid-independent, i.e. entirely EDHF-mediated. In contrast, Suzuki et al (2000) using strips of omental arteries demonstrated almost equal contribution of NO and EDHF to BK and substance P-induced relaxation, and implied that in PE the role of NO, but not EDHF, is reduced [352]. A significant reduction in BK-induced PGI_2-mediated hyperpolarization (but not EDHF-mediated one) in omental arteries from women with PE has been demonstrated by the same group later, suggesting that the function of NO and PGI_2 is down-regulated, whereas EDHF function remains normal [351]. Thus, the discrepancy in results obtained in omental arteries in PE between different research groups deserves consideration and requires further investigation.

Using a wire myography technique Ashworth et al (1994) showed a significant reduction of endothelium-dependent relaxation to BK in myometrial arteries from women with PE [14]. However, after utilization of the pressure myography technique, Kenny et al (2002) failed to reproduce the results [194], although small contribution of EDHF to endothelium-dependent relaxation in PE was compatible to that in arteries from non-pregnant women. It has been suggested that EDHF-dependent compensatory mechanism represents a vascular adaptation of myometrial arteries to NP, which is absent in PE and could has some contribution to the clinical features of the disorder [194].

Endothelium-dependent relaxation in small subcutaneous arteries has also been studied and, in general, the different mechanisms responsible for the reduction in endothelium-dependent dilatation might be related to the agonist used. The degree of reduction in ACh-induced response is modest and accounts for the deficiency in PGI_2-mediated component [250]. In contrast, response to BK is significantly reduced before and after incubation with inhibitor of NO production, suggesting involvement of NO- and presumably EDHF-mediated components [199].

Thus, so far, there has not been enough evidence to suggest that compromised NO-mediated response alone could explain endothelial dysfunction in small arteries in PE and it is possible that changes in EDHF or PGI_2 mediated effects might also be involved depending on the agonist used.

The functional endothelial abnormalities in PE are accompanied by morphological EC layer alterations found in renal [342], myometrial [326, 355] and subcutaneous vasculature [354]. Arteries from NP women have a continuous layer of elongated and tightly connected ECs that provide an optimal environment for gap junction communications. In PE, ECs appear to be considerably increased in size, edematous, separate and detached from neighboring cells and the basal membrane that makes intercellular communications obscure or even unfeasible (Figure 6). It is possible

therefore that in PE, EDHF-mediated responses are hindered or another pathway/s rather than gap junctions might take a part.

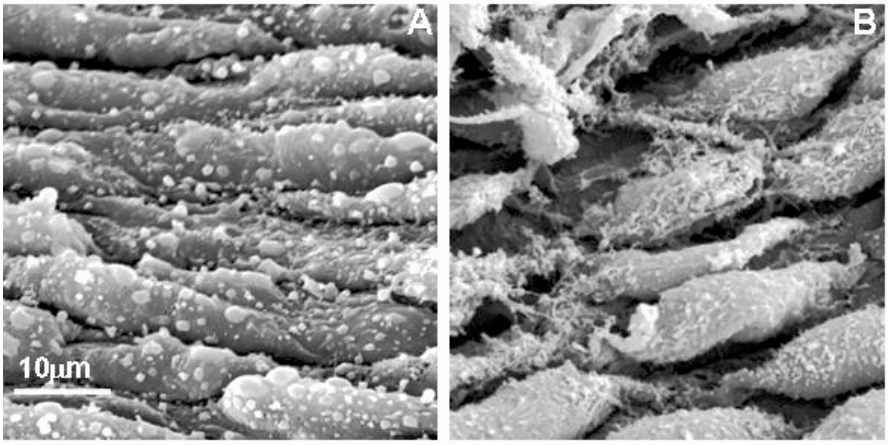

Figure 6. Scanning electron microscopy pictures of endothelial cell layer revealed the striking differences in morphology of endothelium between subcutaneous arteries from pregnant women without (A) and with preeclampsia (B).

1.5 GENDER DIFFERENCES IN EDHF-MEDIATED RESPONSES

The difference with respect to development of cardiovascular diseases (CVD) between females and males is well recognized [277, 312]. Women at reproductive age appear to be more protected from CVD when compared to males [164]. Sex hormones are considered to be of importance but not the sole factor promoting gender-related differences in the development of CVD [16, 176].

It has been assumed that one of the mechanisms underlying cardiovascular protection in females is an enhanced vasodilative capacity of the endothelium [191]. Several studies have demonstrated that basal and agonist-induced NO release from the endothelium is higher in arteries from females compared to males [190]. Estrogen appeared to be responsible for the gender differences in endothelial NO production [277, 406]. In line, production of COX-dependent products [18] and EDHF-mediated responses have been shown to be more pertinent to female vasculature. EDHF appeared to be more important in female arteries to confer endothelium-dependent dilatation, while NO played a predominant role in arteries from males, as reflected in several vascular beds, including mesenteric and tail arteries in rats [251, 279, 395]. These findings suggest that, in the absence of NO, EDHF can mediate vasodilator responses to agonist or mechanical stress in females rather than in males. Accordingly, if under normal physiological conditions the functional role of EDHF is more significant in females, thus during pathological conditions EDHF could compensate for the loss of NO in female rather than in male arteries [251].

Using specific gene-knockout technology to generate animals which lack both eNOS and COX-1, i.e., the "EDHF mouse", Scotland et al (2005) has directly assessed the involvement of EDHF in endothelium-dependent relaxation of small arteries [322]. In eNOS/COX-1 double knockout mice, EDHF-mediated response compensates to the absence of endothelial NO in females but not in males. In female mice, the deletion of eNOS and COX-1 did not affect mean arterial blood pressure, while males become hypertensive [322].

Interestingly, the contribution of EDHF seems to depend on agonist, species and/or anatomic origin of the artery. Gender-specific contribution of EDHF to endothelium-dependent relaxation seems to be different in cerebral vasculature when compared to that in the periphery. In rat middle cerebral arteries the greater part of endothelium-dependent relaxation in response to ATP accounts for EDHF in males, whereas EDHF response is minor in females [144]. Gender *per se* may not necessarily influence EDHF-mediated relaxation, as in certain circulations (i.e. rat kidney) contribution of EDHF to ACh-induced relaxation is similar in both genders [392].

In humans, gender-related differences in the contribution of EDHF *vs* NO are less conclusive and needs further exploration. The enhanced sensitivity to BK was observed in female omental arteries; however NOS inhibition eliminated the gender difference, suggesting the importance for NO rather than EDHF, which indeed conferred up to 90% of dilatation but in gender independent manner. In male subcutaneous arteries, incubation with inhibitors of NO and PGI_2 production increased endothelium-dependent dilatation and, as a result, abolished gender-related differences [318]. In males this could be explained by the antagonistic influence of NO on EDHF-mediated responses [21]. However, it is not clear why antagonistic interplay between NO and EDHF does not emerge in females.

1.6 EFFECT OF ESTROGEN IN VASCULATURE

Pre-menopausal women are protected from CVD, however this protection is lost after menopause. Therefore, CVD develop in women 10-15 years later than in men. The onset of menopause is often followed by adverse cholesterol changes, increased blood pressure, increased glucose, and increased triglycerides. Although a multitude of changes occur at menopause, it has been proposed that the increase in cardiovascular risk factors is mainly due to the loss of estrogen [148, 255, 304]. Estrogen has been introduced not only as a key factor in gender-related differences in incidences of CVD but also in endothelium-dependent maintenance of vascular tone [255], since it has been widely reported that this hormone may enhance NO-mediated relaxation [48, 223, 262, 306].

Nowadays estrogen is not considered as only a female sex hormone but rather as a steroid hormone functioning in both females and males [28]. Two known estrogen receptors (ERs) exist - ERα and more recently discovered ERβ [208]. ERα and ERβ are expressed in ECs and VSMCs of both genders; however ERβ is more prevalent than ERα in female VSMCs, while the expression of the both ER subtypes is equal in male [172]. Whether these gender differences in receptor subtypes expression are functionally important is far from clear.

ERα can be considered as a principal receptor found in association with estrogen-induced modulation of endothelial NO synthesis [90, 264, 347]. The basal release of endothelium-derived NO is decreased in male ERα-knockout (ERαKO) mice compared with wild-type (WT) mice [306]. An important role of ERs (presumably ERα) in human vasculature in males is supported by results from clinical examination in a young man with disruptive mutation in the ER gene (presumably ERα). This patient was found to have a severe endothelial dysfunction and premature coronary atherosclerosis at age of 31 years [345].

ERβ has also been shown to play an important part in cardiovascular effects of estrogen. Endothelial denudation leads to increased levels of ERβ in male rat aorta, while ERα levels remain unchanged [226]. Recent studies indicate that the ER subtypes may also control or counteract activity of each other in the vasculature [50, 86, 306, 412], as ERβ has been shown to offset the rapid ERα-mediated NO release in small arteries from mice or healthy males [85]. It has also been suggested that estrogen-stimulated induction of iNOS appears to be mediated by ERβ but antagonized by ERα [412].

Absence of numerous ERβ-regulated gene products has been suggested to contribute to the abnormal vascular contraction, ion channel function and hypertension in ERβ knockout (ERβKO) mice. These observations have been delivered by Zhu et al (2002) [412], who has reported that prior to the aging period (i.e. 6 month) ERβKO mice develop hypertension in both genders (Figure 7) with blood pressure in males being significantly higher than in ERβKO females, while no difference was observed in WT males and females [412] (Figure 7).

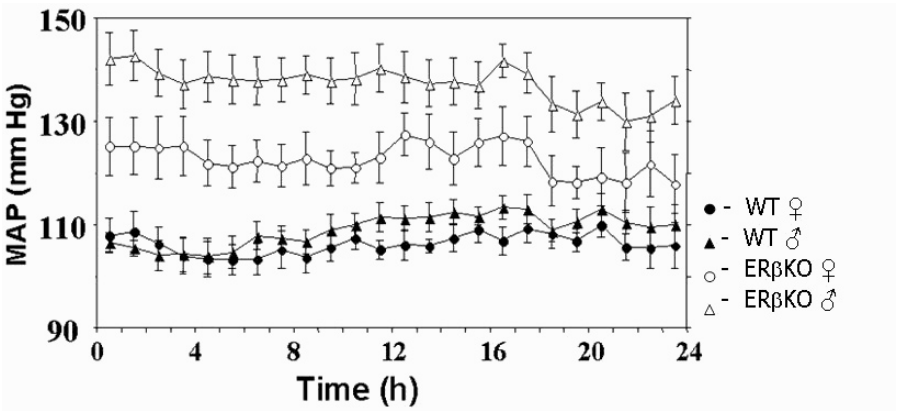

Figure 7. Mean arterial pressure (MAP) in conscious female and male WT and ERβKO mice at 6 months of age. MAP in male ERβKOs (137 ± 4.6 mm Hg, n=9) was significantly higher ($P <$ 0.01) than that in WT males (109 ± 2.7 mm Hg, n=10), or female ERβKOs (123 ± 4.9 mm Hg, n = 10). MAP in female ERβKOs was significantly increased ($P <0.01$) compared to that of WT females (106 ± 2.9 mm Hg, n=10) [412]. (Modified and reprinted with permission from *Prof. Michael E. Mendelsohn* [348]).

Nilsson et al (2001), however, found similar contractile responses to noradrenaline, but enhanced sensitivity to 17β-estradiol (17β-E_2) in aortas from ERβKO *vs* WT male mice [274]. No change in the wall morphology of aorta was observed between WT and ERβKO animals suggesting that ERβ might not be involved in the regulation of vascular structure, at least, in the conduit artery [274]. An increased basal NO release, but decreased sensitivity to ACh has been observed in aortic rings from ovarectomised (OVX) ERβKO and WT mice after 17β-E_2 supplementation. This effect was not observed in ERαKO mice, suggesting that ERα, but not ERβ, is involved in the modulation of basal NO production by estrogen [90].

1.6.1 Role of estrogen in modulating of EDHF-mediated responses

Estrogen may also modulate not only NO but also EDHF-mediated relaxation [178, 227, 356], and this has been attributed as an additional mechanism of cardiovascular protection by hormone replacement therapy [229, 313]. Isolated small arteries from OVX animals are characterized by severely impaired EDHF-mediated hyperpolarization, which is improved by 17β-E_2 replacement therapy [229]. ACh induced EDHF-mediated relaxation is greater in mesenteric arteries from male compared to OVX rats. A possible explanation for this gender difference in EDHF-mediated response has been attributed to the difference in circulating estrogen levels, since concentration 17β-E_2 is higher in male rats than in OVX females due to a metabolism of testosterone by aromatase in the adipose tissue [313]. Furthermore, EDHF-mediated response to ACh is suppressed in arteries from diestrus females, although its extent is less severe than in OVX rats, suggesting that even short-term estrogen deficiency could explicitly diminish EDHF-mediated reactivity [227].

Prolonged administration of 17β-E_2 or isoflavone daidzein to male rats resulted in an enhanced contribution of EDHF to endothelium-dependent relaxation in isolated aorta, in which ACh-mediated dilatation is usually entirely mediated by NO. The level of EDHF-mediated relaxation was up to 30%, and CYP450 metabolite of AA and K^+ ions *per se* have been introduced to serve as mechanisms for the EDHF response.[398].

Estrogen may also modulate EDHF contribution to shear stress responses when NO activity is compromised, as reflected in studies on skeletal muscle arterioles. Flow response in L-NAME-treated male and OVX female rats is solely mediated by prostaglandins, whereas 17β-E_2 replacement switches that to an EDHF-mediated response, recovering the similar profile observed in NO-deficient arterioles obtained from intact female rats[178]. EDHF is also involved in the acute 17β-E_2 -mediated-relaxation in coronary arteries from both female and male rats, and presents a constitutive component of 17β-E_2 mediated response as reflected by inhibition of CYP450 pathway [317].

The mechanisms behind the beneficial effects of estrogen on EDHF-mediated responses have been related to functional and reversible alterations in membrane consistence, ion channels, signal transduction or receptors [227]. Indeed,17β-E_2 has been shown to acutely enhance the activity of the Ca^{2+}-dependent K^+-channels in ECs

of the rabbit aorta [310] and in human coronary arteries [394]. Chronic treatment with 17β-E_2 prevents impairment of EDHF-mediated relaxation in hypercholesterolemic rabbit carotid arteries through activation of both Ca^{2+}-dependent and ATP-sensitive K^+-channels [140]. The K_{IR} channels are also of interest, as K^+-induced cerebral vasodilatation *in vivo* is greater in female than in male rats, and this vasodilatation is reduced after ovariectomy and restored by 17β-E_2 [68].

The MEGJ pathway is also relevant for 17β-E_2 mediated up-regulation of EDHF-responses. The number of gap junctions, at least in myometrial cells, depends on hormonal milieu and their number increases with increasing estrogen and decreasing levels of progesterone [234]. A significant reduction in the expression of Cx 43 protein occurs in mesenteric arteries from OVX rats, while supplementation with 17β-E_2 completely prevents the reduction to a level similar in control animals [229]. Additional details regarding the mechanisms by which estrogen influence EDHF-typed responses in arteries remain to be determined.

"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them."

William Bragg (1862-1942)
The Nobel Prize in Physics 1915

2 AIMS

The overall purpose of this thesis was to further explore functional and morphological features of small arteries by focusing particularly on endothelium-dependent control of vascular tone in human pregnancy and in estrogen receptor β knockout mice.

The specific aims were:

In human studies:

- To estimate the contribution of EDHF *vs* NO to endothelium-dependent relaxation, and to clarify the mechanisms of EDHF-mediated relaxation in isolated small arteries from women with normal pregnancy and preeclampsia.
- To investigate the contribution of different Cx subtypes (Cx37, Cx40 and Cx43) known to compose gap junctions within the vascular wall of small arteries from women with normal pregnancy.
- To compare the ultrastructure of the vascular wall with particular focus on gap junctional communications in arteries from women with normal pregnancy and preeclampsia.

In knockout mice studies:

To estimate the influence of ER subtypes and gender on small artery function by evaluating:

- Relative contribution of NO *vs.* EDHF in endothelium-dependent dilatation, and vascular wall ultrastructure and endothelial morphology.
- Mechanisms of EDHF-mediated response with particular focus on gap junctions, and expression of Cx subtypes.
- Contractile responses and basal NO production.

"I hear and I forget. I see and I remember.
I do and I understand."

Chinese Proverb

3 MATERIALS AND METHODS

3.1 HUMAN SUBJECTS (I-III)

Two groups of pregnant women were recruited from the prenatal care unit at the Karolinska University Hospital, Huddinge: 44 healthy pregnant women and 15 women with PE. The number of women examined in each study is given in Table 1. The investigation was undertaken in accordance with the principles outlined in the Declaration of Helsinki. Local Ethical Committee approval was obtained for the collection of subcutaneous fat biopsies. In all cases informed consent was obtained prior to fat tissue biopsy collection.

Table 1. The number of women and arteries included in the papers I-III.

Pregnancy	Paper I		Paper II		Paper III	
	Women	Arteries	Women	Arteries	Women	Arteries
NP	30	99	14	31	17*	52
PE	–	–	–	–	15	44

* - arteries from the same group of normal pregnant women were studied in Paper I and Paper III.

All NP women were delivered at term by elective cesarean section due to previous cesarean delivery (n=16), psychosocial indications (n=10) or breech presentation (n=18). None had any history of diabetes mellitus, abnormal renal or hepatic function, hypertension or any other significant past medical history.

PE was defined as blood pressure ≥ 140/90 mm Hg and proteinuria exceeding > 300 mg/24 hours in the absence of urinary tract infection after 20 weeks of gestation in previously normotensive, non-proteinuric women. Women with PE who had received anti-hypertensive agents were also excluded. Two women with PE also fulfilled the criteria for the HELLP syndrome at the time of sampling.

3.1.1 Subcutaneous artery preparation

At time of cesarean section, subcutaneous fat biopsies were taken from the incision margin and immediately placed in cold physiological salt solution (PSS, Figure 8A). Two or more arteries preferably from the same artery segment and approximately 200

μm (150-400μm) in diameter with a length of about 2 mm were dissected in ice-cold PSS using a stereomicroscope. Surrounding tissue was carefully removed using microsurgery instruments. Care was taken to make sure that the endothelial layer was not damaged during processing of the dissection as well as vessel mounting.

3.2 MICE (IV-V)

Age-matched (14-22 weeks old) male and female WT mice (ERβ +/+, C57BL/6J) and homozygous mutant mice lacking the gene for ERβ (ERβ -/-, ERβKO) were used. All animal care and procedures used conformed to guidelines on the conduct of animal experiments issued by Stockholm's Södra Djurförsöksetiska Nämnd and the Institute for Laboratory Animal Research *Guide for Care and Use of Laboratory Animals*.

Mice were acclimatized in a temperature- and moisture-controlled room at the animal facilities of Karolinska University Hospital, Huddinge for 1 week before the study. All animals were group housed in cage type 2 (450 cm^2), females and males in separate cages with maximum five mice per cage. Animals were maintained on a 12:12 light/dark schedule at 20-24°C and 50-60% humidity and fed *ad libitum* on a standard rodent diet and tap water. Sawdust was used as a bedding material and different material (i.e. wood blocks, cardboard, paper, etc.) has been used to fulfill the criteria of environmental enrichment.

The same animals were used for Paper IV and V. The mean body weight of males was 31.0±0.9 g (WT, n=15) and 32.5±0.7 g (ERβKO, n=16) compared to 23.0±0.5 g (WT, n=8) and 25.6±1.5 g (ERβKO, n=8) for females.

3.2.1 Small femoral artery preparation

Mice were killed by asphyxiation using a rising concentration of CO_2. The femoral artery (Figure 8B) was carefully removed and dissected free of adherent connective tissues under stereomicroscope, cut to a length ~ 2 mm and immediately placed in iced PSS. Since EDHF tends to play a greater role in relaxation of small rather than large vessels, all arteries included in the Paper IV were divided into two groups according to size. The proximal part of femoral artery (PFA) was included into one group whereas the middle and distal parts (DFA) formed another group. There were significant differences in internal diameter (ID) between the groups. Only DFA has been used in Paper V and mean vessel diameters were as follows: males, 174±5 μm (n=44, WT) and 183±8 μm (n=49, ERβKO); females, 172±5 μm (n=28,WT) and 161±5 μm (n=23,ERβKO).

3.3 WIRE MYOGRAPHY

Small-sized human subcutaneous and murine femoral arteries dissected as described previously were mounted on two stainless steel wires (25 or 40 μm in diameter, depending from the size of artery). One wire was attached to a force transducer and the other to a micrometer in order to measure the vessel tension in the chamber of a

Mulvany's type 4-channel Multi Myograph (Model 610, Danish Myo Technology, Denmark, Figure 9).

A) B)

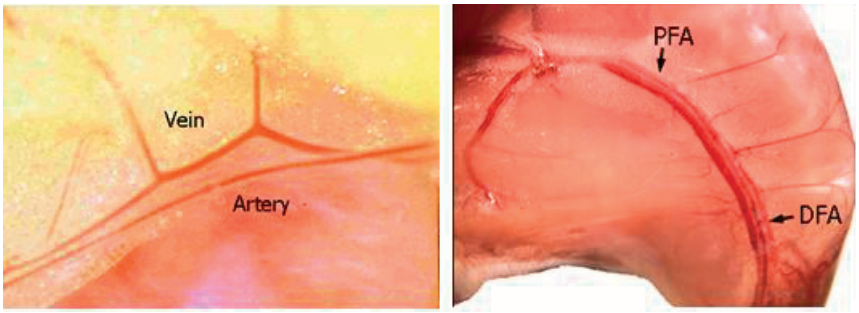

Figure 8. (A) An overview of subcutaneous fat biopsy under stereomicroscope (×10) with representative artery suitable to be isolated and mounted on wires. (B) A leg of mouse without skin with proximal (PFA) and distal (DFA) femoral arteries used in the experiments.

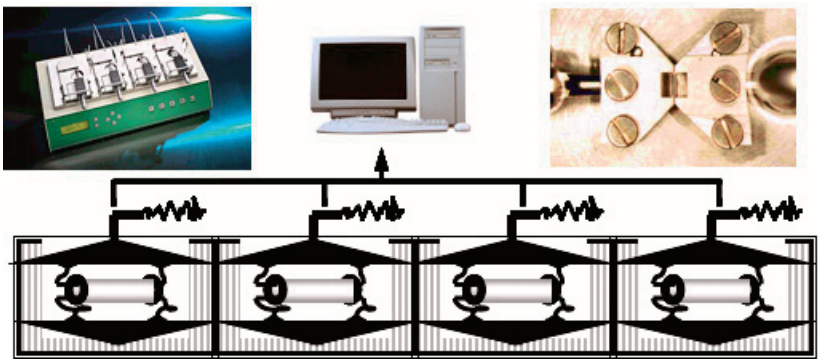

Figure 9. A schematic overview of 4-channel Multi Myograph wire system with mounted artery

After all arteries were mounted, they were allowed to equilibrate for 30 min at 37°C, while continuously being oxygenated with 5% carbon dioxide in oxygen. All solutions, including the incubation solutions, were refreshed every 30 min. Following an equilibration period, a passive circumference–tension curve was created for each segment in order to set optimum resting tension and to allow calculation of the artery diameter. For this purpose arteries were stretched gradually until the calculated internal diameter reached a value equal to the ID that the vessel would have *in vivo,* when fully relaxed and under a transmural pressure 100 mm Hg. Arteries were then set at 90% of this tension to enable optimal contractile conditions with a low resting tension. Myodac Software was used for calibrations and for data registration (version 2.1, Danish Myo Technology, Denmark).

After the normalization procedure the arteries were left to equilibrate for 30 minutes again and up to five reference constrictions were elicited to elucidate if the arteries are suitable for experiments. The first, second and fifth contractions were produced using a

high (124 mmol/L) potassium solution (KPSS, made by equimolar substitution of KCl for NaCl in PSS) containing 1 μmol/L norepinephrine (NE). The third and fourth were obtained with NE (1 μmol/L) or KPSS alone. Arteries that failed to produce active tension equivalent to 100 mm Hg when constricted with KPSS containing 1 μmol/L NE were rejected. Arteries that did not fulfil the viability criteria (i.e. >50% relaxation to ACh (1 μmol/L, murine femoral arteries) or i.e. >60% relaxation to bradykinin (BK, 1-3 μmol/L, human subcutaneous arteries) after pre-constriction with norepinephrine (NE, 1 μmol/L)) were excluded.

3.4 EXPERIMENTAL PROTOCOLS

3.4.1 In human studies (I-III)

All arteries were preconstricted with NE (3 μmol/L) resulting in a contraction level of 90-100% of the initial response to KPSS. In experiments in which K^+-modified PSS (35mmol/l equimolar exchange of NaCl with KCl) was used, the concentration of NE was reduced to 1 μmol/L to achieve a pre-constriction level similar to that used in previous experiments (I).

The concentration-response curves to BK (1 nmol/L to 3 μmol/L) were obtained before and after incubation with N^{ω}-nitro-L-arginine-methyl ester (L-NAME, 300 μmol/L, 30 min) alone (I) or in combination with indomethacin (Indo, 10 μmol/L, 30 min, I-III). The concentration of L-NAME was sufficient to inhibit the production of NO, since additional application of N^{ω}-nitro-L-arginine (L-NNA, 300 μmol/L, 30 min), another inhibitor of NO-synthase, or by addition of the selective guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10μmol/L) had no further inhibitory effect on the BK-induced relaxation. In separate experiments, the concentration-response curves to BK were performed in the K^+-modified PSS (35mmol/l KCl) before and after incubation with L-NAME (I).

3.4.1.1 The contribution of AA metabolites, H_2O_2, MEGJ and connexin sybtypes to BK-mediated relaxation

To study whether AA metabolites play a role as EDHF, the BK-induced relaxation was investigated in the presence of the specific inhibitor of CYP450 epoxygenase sulfaphenazole (10 μmol/L, 30 min) alone or in combination with L-NAME + Indo (I, III).

To evaluate if H_2O_2 is involved in the EDHF-typed responses, an initial concentration-response curve to BK was constructed followed by a second concentration-response curve but in the presence of catalase (1250-6250 U/ml, an enzyme, which dismutates H_2O_2 to form water and oxygen) alone or in combination with L-NAME + Indo (I, III).

To elucidate the contribution of gap junctions to the EDHF-typed responses, the concentration response curves to BK were obtained after incubation either with a reversible inhibitor of gap junctions - 18-α-glycyrrhetinic acid (18-αGA, 100 μmol/L, 15 min, I, III) or with triple combination of newly developed inhibitors - connexin

mimetic peptides (CMPs) - ^{37,43}Gap27 (300 μmol/L), ^{40}Gap27 (300 μmol/L) and ^{43}Gap26 (300 μmol/L, 90 min) with or without L-NAME+Indo (II).

In order to infer the relative functional importance of Cxs 37, 40 and 43 (II), the effects of each of the three CMPs or double combination of them have been evaluated in arteries pre-incubated with L-NAME+Indo: i) ^{37,43}Gap27 plus ^{40}Gap27 (450 μmol/L each); ii) ^{37,43}Gap27 (900 μmol/L); iii) ^{40}Gap27 (900 μmol/L); or iv) ^{43}Gap26 (900 μmol/L). Total peptide concentration in each experiment was kept constant at 900 μmol/L. The marked inhibition of EDHF-mediated dilatation achieved by incubation with ^{43}Gap26 led us to check the effects of ^{43}Gap26 (900 μmol/L) alone on endothelium-dependent relaxation (Paper II). In addition, a full concentration response curve for either sodium nitroprusside (SNP, 0.001-1 μmol/L) or the ATP-sensitive potassium channel opener, pinacidil (0.001-1 μmol/L) before and after incubation with ^{43}Gap26 without L-NAME and Indo was constructed (II).

3.4.2 In knockout mice studies (IV-V)

3.4.2.1 Vasodilator responses

After the standard start procedure, as described previously, the cumulative concentration response curve in response to non-selective agonist of β-adrenergic receptors isoproterenol (ISO, 10^{-6} - 3×10^{-5} mol/L, V) and to endothelium-dependent agonist ACh (10^{-9} - 10^{-5} mol/L, IV) were obtained after achieving a stable tension plateau induced by phenylephrine (PhE, 10 μmol/L). In our study, along with previous ones [53], a sustained level of tension after constriction was difficult to obtain in murine arteries because of the spontaneous decrease in tone which achieved up to 30% from the level of initial pre-constriction with maximal concentration of PhE. So when a stable tension plateau after pre-constriction with 10μM PhE was reached, the level of tension still was ~50-70% of the maximum response to KPSS.

After the first concentration-response curve to ACh, the artery was washed with PSS and the procedure repeated following incubation with cocktail of inhibitors: L-NAME (100 μmol/L) + L-NNA (300 μmol/L) + Indo, 10 μmol/L). The term "EDHF" used in this study refers to the L-NAME and Indo-insensitive component of endothelium-dependent vasodilatation to ACh (IV).

Differences in the EDHF-mediated components of relaxation to ACh between arteries from WT and ERβKO males prompted us to clarify pathways contributing to EDHF-induced relaxation in arteries from male mice. A single supramaximal concentration of ACh (1μmol/L) was chosen, since it produced rapid relaxation. The contribution of epoxygenase products of AA, H_2O_2 or gap junctions to EDHF–mediated relaxation was investigated in the presence of the specific inhibitor of CYP450 epoxygenase - sulfaphenazole (10 μmol/L, 30 min), catalase (1250 U/ml, 15 min) or 18-αGA (100 μmol/L, 15 min), respectively (IV).

3.4.2.2 Contractile responses (V)

The cumulative concentration-response curves were made for either phenilephrine (selective agonist of α_1-adrenoceptors, PhE, 10^{-8} - 5×10^{-5} mol/L), norepinephrine (non-selective agonist of adrenoceptors, NE, 10^{-8} - 5×10^{-5} mol/L) or thromboxan A_2 mimetic (U46619, 10^{-9}-10^{-7} μmol/L) before and after incubation with NOS inhibitors L-NAME (100μmol/L) plus L-NNA (300 μmol/L) and PGI_2 production inhibitor Indo (10μmol/L). Constriction of the arteries during incubation with L-NAME (100μmol/L) + L-NNA (300μmol/L) + Indo (10μmol/L) was considered as an index of vasoactive properties of the endothelium. In a separate set of experiments, cumulative concentration response curves to NE (10^{-8} - 10^{-5} μmol/L) were generated before and after pre-incubation (for 20 min) with yohimbine (1μmol/L) or pronethalol (1μmol/L) to block α_2- or β- adrenergic receptors, respectively.

3.5 IMMUNOHISTOCHEMICAL ANALYSIS

3.5.1 For Cx37, Cx40, Cx43 (II, IV)

Freshly isolated arteries were cryopreserved in optimal cutting temperature (OCT) compound cooled by liquid N_2. Transverse 10-μm-thick cryosections were prepared and mounted onto slides, air-dried, and stored at -20° C. Immediately before immunostaining, the sections were fixed in 4° C acetone for 10 min and then rehydrated in Tris buffer solution, pH 7.6. Endogenous peroxidase was blocked by 1% H_2O_2 in Tris buffer for 10 min. Permeabilization and blocking was performed in Tris buffer containing 0.2% triton-X100 and 2% bovine serum albumin (BSA) for 60 min at room temperature. Sections were immunostained with polyclonal rabbit antibodies against mouse connexins (Cx37, Cx40 and Cx43 1:50 dilution (IV) but for subcutaneous arteries Cx37 and Cx40 (1:50 dilution) or Cx43 (1:100 dilution, II) at 4°C overnight; (Zymed Laboratories, Inc., San Francisco, CA, USA). The specificity of these antibodies has been reported previously [221, 268]. Negative control sections were incubated with non-immune goat IgG (SDS, Falkenberg, Sweden). Sections were washed in Tris buffer solution and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) for 1h at room temperature and diluted 1:300. After rinsing with Tris buffer solution, the bound antibodies were visualized by means of avidin-biotin complex with peroxidase (Vectastain ABC Elite, Vector Laboratories), for 30 min, following application of 3, 3-diaminobenzidine in H_2O_2 (DAB-kit, Vector Laboratories). All slides were counterstained with hematoxylin, dehydrated and mounted with Pertex (Histolab, Gothenburg, Sweden).

3.5.2 For ERα and β (V)

WT and βERKO femoral arteries were fixed in 4% formaldehyde for a maximum of 24h and then stored in 70% ethanol until embedding. Paraffin-embedded sections were cut in slices 4 μm thick, deparaffinized, washed and subsequently incubated for 10 min at 750 Watts in 10 mM citrate buffer (pH 6.0) to induce antigen retrieval. After cooling (20 min), sections were washed in phosphate buffered saline (PBS). Endogenous peroxidase was blocked by 3% H_2O_2 in methanol for 10 min. Sections for ERα and β staining were incubated with 10% blocking normal goat serum

(Vector Laboratories, Burlingame, CA, USA) in PBS for 1h. Polyclonal rabbit antibodies against mouse ERα and ERβ (Santa Cruz Biotechnology, Inc., CA, USA) were used at the dilution 1:100 at 4°C overnight in normal goat serum with detergent. The specificity of these antibodies has been described elsewhere [65, 324]. Negative control sections for both ERα and ERβ were incubated with non-immune goat IgG (SDS, Falkenberg, Sweden). Normal human endometrial tissue sections served as a positive control for ERα [235, 358] and ovarian tissue was used as a positive control for ERβ [358]. Sections were washed in PBS and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) for 1h at room temperature and diluted 1:200. After rinsing with PBS and detergent, the bound antibodies were visualized by means of avidin-biotin complex with peroxidase (Vectastain ABC Elite, Vector Laboratories), for 30 min, following application of 3,3-diaminobenzidine in H_2O_2 (DAB-kit, Vector Laboratories). All slides were counterstained with hematoxylin, dehydrated and mounted with Pertex (Histolab, Gothenburg, Sweden).

3.6 SCANNING ELECTRON MICROSCOPY (IV)

Femoral arteries were cut longitudinally, rinsed in PSS, fixed and kept for at least 48 h in a 2.5% (wt/vol.) glutaraldehyde solution in PSS, and postfixed in a solution of 1% (wt/vol.) osmium tetroxide in a sodium cacodylate buffer (0.15 M, pH 7.3) containing 75 mM sucrose. Samples were dehydrated in acetone series, dried in a critical-point drier using carbon dioxide, mounted on the specimen holder, and coated with gold palladium and examined for the morphological changes in EC layer under scanning electron microscope Jeol JSM-820 (Jeol Ltd., Tokyo, Japan).

3.7 TRANSMISSION ELECTRON MICROSCOPY (III, IV)

Artery segments were fixed in 2% glutaraldehyde + 0.5% paraformaldehyde in 0.1M sodium cacodylate buffer containing 0.1M sucrose and 3mM $CaCl_2$, pH 7.4 at room temperature for 30 min followed by 24 h at 4°C. Specimens were rinsed in 0.15 M sodium cacodylate buffer containing 3mM $CaCl_2$, pH 7.4, and postfixed in 2% osmium tetroxide in 0.07 M sodium cacodylate buffer containing 1.5 mM $CaCl_2$, pH 7.4, at 4°C for 2 h and dehydrated in ethanol followed by acetone and embedded in LX-112 (Ladd, Burlington, Vermont, USA). Semithin sections were cut and stained with toludinblue and used for light microscopic analysis. Ultra thin section (approximately 40-50 nm) were contrasted with uranyl acetate followed by lead citrate [129]. Sections were examined in a Tecnai 10 transmission electron microscope at 80 kV and digital images were captured by a Mega View III digital camera (Soft Imaging System, GmbH, Münster, Germany).

3.8 SOLUTIONS AND CHEMICALS

During all biopsies handling, and in all experimental procedures isolated arteries were constantly kept in PSS of the following composition (mmol/l): NaCl 119, KCl 4.7,

$CaCl_2$ 2.5, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.026 and glucose 5.5. All drugs and chemicals except the CMPs and antibodies were obtained from Sigma-Aldrich, Sweden. The CMPs, ^{37,43}Gap27 (SRPTEKTIFII), ^{40}Gap27 (SRPTEKNVFIV) and ^{43}Gap26 (VCYDKSFPISHVR) were purchased from American Peptide Company, Inc. (Sunnyvale, CA, USA). To prepare stock solution, the substances were dissolved in distilled water. Indo, ODQ and U46619 were dissolved in ethanol. 18-αGA and sulfaphenazole were diluted in dimethyl sulphoxide (DMSO) at a concentration of 10^{-2}mol/l. The highest concentration of ethanol and DMSO in the chamber was 1% (vol/vol), and the final bath concentration of combined solvents used simultaneously was not higher than 3% (vol/vol). CMPs were dissolved directly in PSS before every experiment. All concentrations represent the final steady-state concentrations in the chamber. The solvent used did not affect the mechanical responses at their final bath concentrations.

3.9 DATA ANALYSIS

The force developed by the artery per millimeter of artery segment during application of a certain concentration of a vasoactive substance was calculated using Myodata (version 2.1, Danish Myo Technology). Data were then transferred to STATISTICA (version 7.0, StatSoft, Uppsala, Sweden), in which all statistical analyses were performed. All absolute measurements were corrected for the baseline force developed by the arteries. Contractile responses were expressed as a percentage of the maximal constriction with KPSS (Paper V). The relaxation to agonists was calculated as a percentage of the contraction induced by vasoconstrictors used. Negative log concentration (in mol/l) required to achieve 50% of the maximum response (pEC_{50}) was calculated by nonlinear regression analysis (BioDataFit 1.02, Paper I, III-V).

Multivariate ANOVA for repeated measures was used to compare BK concentration-response curves before and after incubation with particular inhibitors and for differences in BK-mediated relaxation between experimental groups. Paired and unpaired Student's *t*-test as appropriate was used to compare pEC_{50} value, internal diameter (ID) and preconstriction before and after incubation with different substances in arteries used for different experimental protocols. All data is presented as mean ± standard error of the mean (SEM), unless indicated in the text. The evaluation of connexin's expression was performed using semi quantitative analysis (Paper IV). The staining was scored blindly by 4 investigators: 1 if < 5-25 % staining, 2 if 25-50 %, 3 if > 50-70 %. Statistical significance was taken at the 5% level.

"The great tragedy of science: the slaying of a beautiful hypothesis by an ugly fact."

Thomas Henry Huxley (1825-1895)
English biologist

4 RESULTS AND DISCUSSION

4.1 HUMAN STUDY

4.1.1 Rationalization of Hypothesis, aims and methodology

EDHF's contribution to endothelium-dependent relaxation is greatest in the resistance-sized arteries and, as such, it is ideally suited to the control of systemic blood pressure and local organ perfusion that are altered in normal pregnancy (NP) and disturbed in PE [381]. Based on previous data (please see "Introduction" for details) we hypothesized that contribution of EDHF to the endothelium-dependent dilatation in small arteries is altered due to required situation in order to preserve the vasodilative capacity of the endothelium. Therefore, the role and mechanisms of EDHF-mediated responses may differ in normal versus diseased state (NP *vs* PE).

Kenny et al, (2002) confirmed the presence of a novel EDHF-dependent mechanism in relaxation of small myometrial arteries in NP, an effect that disappeared in PE [194]. Whether disappearance of EDHF-dependent compensatory mechanism is specific for the uterine circulation or is a systemic phenomenon in PE conferred the main aim of current investigations. Our studies were designed to compare the role of EDHF and mechanism of its action in small arteries from non-reproductive vascular bed in pregnant women with *vs* without PE. In order to reveal the mechanisms of maternal cardiovascular adaptation in non-reproductive vascular bed, subcutaneous small arteries were considered as an optimal target. Twofold increase in skin blood flow in NP is comparable to that obtained in kidney circulation, and this increase is only less pronounced when compared to changes in blood flow in uteroplacental vascular bed [303]. The circulation of the skin, therefore, plays an important role in the reduction of maternal peripheral resistance. Moreover, subcutaneous small arteries are widely used for investigation of vascular function in a variety of conditions, including NP and PE [75, 199, 380], hypertension [224, 276, 294], diabetes [10, 301, 388], glaucoma [41, 73], congestive heart failure [11], hypercholesterolemia [145, 219], Raynaud's disease [339], coronary heart disease [87], and menopause [206].

The question arises if results obtained in our studies (I and II) are unique to the pregnant state *per se*, implying that the comparison with arteries from non-pregnant women would be desired. However, we were reluctant for such an inclusion, as we did consider an obligatory acquisition of healthy female volunteers' precisely matched for age and phase of menstrual cycle due to the evidence that both age and hormonal environment seem to play an essential role in the EDHF contribution [44, 231, 313]. Indeed, ERβKO studies presented within this thesis suggest that hormonal milieu and ERs are involved in the control of EDHF contribution to endothelium-dependent dilatation. Although several studies collected subcutaneous [10] or myometrial arteries [194] from non-pregnant women, it is difficult to define if these participants were really healthy. These biopsies were obtained during the gynecological or abdominal surgery

due to several diseases (e.g. cancers and ovarian cysts), which may be associated with alterations in the hormonal environment that could influence the contribution of EDHF. It was doubtful whether comparisons with data on such kind of biological material will increase insight into the mechanism of underlying pregnancy-induced changes in the peripheral resistance, however, future studies involving healthy female volunteers are encouraged.

In this thesis EDHF-mediated relaxation is defined as a relaxation resistant to combined blockade of COX and eNOS, however as it has been described in the "Introduction", EDHF action is associated with endothelium-dependent hyperpolarization of the vascular SMCs and it is sensitive to inhibition of K^+ conductance either by high extracellular K^+ or K^+-channels blockers. Since changes in membrane potential (E_m) are peculiar to EDHF-mediated relaxation, the electrophysiological measurement could be sensible for providing an additional support for EDHF responses in studied arteries. However, an electrophysiological measurement of alterations in E_m is associated with meticulous technical difficulties when applied to these vessels. It is difficult to measure E_m in EC, and even more complicated in ECs and SMCs simultaneously, as smooth muscle E_m recording alone does not provide a comprehensive idea of EDHF-mediated response. Therefore, we used a pharmacological approach, i.e. raising extracellular K^+ or inhibiting the production of NOS and COX products. This is a widely accepted approach [1, 74, 194, 286]. The experiments were based on the evidence that endothelium-dependent hyperpolarization of SMC, as a mechanism of EDHF-mediated vasodilator response, depends on the opening of K^+ channels. An increase in extracellular K^+ should then reduce the electrochemical gradient for K^+ and inhibit the EDHF responses [252]. Previous studies confirmed that high K^+ specifically antagonizes the action of EDHF by counterbalancing SMC E_m and, it has also minimal effect on NO and PGI_2-mediated vasorelaxation [1, 74]. Even higher K^+ (up to 50 mM) levels have no effect on ACh-induced increase in NO concentration, at least, in rat mesenteric arteries [343].

The other way to confirm the involvement of EDHF is to use blockers (i.e. apamin and charybdotoxin) of small and intermediate-conductance Ca^{2+}-activated K^+ channels, which have been considered as a unique characteristic of EDHF-mediated response [119, 414]. However, the agonist-induced increase in EC $[Ca^{2+}]_i$ could also activate apamin- and charybdotoxin-sensitive K^+ channels with hyperpolarization and release of NO [343], and it might be that apamin and charybdotoxin action will be associated with blockade of both EDHF and NO-mediated response. In rat mesenteric arteries, ACh-induced NO release was reduced by 85% in the presence of apamin plus charybdotoxin. [343].

As indicated in the "Introduction", it might be a question if the residual endothelium-dependent relaxation in PSS after NOS and COX inhibition still reflects the existence of EDHF-mediated response [119]. Evidence exists that endothelium-derived NO and PGI_2 can hyperpolarize the underlying SMC [283, 403]. However, predominantly in human subcutaneous arteries NO-induced relaxation does not involve hyperpolarization, since maximal SMCs hyperpolarization induced by ACh is far above that achieved by high concentration of NO *per se* after stimulation with NO donor (11 mV *vs* 2 mV) [45]. Thus, in this thesis we used the pharmacological approach to confirm the involvement of EDHF to endothelium-dependent relaxation in

subcutaneous arteries in human pregnancy. Based on the above considerations, we believe this rigorous approach to be optimal. Furthermore, comparable relaxations to BK after different incubational conditions (L-NAME+Indo, ODQ+L-NAME+Indo; K^+-modified PSS (35 mmol/l equimolar exchange of NaCl with KCl, Figure 10)) further strengthened the significance of EDHF to endothelium-dependent relaxation in small arteries from NP women, and the EDHF and NO contribution seems to be equally important.

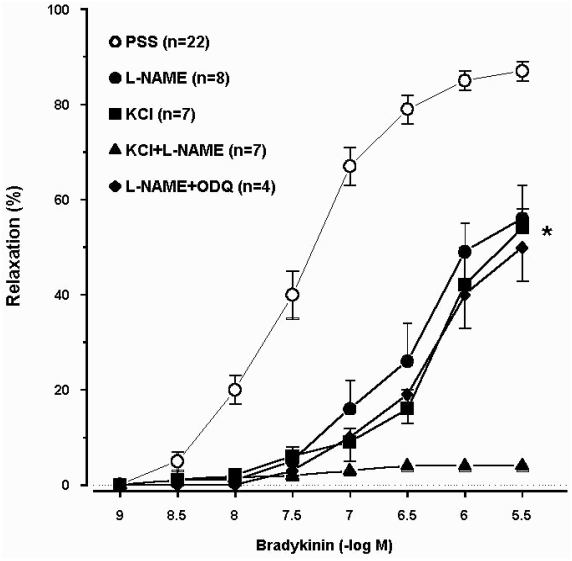

Figure 10. Concentration-response curves to bradykinin in physiological salt solution (PSS) and with different combinations of inhibitors to block endothelium-derived vasodilators. L-NAME - after pre-incubation with N^w-nitro-L-arginine methyl ester (L-NAME, 300 μmol/L); KCl - in the presence of KCl (35 mmol/L) made by equimolar substitution of KCl for NaCl in PSS; KCl+L-NAME - in combination of KCl (35 mmol/L) and L-NAME (300 μmol/L); L-NAME+ODQ – after pre-incubation with combination of L-NAME with selective guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10μmol/L). $^*P < 0.001$ vs PSS.

Previous studies indicated the predominance of EDHF-mediated responses (up to 90% from the level of relaxation in PSS) in subcutaneous arteries from humans [45, 73, 74] including pregnant women [10]. The discrepancy between studies in the relative contribution of endothelium-derived factors could be attributed to the agonist used or state of pregnancy *per se*. In the above studies, ACh has been applied as the agonist to endothelium-dependent relaxation, while in our studies BK was used. Differences in responses to BK and ACh in subcutaneous arteries have previously been reported [4, 199, 249], and BK-induced relaxation has been shown to be enhanced in arteries from pregnant *vs* non-pregnant women [199], whereas ACh-induced relaxation remained similar [249]. Subcutaneous arteries from pregnant women also exert a higher contribution of EDHF in carbachol (synthetic analog of ACh) induced relaxation ~70% [10], whereas in BK-induced response - only ~50% [199]. NP is associated with

enhanced production of NO [338], therefore it is possible that NO may down-regulate the EDHF production or mechanism of its action.

For clarification of the mechanisms responsible for EDHF-induced relaxation in subcutaneous arteries we have chosen three the most frequently implicated pathways. The mechanism of EDHF-induced relaxation may differ in subcutaneous arteries depending on the disease conditions, as CYP450 enzyme-dependent products of AA metabolism have been suggested to predominate in arteries from healthy non-pregnant female and male volunteers [74]. This contrasted to arteries in patients with cancer or CVD [45]. In addition to epoxygenase products of AA, we anticipated endogenous H_2O_2 or gap junctional communications to account for EDHF activity in NP and PE. We deliberately excluded K^+ *per se*, since three recent independent studies failed to prove it [45, 74, 77]. We have also neglected CNP, as a recent review paper seriously questioned if CNP could act as a diffusible candidate for EDHF [315].

"When you have eliminated the impossible,
what ever remains, however
improbable must be the truth"

Sir Arthur Conan Doyle (1859-1930)

4.1.2 Normal pregnancy (I, II)

In this thesis we report a significant impact of EDHF to endothelium-dependent dilatation in small subcutaneous arteries from NP women. Our findings are in line with earlier observations [199], however we are likely to provide a more comprehensive support. The contribution of NO and EDHF in BK-induced dilatation seems to be almost equal, since L-NAME and KCl reduced the dilatation to a similar level, whereas a combination of both abolished the response (Figure 10). Moreover, we show that endothelium-derived vasodilator PGI_2 appears to play no role in BK-induced responses in arteries from NP women, as similar responses to BK have been observed after incubation with L-NAME alone or L-NAME+Indo (Figure 2, Paper I). This is an additional confirmation to previous reports in pregnant [199] and non-pregnant women [45, 74, 253].

To our knowledge we performed the first attempt of clarifying the mechanisms behind the EDHF-mediated response in human pregnancy (Paper I), although several other studies have estimated the mechanism of EDHF-typed responses in human subcutaneous arteries but from non-pregnant subjects [45, 74, 253]. Since a product of CYP450-dependent metabolism of AA has been identified as EDHF in one report [74], our initial step was based on this pathway. Ketoconazole, a selective inhibitor of CYP3A isoform [66] has been implemented previously [74], however since CYP450-2C as an EDHF synthase was found at least in the porcine coronary artery [125] and this CYP450 isoform has been shown to exist in human ECs [12, 124], we applied sulfaphenazole as a specific inhibitor of CYP450-2C [66]. In our study, this inhibitor alone had no detectable effect on BK-induced relaxation in PSS, or in the presence of L-NAME, suggesting that CYP450-2C metabolites of AA do not account for the EDHF-mediated responses in arteries from NP women (Figure 4, Paper I). Our results are in line with those in subcutaneous arteries from non-pregnant humans [45, 373], in

which different inhibitors of CYP450 superfamily (17-octadecynoic acid, econazole or *N*,*N*-Diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF 525a, non-selective inhibitor of CYP450)) were used. The inconsistency in results between studies could be partly related to CYP450 inhibitors used that have their own limitations. Nonspecific inhibitors of CYP450 such as micanazole and other imidazoles have been shown to block K^+ channels [113] or in ECs impair agonist-mediated increase in intracellular Ca^{2+} [165].

Pregnancy *per se* is a state of oxidative stress [397], defined as an imbalance between free radical damage and antioxidant protection, which occurs from the increased metabolic activity and reduced scavenging power of antioxidants [267]. In early pregnancy intrauterine oxygen tension is extremely low, however it increases up to 2.5 times at the end of the first trimester when blood flow into the intervillous space is established [185]. The hypoxia/reoxygenation of the placenta is associated with a burst of oxidative stress [185], and hypothetically endogenous level of H_2O_2 could increase in NP, and possibility exists that free radicals may control the vessels' tone. The choice of catalase, a scavenger of extracellular H_2O_2, in EDHF-mediated responses was apparent; however it (1,250 U/ml or 6,250 U/ml) had no significant effect on BK-induced relaxation in the absence or presence of L-NAME (Figure 5, Paper I). This suggests that endogenous H_2O_2 is not involved in the EDHF-mediated responses in arteries from NP women. Our result contrasts with those indicating contribution of endothelium-derived H_2O_2 to BK- [242, 263] and flow-induced [260] dilatation of human arteries but in non-pregnant subjects and in different vascular bed. It has been suggested that H_2O_2 may serve as EDHF in conditions with insufficient NO production [115], and it is more likely that such pathway could occur in PE, the condition with profound failure in pro- and anti- oxidative balance [180].

Currently, the role of myoendothelial gap junctions (MEGJ), as a central component of the EDHF mechanism, has been increasingly appreciated [95, 314, 340]. We have employed two known inhibitors: glycyrrhhetinic acid metabolites (18-α-glycyrrhetinic acid, 18-αGA, a lipophilic steroidal aglycone derived from glycyrrhizic acid found in the liquorice root *glycyrrhizia glabra* [151]) and newly developed, highly selective and specific [151, 240] inhibitors - connexin mimetic peptides (CMPs).

We found that gap junction communication apparently relies upon the EDHF-mediated relaxation in subcutaneous arteries from NP women. This was confirmed by evidence that incubation with either 18-αGA or triple combination of ^{37,43}Gap27, ^{40}Gap27 and ^{43}Gap26 basically abolished EDHF component in BK induced relaxation (Figure 11). Moreover, a morphological prerequisite for gap junctions found in these arteries further strengthens a pivotal role for gap junctions in EDHF-mediated relaxation in these vessels.

In arteries with an ID, corresponding to that used in functional studies, ECs send long projections through internal elastic lamina (IEL) towards SMCs to set up a tight connection with each other and provide an ideal background for MEGJ (Figure 12). Classically, in transmission electron micrographs, gap junctions are identified as pentalaminar structures at points of close membrane apposition [122]. Compatible structures were observed in our study as well, although relatively rare, as we did performed limited sections. The difficulty to visualize MEGJ could be also related to a lack of adherent junctions, which organize gap-junctional plaques to form pentalaminar

structure and, as result, at MEGJ level the junctional membranes will be relatively roughly separated. It is also possible that the individual junctional channel at the level of MEGJ provides the pathway for EC-SMC communication [122]. Based on this we consider the close association between EC and SMC (Figure 12 and Figure 6 in Paper III) as typical MEGJs.

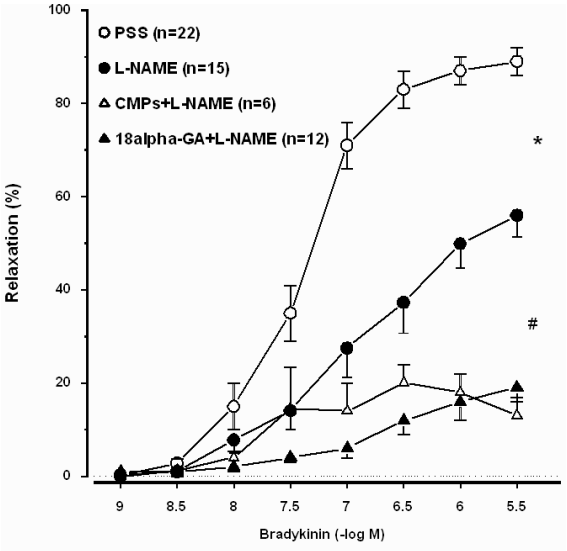

Figure 11. Concentration-response curves to bradykinin in physiological salt solution (PSS) and with different combinations of inhibitors to reveal the contribution of myoendothelial gap junctions in EDHF mediated response. L-NAME - after pre-incubation with N^{w}-nitro-L-arginine methyl ester (L-NAME, 300 μmol/L); CMPs+L-NAME - in triple combination of connexin mimetic peptides (CMPs, ^{37,43}Gap27, ^{40}Gap27 and ^{43}Gap26, 300 μmol/L of each) and L-NAME; 18α-GA+L-NAME– after pre-incubation with combination of 18α-glycyrrhetinic acid (18α-GA, 100 μmol/L) and L-NAME. $^{*}P < 0.001$ *vs* PSS; $^{\#}P < 0.001$ *vs* L-NAME.

We also assessed the relative functional importance of Cx subtypes, i.e. Cxs 37, 40 and 43 [56] by using three CMPs separately, which had no influence upon the endothelium-independent responses evoked by SNP or pinacidil (opener of ATP-sensitive K^{+}-channels). Our results indicated the predominant role of Cx 43 rather than Cxs 40 and 37, since only incubation with ^{43}Gap26 (900 μmol/L) exerted marked inhibition of EDHF-mediated relaxation to BK, which was similar to that after incubation with the triple combination of CMPs (see Fig 3, paper II).

Thus, the additional and novel finding that we have provided in this thesis (Paper II) is that the MEGJ involved in EDHF-mediated responses rely largely upon Cx43 in subcutaneous arteries from NP women.

Immunohistochemistry experiments have confirmed the presence of Cx37, Cx43 and Cx40 expression in both the endothelium and smooth muscle of studied arteries, and typical staining of them within the artery wall and is presented in Figure 5 (paper II). Cx40 had a higher expression in the endothelium when compared with Cx43 (2.3±0.6

vs 1.4±0.7, p=0.05, n=6) and Cx37 (2.3±0.6 *vs* 0.9±0.5 p=0.02, n=6). In the media, Cx43 was predominant when compared to Cx40 (2.5±0.2 *vs* 1.9±0.4 p=0.02, n=6) and Cx37 (2.5±0.2 *vs* 2.0±0.3, p=0.02, n=6).

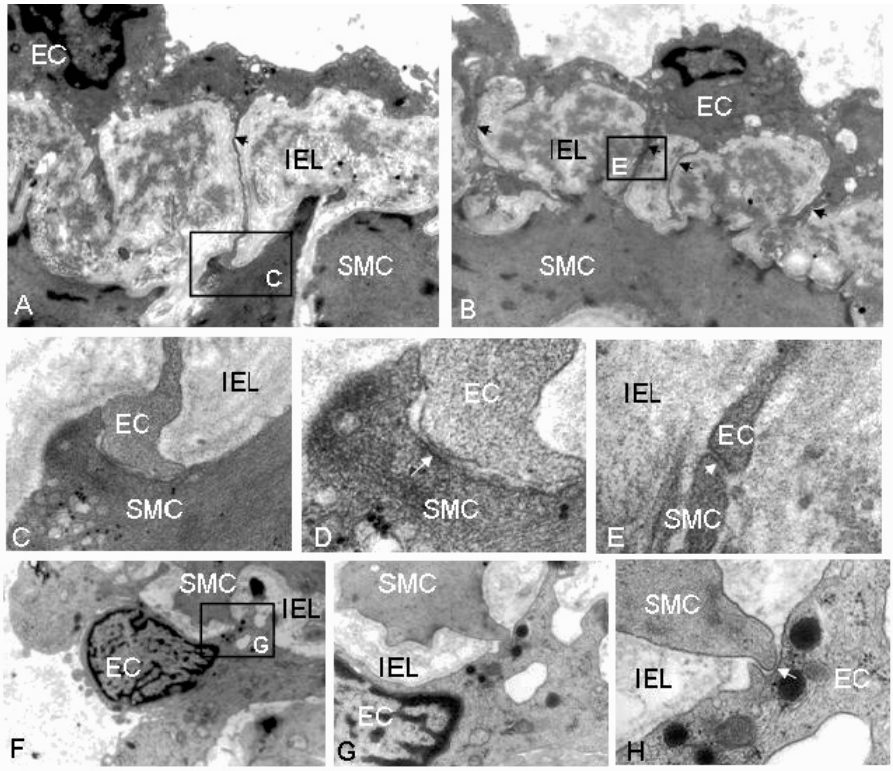

Figure 12. Transmission electron microscopy pictures of arterial wall of subcutaneous arteries from normal pregnant women. (A,B) The low magnification pictures show an overview of the vascular wall with endothelial cells (EC) and vascular smooth muscle cells (SMC) separated by the internal elastic lamina (IEL) that has penetrations allowing protrusions from both EC and SMC to meet with each other. The areas denoted by the boxes are magnified and showing the close contact between the cells. (C-E, G-H) Protrusions from both EC and SMC in tight association with each other are considered as prerequisites for myoendothelial gap junction. The width of the gap is ~12 nm (D, arrows), ~10 nm (E, arrow) and ~ 10-13 nm (H, arrows).

It should be noticed however, that Cxs expression at the level of myoendothelial gap junctions is histologically most challenging and probably untraceable due to their small size and anatomical proximity to much larger interendothelial gap junctions [240], some complications may also arise from differential binding efficiencies of different antibodies used [411].

If the pattern of Cxs expression/function observed in subcutaneous arteries from NP women could be extrapolated to arteries in non-pregnant subjects, particularly with

respect to Cx43, needs further consideration. Indeed, the expression of Cx43 has been shown to be actively regulated by physical forces, such as shear stress or mechanical load [309] and this could contribute to an increased function of Cx43 in subcutaneous arteries in NP women [303]. Although we failed to differentiate if Cx43 is located principally at the level of MEGJs, the present study has provided a critical support for this particular Cx subtype in the EDHF-typed of endothelium-dependent relaxation.

"Errors using inadequate data are much less than those using no data at all."

Charles Babbage (1792-1871)
English mathematician

4.1.3 Preeclampsia (III)

Life threatening consequences make PE as a major cause of maternal and perinatal morbidity and mortality worldwide. Vascular malfunction confers maternal syndrome of PE, and both hypertension and proteinurea, as main maternal symptoms, could be partly explained by generalized endothelial dysfunction. Experimental evidence, which supports the role of EDHF in blood pressure and blood flow control, led us to anticipate that in this disorder EDHF-mediated responses in small arteries could be compromised. Our group has reported that PE is associated with pronounced alterations in small artery function by means of reduced dilatory response to shear stress and in the endothelial morphology [354,355] which could potentially cause an impairment of intercellular communication. Consequently, we hypothesized that, in arteries from women with PE, EDHF activity would be mediated by one or several pathways, which do not require an intercellular communications via MEGJs.

Despite an evidence for maternal endothelial dysfunction in PE provided in several previous studies (see "Introduction" for details), in this thesis we show that the overall responses to endothelium-dependent relaxation to agonist BK are similar in subcutaneous arteries from women with and without PE. However, the NOS and COX inhibition revealed small but significant difference in BK-induced relaxation between arteries from NP women and women with PE (Figure 1, Paper III) indicating that the component of endothelium-dependent relaxation insensitive to NOS and COX inhibition (i.e. EDHF) is reduced in PE.

Again using a pharmacological approach, we further estimated the contribution of MEGJ *vs* H_2O_2 or metabolites of AA to EDHF-mediated relaxation in subcutaneous arteries from women with PE. This cohort appeared to be heterogeneous; as we anticipated after dividing these women based on the level of MEGJ inhibition that we obtained in EDHF-mediated relaxation of these arteries. Thus, the MEGJs were preserved in ~60% of women with PE, and this was considered as a central mechanism for the EDHF-mediated response, and a reduced contribution of EDHF to endothelium-dependent relaxation was also obvious for this subgroup (PE-1). In the rest of PE women (PE-2), MEGJs appeared to play a minor role and H_2O_2 and CYP450 metabolites of AA emerged as alternative and/or additional candidates for EDHF-typed responses. In contrast to the PE-1 subgroup, the arteries from the PE subgroup (PE-2) demonstrated similar EDHF contribution to BK induced relaxation as in NP women. The reasons behind the discrepancy in contribution of EDHF and MEGJs *per se* to

endothelium-dependent relaxation remain unknown and warrant further investigation. It is possible that the similar contribution of EDHF to BK-induced relaxation between NP and some women with PE, namely PE-2, is maintained due to manifestation of additional pathways (e.g. H_2O_2 or CYP450 metabolites of AA), which are invoked to compensate for the compromised MEGJ-mediated, EDHF component in PE.

Some support exist that H_2O_2 and CYP450 metabolites of AA might be involved in the control of vascular tone particularly in PE. An oxidative stress in PE [180, 316] may contribute to the increased production of H_2O_2, which may act as an inhibitor of gap junctions *per se* [366]. At the same time endothelial H_2O_2 could serve as EDHF to compensate the compromised MEGJs-mediated response [242]. It has been shown that the urinary excretion of EETs increases significantly in pregnant women with hypertension [49], therefore there could be a hypothetical prerequisite for EETs to serve as EDHF *per se* or as modulators or amplifiers of EDHF [128].

Our findings suggest that in PE disturbances at MEGJ occur that lead to a reduction of EDHF-mediated relaxation but only in those women, in which additional pathways for EDHF have not emerged to compensate a diminished contribution of EDHF. However, analysis of demographic data of PE patients has not provided a clue for the observed heterogeneity in pathways involved in EDHF-mediated relaxation (Table 2).

Table 2. Patients' characteristics

	PE-1	PE-2
N	7	5
Age (years)	28 (31-34)	33 (25-39)
Nulliparity (%)	40	20
Gestational length (wk)	33 (31-37)	34 (33-37)
Systolic blood pressure, mmHg	161±8	165±12
Diastolic blood pressure, mmHg	102±2	96±3
HELLP syndrome	0	2
Previous PE	1	0
Birth weight, g	2055±200	2808±465

The ultrastructural analysis of the vascular wall among patients with PE has revealed complex alterations that could aggravate the establishment of tight contact between ECs and SMCs. The thickness of IEL varied and IEL *per se* was not homogenous. ECs became thin due to the large intracellular vacuoles and detachments, and even if ECs were able to form the projections, they did so very seldom. SMCs occasionally sent relatively long projections toward EC and, in cases of the denuded areas, the sprouts of SMCs reached the lumen. We have not observed this pattern in arteries from normal pregnant women. The attachment of blood cells was predominantly observed in arteries from PE but never in NP (please see Figures 7 and 8 in the Paper III).

Thus, PE is associated with changes in small artery ultrastructure that could substantiate the aggravation of intercellular communication particularly between EC and SMC; and morphological signs of increased ECs death in PE further support the abnormalities present. These findings are in agreement with our previous report in both subcutaneous [354] and myometrial [355] arteries in PE by using scanning electron microscopy technique instead. Compatible changes in vascular wall morphology have been seen in other vascular beds including umbilical, fetal villous and omental arteries from women with PE [93, 353, 409].

Importantly, observed morphological changes of peripheral vasculature in PE might have implications in understanding the pathogenesis of earlier CVD in women with history of PE in comparison to those with normal pregnancy [3, 30, 139]. Indeed, the regeneration of endothelial injury with the help of endothelial progenitor cells or replication of neighbouring cells is associated with normalization of some, but not all, aspects of endothelial function [119]. Progressive impairment of the endothelium-dependent responses after endothelial regeneration has been reported [328]. Whether functional and morphological alterations within the vascular wall in PE would predispose these women to develop CVD later in life is still unknown. Hopefully, ongoing investigations on small artery morphology and function in women with an adverse pregnancy history will bring some clarity on these issues.

In summary, we have demonstrated that PE is associated with functional and morphological alterations at the level of small peripheral arteries. Although the overall endothelium-dependent response to BK was preserved in PE, EDHF's contribution is reduced. Heterogeneity within PE group with respect to EDHF contribution and involvement of MEGJ, H_2O_2 and CYP450 epoxygenase metabolites of AA has been observed. It differs from NP where MEGJs are the principal pathway involved in EDHF-mediated responses in small subcutaneous [215, 233] or myometrial [195] arteries. Our results indicate that H_2O_2 or CYP450 epoxygenase metabolites of AA may act as back-up mediators of EDHF, emerging when communications via gap junctions are compromised.

"Science... never solves a problem without creating ten more."

George Bernard Shaw (1856-1950)
The Nobel Prize in Literature 1925

4.1.4 Limitations and potential for future research

There are several limitations of the current projects, and those need to be revealed in order to consider promotion of future experiments. First of all, as it was already mentioned above, the measurements of E_m were not performed, and it would be more correct to use the term NO/PGI_2-independent component of endothelium-dependent relaxation rather than EDHF. However, we used the "EDHF" term due to its "historical" title for the third endothelium-derived vasodilator to which the study was devoted.

By implying the pharmacological approach, we have emphasized the importance of MEGJs in EDHF-mediated relaxation of subcutaneous arteries from NP women at

term. Use of currently available inhibitors of gap junctions, including CMPs, made the clarification of the relative role of MEGJ *vs* gap-junctional coupling within the endothelial or smooth muscle layers (and not between the different layers) impossible. Therefore, by blocking gap junctions we virtually abolished spread of electronic signal through both MEGJs and intercellular communication within ECs or SMCs. It is important to consider this issue when significance of longitudinal transfer of the signal through endothelial and smooth muscle layers for the control of vascular tone at level of small arteries is considered.

Incubation of arteries with different combinations of CMPs led us to suggest the importance of Cx43 in the endothelium-dependent relaxation resistant to inhibitors of NOS and COX products. Immunohistochemical analysis of staining for Cx subtypes indirectly revealed the predominance of Cx43 in media, although the limitations of immunohistochemical analysis, particularly at the level of MEGJs, have to be considered. However, we believe that we have good implications to rely on our results from pharmacological experiments, which indicate the importance of Cx43. Up to now, the mechanisms behind the inhibitor action of CMPs are far from clear, and speculations about the role of particular Cx subtypes for vascular maintenance should still be taken with caution.

Despite the fact that we have provided pharmacological evidence for the involvement of H_2O_2 and CYP450 metabolites of AA into endothelium-dependent relaxation resistant to NOS and COX inhibitors (i.e. EDHF-mediated relaxation) in arteries from only some PE women, it is unclear if this part of relaxation is associated with endothelium-dependent hyperpolarization. For example, the production of both H_2O_2 and EETs seems not necessarily require hyperpolarization of ECs or even SMCs. The question arises if these candidates serve as EDHF *per se* or act as modulators and messengers of EDHF (please see "Introduction" for the details). Therefore, H_2O_2 or CYP450 epoxygenase metabolites of AA may alternatively act as back-up mediators of EDHF rather than EDHF *per se*, becoming evident when communication via gap junctions is disrupted.

It is of growing interest to define the mechanism behind EDHF-mediated relaxation in other vascular beds in PE, e.g. myometrial arteries. The relative contribution of EDHF *vs* NO in the uterine circulation in PE has been reported [194], although studies to clarify the mechanism behind the EDHF-typed responses are warranted.

The reasons that bestow the heterogeneity of EDHF responses between women with PE are not clear, and the extended cohort with detailed demographic characteristics might help us to elucidate any apparent correlation. A follow-up study comparing endothelial function in women with a history of PE and normal pregnancy and the relative contribution and the mechanisms of EDHF in these responses will hopefully help us to clarify all these issues.

"What is now proved was once only imagined."

William Blake (1757-1827)
English poet

4.2 KNOCKOUT MICE STUDIES

4.2.1 Hypothesis, aims and methodological considerations

Recently EDHF has been implicated in gender-related differences in blood pressure control [322]. As it has been described in the "Introduction" (Figure 7), aging hypertensive ERβKO mice develop gender-related differences in blood pressure [412]. Therefore, EDHF-mediated responses in small femoral arteries isolated from young WT and ERβKO mice prior to the development of hypertension were studied (Paper IV). A modified vascular adrenergic reactivity has been suggested to be an important factor in the pathogenesis of hypertension [155], consequently gender-related differences in blood pressure in aging ERβKO mice could also be attributable to modified adrenergic response in peripheral circulation (Paper V).

A large body of experimental studies and epidemiological evidence based on observational studies in postmenopausal women with and without estrogen replacement therapy have demonstrated the beneficial cardiovascular action of estrogen. However, data from controlled, prospective clinical trials for primary [305] and secondary prevention [181] indicate a trend towards increased cardiovascular risk. Although these trials can be criticized on the basis of their recruitment of older patients with pre-existing atherosclerotic disease [278], there are some adverse effects of hormonal treatment. Those include oncogenic risk [295, 402] and potential negative consequences on the circulation, such as procoagulant and plaque-destabilizing effects [162]. This led many people to believe that the development of pure agonists or antagonists for ER subtypes might provide benefits of estrogen replacement therapy with reduced side effects. Indeed, selective activation of ERα and ERβ, both of which differ in tissue-specific expression and biological function, appears as a novel pharmacological principle to improve the safety and efficiency of estrogenic compounds in the prevention of CVD [396]. Hence, the role of ER subtypes in multiple mechanisms involved in the regulation of cardiovascular system should be examined more comprehensively.

Due to the lack of highly selective receptor antagonists or agonists, the investigations of ERα and ERβ function on vascular maintenance are mainly confined to animal models in which the receptors are selectively "knocked out". Hypertension reported in ERβKO [412], but not in ERαKO, mice is an indirect indication that a certain ER subtype is involved in the control of blood pressure. Several studies showed that an elevated blood pressure develops in OVX rats [256, 364]. In addition, an increased rate of hypertension has been reported after menopause [298], although it did not occur during the time when the ovary became senescent, but rather over a number of years [15]. Mice double deficient in eNOS and apoprotein-E are hypertensive and atherosclerotic, however gonadectomy reduces blood pressure and atherosclerosis in animals of both genders [173]. Levin (2002) suggested that ERα might mediate the hypertension-promoting actions of estrogen when ERβ or eNOS is perturbed [217]. Taken together, the following questions need to be answered: 1) what is the mechanism behind the

hypertension in ageing ERβKO animals, 2) why the blood pressure in ERβKO males is higher than in females? It must be emphasized, however, that gender-related difference in blood pressure is not unique to hypertensive ERβKO mice, such difference has been reported previously in spontaneously hypertensive rats (SHR) [63].

In this thesis the homozygotous mutant mice lacking the gene for ERβ (ERβ -/-, ERβKO) and their WT littermates were used. Changes in blood pressure were confirmed prior to the initiation of the functional studies [412]. Normal blood pressure was found in intact ERβKO animals until 5 to 6 months (22-26 weeks) of age and increased thereafter. As we intended to study prerequisites for the development of hypertension in ERβKO animals, we deliberately recruited mice from 14 to 22 weeks of age only [412].

Contrary to previous reports that involved investigations on isolated aorta [90, 274, 412], we concentrated on peripheral circulation (i.e. small femoral arteries). We anticipate, these are more likely to be affected by hypertension, or relevant to study pathogenesis of hypertension [412]. Studies using large conduit arteries may not be directly applicable to understand the physiology and pharmacology of small arteries [265], which are indeed involved in the regulation of peripheral resistance, and as such are ideally suited to control blood pressure. Although in our study the definition of femoral artery as resistant is far from accurate, while the size of it (~200μm) is equivalent to small arteries defined as resistant in other species [88]. Moreover, more similarities exist in the physiology and pharmacology between small and resistance arteries rather than in comparison to the aorta (internal diameter for mouse ~1100μm). Furthermore, the aorta is defined as an elastic artery, whereas the femoral artery is defined to be a muscular type [84]. It has been shown that elastic arteries contain mostly vimentin positive-desmin negative SMCs, while muscular arteries also contain vimentin positive–desmin-positive SMCs [23], and this phenotypic diversity could have consequences on the contractile capacity of the vessel.

Prior to the functional study ER subtype expression in femoral arteries was determined by immunohistochemistry. ERα and β were localized within the vascular wall including both endothelium and VSMC. No staining for ERβ was observed in arteries from ERβKO female or male mice (Paper V, Fig.1).

In this thesis BK has been used in human studies, as it is a more physiologically relevant stimulus for the evaluation of endothelial function [237]. In contrast to the human studies, we applied ACh to the mouse arteries. ACh is the most commonly used endothelium-dependent vasodilator, although the physiological relevance of it has been questioned by some researchers [199]. Pilot studies revealed that BK failed to induce any relaxation in femoral as well as in mesenteric arteries or aorta of the studied mice. These findings concur with others, in which lack of dilator response to BK in murine arteries was reported [35, 232]. However, some reports exist showing a pronounce relaxation to this agonist in mice [205, 281, 322]. It is likely that a strain-dependent variation in vascular responsiveness to BK in mice takes place, while dilator responses to ACh are most frequently reported in the murine arteries. The physiological role of ACh in vascular reactivity experiments is now increasingly appreciated, since the localisation of choline acetyltransferase (the enzyme involved in the ACh synthesis) was confirmed in the vascular ECs [192, 284]. Therefore, ACh could be released from

the ECs to cause NO production from adjacent ECs [42]. ACh could also be released from ECs in response to shear stress [258].

A modified vascular adrenergic reactivity has been suggested to be important in the pathogenesis of hypertension [155]. In addition, investigations in several experimental animal models, in which abnormalities in sympathetic nervous system were related to hypertension, have indicated clear gender-related aspects [170]. In addition, and in accordance to several studies, two subtypes of adrenoceptors, α and β, are involved in the pathogenesis, or maintenance of different types of hypertension. In the resistance vasculature of young pre-hypertensive and adult hypertensive rats, a high density of α_1-adrenoceptors was found [155, 399], and the maximum contractile response to selective α_1-adrenoagonist [182, 384] was increased in endothelium-denuded arteries from SHR. On the other hand, salt loading could cause hypertension via a mechanism involving α_2-adrenoceptors [155]. Furthermore, β-adrenoceptor-mediated relaxation is diminished in peripheral arteries from SHR [131]. Overall, all adrenoceptors responsible either for constriction (α_1 and α_2 on SMC) or, straight conversely for relaxation through β, both on ECs and SMCs, and endothelial α_2-adrenoceptors (Figure 13), might act in concert to confer modified adrenergic reactivity in hypertension.

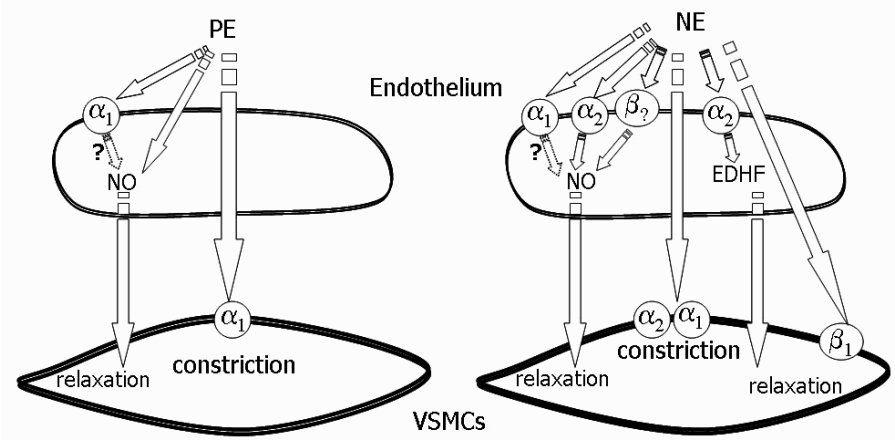

Figure 13. A schematic presentation of all possible pathways involved in response of vascular wall to adrenergic agonists.

We therefore compared constrictor response to non-specific agonist of α and β adrenoceptors – NE, before and after incubation with specific antagonists of α_2 or β-adrenoceptors in small femoral arteries isolated from pre-hypertensive ERβKO and age-matched WT mice. We also studied constriction to phenylephrine (PhE, selective agonist of α_1-adrenoceptors) and thromboxan mimetic (U46619) in these animals. To check the influence of endothelium-derived vasodilators on contractile responses, incubation with inhibitors of NO and PGI_2 production were used. In addition, response to the agonist of β-adrenoceptors – ISO was obtained in arteries from male mice.

"In theory, there is no difference between theory and practice. But, in practice, there is."

Albert Einstein (1879-1955)
The Nobel Prize in Physics 1921

4.2.2 EDHF in ERβKO mice (IV)

In this thesis and in line with previous reports [84, 387], we demonstrated a significant contribution (up to 60% from the level of full response to 30 μmol/L ACh) of a non-NO, non-prostanoid relaxing factor, previously defined as EDHF-mediated response (Figure 14), further strengthening the importance of EDHF in endothelium-dependent dilatation in murine femoral arteries.

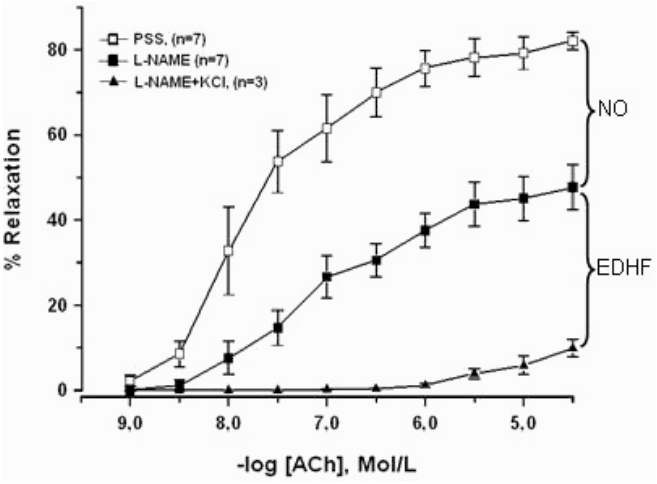

Figure 14. The relative contribution of endothelium-derived vasodilators (NO *vs* EDHF) in ACh-induced relaxation of small femoral arteries isolated from WT female mice.

Our results concur with previous reports of an enhanced endothelium-dependent relaxation in females when compared with males (see review [277] for details, Fig 1a, Paper IV), but in contrast to our own study, in which we failed to obtain the gender-related differences in ACh-induced relaxation in small (ID~200μm) arteries from the mesentery in the same animal model [85]. The reasons for these inconsistencies between murine femoral and mesenteric arteries with respect to endothelium-dependent relaxation are unclear, however it may be attributed to vascular bed specificity or the different experimental system used (wire *vs* pressure myograph). Also in general, endothelium-dependent response to ACh was higher in femoral (75-82%) *vs* mesenteric (39-53%) arteries from WT mice [85].

It is important to stress that in WT mice the gender-related difference in endothelium-dependent relaxation is a distinguishing feature of arteries with ID<200μm, as in larger arteries such differences do not occur (Fig.1b, Paper IV). Since gender related differences in ACh-induced relaxation persists after inhibition of NOS and COX products, we suggest that EDHF, rather than NO or PGI_2, is involved. Moreover, the

sensitivity to ACh after *vs* before the incubation is significantly reduced in arteries from males, but not from females, further indicating that EDHF could serve as a back-up endothelium-derived vasodilator more prominently in females, when other endothelium-derived pathways are blocked or lost [8, 322].

In this thesis (Paper IV) we also suggest that ERβ has influence on the gender-related difference in ACh-induced relaxation through EDHF rather than NO. This is based on observations that endothelium-dependent dilatation is similar in female and male ERβKO mice, and the sensitivity to ACh after *vs* before incubation with L-NAME is similarly reduced in arteries from both genders. In contrast, in WT mice the reduced sensitivity is observed only in males.

Based on differences in sensitivity to ACh before *vs* after incubation with "L-NAME" between WT and ERβKO females, but not males, one could anticipate that more pronounced EDHF-mediated responses in females is regulated through ERβ. However, the comparison of ACh-induced relaxation between arteries from WT and ERβKO females shows a full identity in concentration-response curves to agonist before and after inhibition of NOS and COX products (Fig.3a, Paper IV), thus opposing such a suggestion.

In contrast, arteries from ERβKO males are more sensitive to ACh *vs* WT males (Fig.3b, Paper IV), and this difference in sensitivity to ACh is evident before and after COX and NOS inhibition. These results imply the male gender-specific involvement of ERβ into the down-regulation of EDHF-mediated responses in small arteries. The absence of ERβ in the murine circulation therefore appears to enhance sensitivity to ACh through EDHF-mediated mechanism in males but not in females.

The mechanisms behind the EDHF mediated pathways are of interest, and to our knowledge we are the first to explore them in femoral arteries from male mice. Based on a study by Chataigneau et al. [53] we used a single, supramaximal concentration of ACh (1 μmol/L) to evaluate the mechanism of EDHF-mediated response, since the response to this single concentration of ACh is rapid and sharp and could not be mistaken for a spontaneous reduction in tone.

Gap junctions but not metabolites of AA or endogenous H_2O_2 are involved in the EDHF responses in WT and ERβKO male mice (Fig.4, Paper IV). This is further supported by a morphological approach, which has been applied to look at structural prerequisites for MEGJ in arteries from ERβKO male mice. Again, as for gender differences with respect to endothelium-dependent dilatation and artery size, a discrepancy was found in ultrastructure depending on artery size. A relatively big IEL separating ECs from underlying SMCs obstructed the occurrence of tight connection in PFA (Figure 6A, Paper IV), and it is unlikely that MEGJ could serve for EDHF-mediated response in those vessels.

In contrast, in DFA, frequent penetrations in IEL have provided a setting for numerous EC and SMC projections to form tight connections that could be considered as prerequisites for MEGJ (Figure 6B-F, Paper IV). Morphological data concurs with the common hypothesis that incidence of MEGJ is inversely correlated with internal diameter and the number of SMC layers in the media [240, 309].

We hypothesised that estrogen through the nuclear receptor α or β constantly activate expression of Cx proteins that have a profound influence on EDHF-mediated hyperpolarization and relaxation and could be responsible for the gender-related differences observed. Indeed, it has been shown that an ovarectomy significantly reduces expression of Cx43 [228] and Cx40 [270] in mesenteric arteries, but not in the aorta [175] in rats. Replacement therapy with 17β-E_2 completely prevented this reduction and it remained similar to that in control animals [227, 228, 270]. Also, the expression of Cx37 was up-regulated by 17β-E_2 in M-phase of the ECs of human endometrium [46]. Moreover, in rats, the half-palindromic estrogen responsive elements in the promoter region of the Cx43 gene have been shown to be sufficient to activate transcription of this gene by estrogen [227, 408]. In our study however, immunostaining for Cx 37, 40 and 43 subtypes has not revealed any difference in expression level between arteries from WT and ERβKO male mice (Fig.7,8, Paper IV). Cx 37 seemed to be the predominant subtype expressed in arteries from both WT and ERβKO mice (Fig.7,8, Paper IV). On the other hand, as it was mentioned above, the comparison of staining between different antibodies is more difficult due to complications which may arise from differential binding efficiencies [411]. Poor expression of Cx 40 and 43 was consistent with previous reports showing abundant Cx37 expression in murine vasculature [184, 411].

Cx37 could play an important role in EDHF-mediated responses in rodents, as a decrease in the contribution of EDHF was accompanied by reduced expression of Cx37 but not Cx 40,43 or 45 in small mesenteric arteries from apoE-deficient streptozotocin-diabetic mice [100]. A similar pattern was observed in the mesenteric artery from SHRs [309].

Overall, ERβ confers gender differences in endothelium-dependent relaxation in small femoral arteries from mice by reducing the contribution of EDHF-induced relaxation through gap junction communications in male arteries. Absence of ERβ has no influence on the expression of main Cx subtypes within the vascular wall as well as on ultrastructure and morphology of the EC layer. The up-regulation of the EDHF pathway in ERβKO males with no change in female arteries, does not support the suggestion that an alteration in endothelial function may predispose these animals to the development of hypertension as they age.

"The opposite of a correct statement is a false statement. But the opposite of a profound truth may well be another profound truth "

Niels Bohr (1885-1962)
The Nobel Prize in Physics 1922

4.2.3 Contractile responses in ERβKO mice (V)

It is widely recognized that the sympathetic system plays an important role in blood pressure control through modulation of contractility in response to activation of adrenergic receptors, the significance of which is emphasized by the efficiency of α_1- and β-adrenoceptors antagonists or α_2-adrenoceptors agonists in the treatment of human hypertension [155, 288].

Increased PhE-induced constriction in small femoral arteries from ERβKO males *vs* ERβKO females and WT animals of both genders is a key finding in this study (Figure 15). The mechanism involved is currently unknown, although our results indicate gender-related changes in constriction to be specific for α_1-adrenergic receptors activation, as NE- and U46619-induced constrictions were unaltered (Figure 16). We therefore suggest that enhanced α_1-adrenergic reactivity of small arteries in normotensive male ERβKO mice might initiate the development of hypertension.

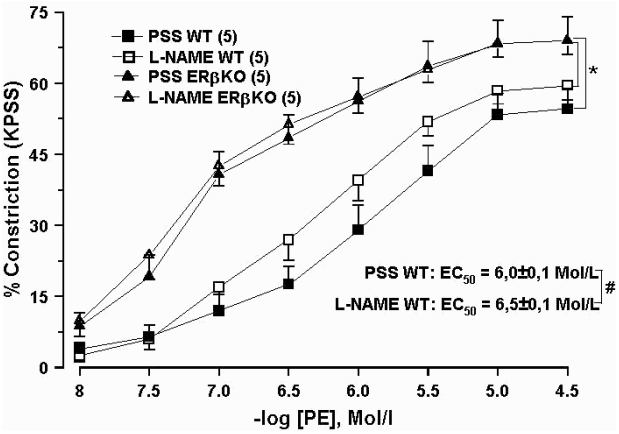

Figure 15. Contractile responses to selective α_1-adrenergic receptor agonist phenylephrine (PE) before and after incubation with inhibitors of NO and PGI_2 production (L-NAME, 100μM +L-NNA, 300μM +Indo, 10 μM) in small femoral arteries from WT and ERβKO male mice.
Data are reported as means ± SEM for the number of animals indicated in parentheses. *, $p < 0.05$ between the responses in arteries from ERβKO *vs* WT mice; #, $p < 0.05$ between the responses before *vs* after incubation with inhibitors of endothelial function.

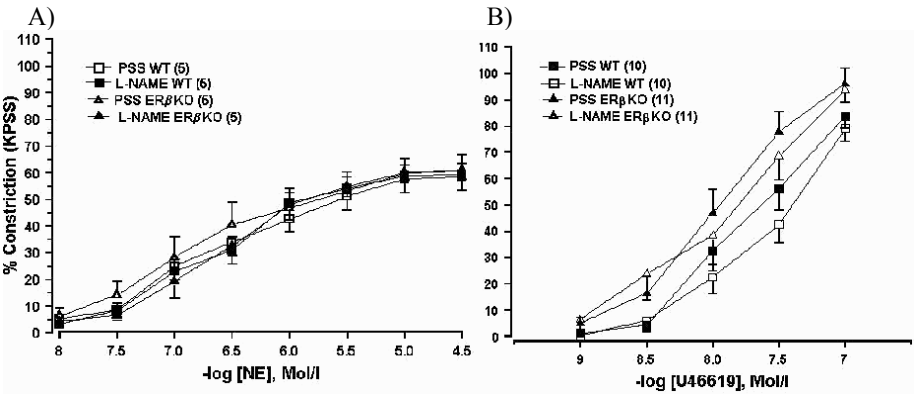

Figure 16. Contractile responses to a non-selective agonist of α and β adrenergic receptors, norepinephrine (NE, A), and thromboxane mimetic (U46619, B) before and after incubation with inhibitors of NO and PGI_2 production (L-NAME, 100μM +L-NNA, 300μM +Indo, 10 μM) in small femoral arteries from WT and ERβKO male mice.
Data are reported as means ± SEM for the number of animals indicated in parentheses.

In OVX rats, for example, hypertension is accompanied by increased reactivity to PhE in small cerebral arteries, and therapy with tamoxifen (the partial ER agonist) abrogates PhE-induced constriction and moderately reduces systolic blood pressure [364]. On the other hand, contraction to PhE has been shown to be similar in isolated aorta [412] and mesenteric arteries [85] between ERβKO and WT mice, or in aortas from WT, ERβKO and ERαKO female mice after OVX and 17β-E_2 supplementation [90]. Thus, the vascular bed specific (femoral *vs* mesenteric, aorta) enhancement of PhE responses occurs in ERβKO male mice. It is important to stress that the femoral artery is mainly under adrenergic control, while mesenteric arterial tone is predominantly maintained by the rennin-angiotensin system, and different reactivity of the femoral arterial bed to exogenous agonists in comparison to mesenteric bed has been reported [166].

Male gender specific enhancement of PhE-induced constriction in ERβKO mice is in line with observations in small arteries from SHR, in which amplified adrenergic nerve stimulation was accompanied by higher blood pressure in males [63, 266] *vs* females [36]. Since after OVX the α_1-adrenoceptor-mediated response to adrenergic nerve stimulation was significantly increased in arteries from SHRs females and estrogen supplementation reversed it back to the initial level, estrogen has been implicated for gender discrepancy in adrenergic response [36].

Endothelium-derived factors could be involved in the enhanced α_1-adrenergic responses in ERβKO males. Indeed, inhibitors of endothelium-derived vasodilators increased sensitivity to PhE in arteries from WT, but not ERβKO mice, suggesting that NO and possibly PGI_2 are released to suppress the contraction in WT animals, whereas in ERβKO males vasodilative capacity of the endothelium seems to be compromised (Figure 15).

A few studies suggested that the release of endothelium-derived NO could be linked to endothelial α_1-adrenoceptors [31, 371]. However, direct evidence for this is still ambiguous and the current accepted view suggests that endothelial α_2- rather than α_1-adrenoceptors are linked to NOS [378]. The alternative hypothesis implies the mechanism involving MEGJs, which may provide a mode for Ca^{2+} efflux from constricted SMCs to the endothelium [101]. In more details, the activation of α_1-adrenoceptors leads to an increase in SMC $[Ca^{2+}]_i$ followed by passive diffusion of Ca^{2+} or triphosphoinositol (IP_3) down their concentration gradients from SMCs to ECs through MEGJs [102]. Since elevation of endothelial $[Ca^{2+}]_i$ is known to increase the production of NO, PGI_2 and EDHF, it could suppress α_1-adrenoceptor-mediated constriction. This hypothesis has also a critique point, because theoretically, any increase in SMC $[Ca^{2+}]_i$ could lead to an increased production of endothelium-derived vasodilators if MEGJs are present. In our study, however, endothelium-dependent reduction in constriction either to U46619 or NE was not observed (Figure 16). Our findings about enhanced EDHF-mediated relaxation through MEGJ in ERβKO males also add some evidence refusing such a possibility.

An alternative mechanism relevant to endothelium-dependent modulation of α_1-adrenergic responses may involve the release of endothelium-derived endothelin-1 (ET-1), the constrictor effect of which is normally compensated by basal NO production [362]. ET-1 has been suggested to be responsible for increased sensitivity of arteries to PhE after NOS inhibition [273, 362]. Epinephrine-induced release of ET-1 in cultured

porcine ECs is blocked by α_1- but not α_2- or β-adrenergic antagonists, indicating that α_1-adrenoceptors are involved in ET-1 release [200]. Furthermore, coronary arteriolar constriction in response to activation of α_1-adrenoceptors mostly results from the release of ET-1 [98].

Gender related difference in vascular effects to ET-1 was also observed. The ET-1 concentration is usually higher in men *vs* women [289], and it is reduced in male to female transsexuals, but increased in female to male transsexuals indicating that sex hormones are of importance [257]. Estrogen also inhibits constrictions to ET-l in coronary microvessels isolated from male and female dogs [213]. Long-term blockade of ET_A receptors reduces blood pressure in post-menopausal SHR to a level found in young females [404], and the expression of preproendothelin-1 is significantly up-regulated in OVX pigs [391]. Estrogen appears to decrease plasma ET-1 level in OVX rabbits [410] and in post-menopausal women [27]. Akishita et al [6] demonstrated that the inhibitory effect of estrogen on ET-1 production and its mRNA expression could be blocked by an estrogen receptor antagonist, ICI 182,780, suggesting an ER-dependent pathway. The importance of ERβ has been suggested since 17β-E_2, the ERβ agonist DPN (diarylpropionitrile), but not the ERα agonist PPT, attenuated the ET-1-induced constriction of the aorta from male rats [17]. However, it is important to note that over expression of ERα in ECs dramatically decreases the ET-1 secretion [7].

Thus, all above-mentioned findings led us to anticipate a possible role of ET-1 in the increased α_1-adrenergic constriction after inhibition of NOS and COX products in arteries from WT mice. The lack of effect of NO inhibition on PhE-induced constriction in femoral arteries from ERβKO males may be due to, either increased production of ET-1, inadequate release of NO, or both. Our hypothesis that ET-1 might be involved in observed alterations in PhE-induced constriction is strengthened by evidence that chronic treatment of male SHR with an inhibitor of ET_A receptors also decreases PhE-induced constriction in isolated aorta [183], and ET_A receptor inhibition prevents the rise in blood pressure after OVX [256].

An increased response to inhibitors of NO and PGI_2 production (L-NAME+L-NNA+Indo) has also been observed in arteries from ERβKO males but not in females. The inhibition of endogenous production of NO may increase basal tone through at least two possible ways: directly as a consequence of abolishment the basal NO-mediated relaxation and indirectly through uncovered constriction in response to endothelium-derived contractile factors, namely ET-1 [273]. ET-1 and NO are known to counteract each other by a physiological antagonism, one factor being a constrictor and the other a dilator [142]. It has been shown that endogenous production of ET-1 contributes to the maintenance of vascular tone [163] and altered expression and activity of ET-1 could contribute to the development of hypertension [321].

Thus, the enhanced response to inhibitors of endothelial function in femoral arteries from ERβKO males is in line, at least partly, with our above-mentioned speculation for the role of ET-1 in the enhanced PhE-induced constriction. The higher constriction in response to complex of endothelial inhibitors in arteries from ERβKO males could therefore be caused by an increased basal release of ET-1 rather than by abolishment of enhanced production of NO *per se*. Indeed, the increase in basal NO production in femoral arteries from ERβKO males is in poor agreement with the simultaneous

absence of NO-mediated influence on PhE-induced constriction. Also, increased reactivity of cerebral arteries from OVX rats in response to NOS blocker (L-NNA) is associated with enhanced reactivity to PhE and hypertension [364] which could be prevented by ET_A inhibition [183, 256, 320].

Based on our results and the data presented in the literature we speculate that alterations in the relative release between ET-1 and NO could partly explain an increased contractile response to Phe in small arteries from ERβKO males. Moreover, our ET-1-related hypothesis could also be relevant for enhanced EDHF-mediated responses found in ERβKO males (Paper IV), since a higher ET-1 level has been shown to increase gap junctional communication [290] that is in charge for EDHF-mediated responses in the small femoral arteries. Obviously, further investigations are required to finalize the role of endothelium-derived factors, principally ET-1, in enhanced contractility at the level of small arteries in ERβKO males and in the development of increased blood pressure with age.

Similar responses to NE in ERβKO and WT males observed in this thesis suggest that ERβ-dependent modulation of adrenergic receptors may occur to prevent enhanced α_1-adrenoceptors-mediated constriction in ERβKO males. NE is a non-specific agonist of adrenergic receptors and activates α- and β-adrenoceptors located on endothelium and smooth muscle, whereas PhE constricts arteries solely through α_1-adrenoceptors located on SMCs (Figure 13). It is known that NE-induced constriction could be modulated by the activation of endothelial α_2-adrenoceptors linked to the release of NO [378], EDHF [363], vasoconstrictor cyclooxygenase-derived prostanoids [121] and ET-1 [365]. Kim et al. (1999) also suggested that α_2-adrenoceptors mediate both an endothelium-dependent and endothelium–independent relaxation of rat aorta through the release of NO and the opening of glibenclamide (ATP) sensitive K^+ channels in SMCs, respectively [197]. In the mouse denuded aorta, contractions to NE could be increased by deletion of α_2-adrenoceptors or by α_2-adrenoceptor antagonists but not by glibenclamide, suggesting endothelium- and ATP-sensitive K^+ channels- independent inhibitory effect on α_2-adrenoceptor in NE-induced contraction [375]. Furthermore, in contrast to large conductance arteries in which response to α_2-adrenoceptors triggers the release of endothelium-derived NO, inhibitors of NOS do not prevent relaxation mediated by α_2-adrenoceptors agonists in small arteries [275, 365] and EDHF seems to be of particular importance [363]. On the other hand, in small arteries in contrast to large, α_2-adrenoceptors are located on SMCs and mediate constriction [67].

Considering the evidence that an increased number of α_2-adrenergic receptors at the level of smooth muscle is associated with the development of hypertension [385], and lower sensitivity to α_2-agonist stimulation has been reported in OVX rats [121] we further explored the contribution of α_2-adrenoceptor to NE-induced constriction in femoral arteries from WT and ERβKO males. We found that α_2-adrenoceptors linked to endothelium-derived factors did not modulate the NE-induced responses in WT and ERβKO male mice, since concentration-response curves to NE were similar before and after incubation with eNOS and COX inhibitors (Figure 16). However, yohimbine significantly inhibited constriction, suggesting the contribution of α_2-adrenoceptors located on SMC (Figure 17), although the response to NE after incubation with

yohimbine was still significantly lower than that to PhE in arteries from ERβKO males (Figure 4, Paper V).

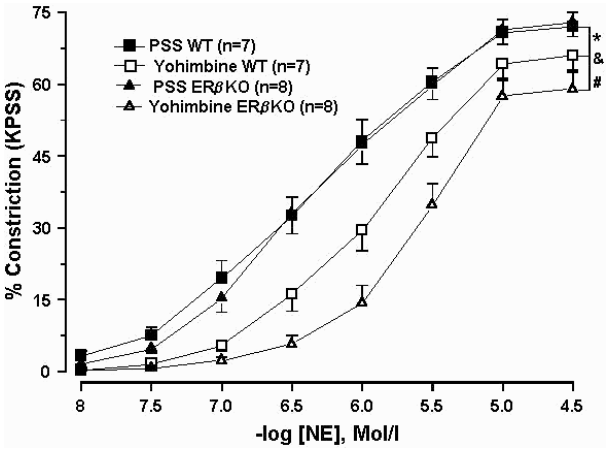

Figure 17. Contractile responses to a non-selective agonist of α and β receptors, norepinephrine, before and after incubation of α_2-adrenoceptors inhibitor- yohimbine (1 μMol/l) in small femoral arteries from WT and ERβKO male mice.
Data are reported as means ± SEM for the number of animals indicated in parentheses. *, $p <$ 0.05 between the response before *vs* after incubation with yohimbine in arteries from WT mice; #, $p < 0.05$ between the responses after incubation with yohimbine in arteries from WT *vs* ERβKO male mice; &, $p < 0.05$ between the response before *vs* after incubation with yohimbine in arteries from ERβKO mice.

Therefore, it seems reasonable that the events occurring at the level of β-adrenoceptors may explain the discrepancy in contractile effects between NE and PhE in arteries from ERβKO males. Indeed, NE, as an agonist of β-adrenoceptors, has been implicated in hyperpolarization and relaxation through Gs protein/adenylate cyclase/cAMP signaling cascade and it has been suggested to play an important role in the sympathetic control of smooth muscle tone by opposing α-adrenoceptor-mediated constriction [146, 269].

Additional experiments using a nonselective inhibitor and agonist of β-adrenoceptors – pronethalol and ISO, respectively, substantiated the implication for compensatory up-regulation of β-adrenoceptor-mediated relaxation to diminish enhanced constriction to α_1-adrenoceptor stimulation in arteries from ERβKO male (Figure 18, 19). Indeed, concentration-response curves to NE after pre-treatment with pronethalol were similar to that in response to PhE, suggesting that β-adrenergic relaxation opposes the enhanced α-adrenergic constriction in ERβKO males. This β-Adrenoceptor-mediated modification of NE-induced response seems to be peculiar to ERβKO mice, since in WT animals incubation of arteries from pronethalol did not influence the NE response. On the other hand, the difference in responses to ISO between ERβKO and WT males were only different at a higher concentration of β-adrenergic agonist (Figure 19).

There are, at least, three subtypes of β-adrenoceptors - β_1, β_2 and β_3 and those are located on smooth muscle and/or endothelium. Based on the experiments with β-adrenoceptor knockout mice, Chruscinski et al, (2001) suggested that vascular

relaxation of the murine femoral artery is solely dependent on β_1-adrenoceptor subtype and independent from the endothelium [70]. ISO-induced relaxation in the femoral arteries from both WT and ERβKO animals was, however, modest with a threshold concentration around 0.1μMol/l and the minor relaxation to ISO might be explained by the pre-constrictor *per se*, since PhE by activating protein kinase C may reduce β-adrenoceptor coupling to adenylate cyclase [141].

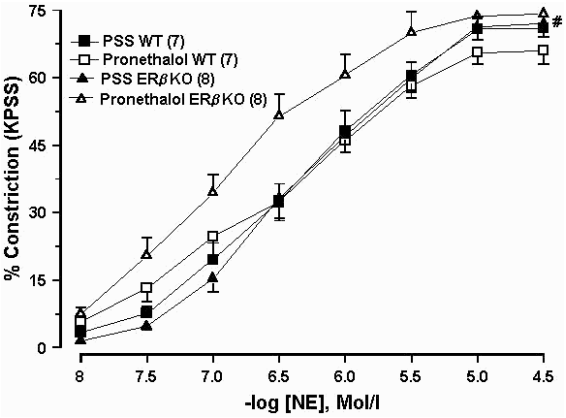

Figure 18. Contractile responses to non-selective agonist of α and β receptors, norepinephrine, before and incubation with β-adrenoceptors inhibitor pronethalol (1 μMol/l) in small femoral arteries from WT and ERβKO male mice.
Data are reported as means ± SEM for the number of animals indicated in parentheses. $^{\#}$, p <0.05 between the responses after incubation with pronethalol in arteries from WT *vs* ERβKO male mice.

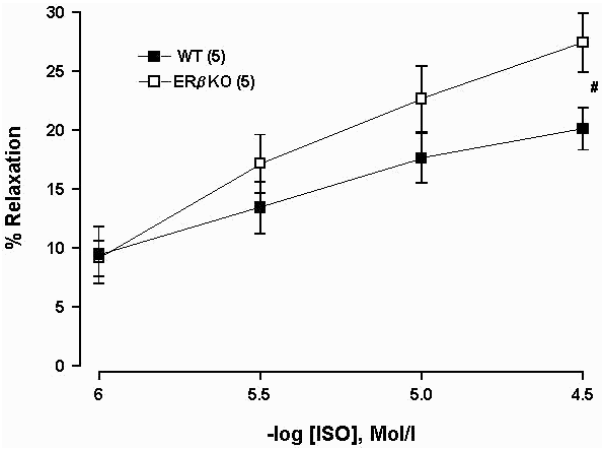

Figure 19. Concentration-response curves to non-selective agonist of β-adrenoceptors isoprenaline (ISO) in small femoral arteries isolated from WT and ERβKO male mice. Data are reported as means ± SEM for the number of animals indicated in parentheses. $^{\#}$, p <0.05 difference in response to 30 μmol/l of ISO between ERβKO and WT males.

Several reports have indicated the relationship between estrogen and β-adrenoceptors in the control of vascular tone. NE-induced relaxation in coronary arteries has been found to be enhanced by acute exposure to physiological levels of 17β-E_2 [22]. Also ISO-induced relaxation has been reported to be impaired in OVX rats, and β-adrenoceptor-mediated relaxation was restored by estrogen substitution [79, 120]. Increased release of NO and improved Gs-protein function within the vascular wall have been suggested as a mechanism behind the beneficial estrogen effect on β-adrenoceptor-mediated responses on isolated aorta [79]. Chan et al (2002) provided support for the synergistic interplay between β-adrenoceptor activation and 17β-E_2, since the enhancement of 17β-E_2-induced relaxation was induced by ISO in rat mesenteric arteries [52]. However, an impaired β-adrenoceptor-mediated relaxation in both conduit and small arteries from SHR has been reported [13, 64, 131, 327], and such abnormalities have been suggested to precede the development of hypertension [64, 131, 146, 327].

The mechanisms responsible for enhanced contribution of β-adrenoceptors to inhibit α_1-adrenergic constriction in ERβKO males is currently unknown, although again the possible role of ET-1 to alter vascular reactivity is of relevance, as in cultured vascular SMCs chronic ET-1 receptor activation increased β-adrenoceptor density and adenylate cyclase activity [37, 38]. It has been shown that ISO induces a greater response in rabbit aorta if pre-constricted with ET-1 but not PhE [141]. ET-1 has also been implicated in the amplification of β-AR/G protein coupling to adenylate cyclase [141].

Overall, the alteration of vascular reactivity in small femoral arteries from male mice deficient in ERβ are in close agreement with the proposed pathogenetic role of ET-1 (Figure 20). The enhanced basal tone in response to inhibitors of endothelial function indirectly suggest the rise in ET-1, which might be responsible for increased constriction to α_1-adrenoceptor agonist PhE and the up-regulation of β-adrenoceptors-mediated effects to inhibit the constriction in response to endogenous adrenergic agonist NE. This speculation is strengthened by fact that hypertension in OVX rats is prevented by ET_A-receptor inhibition [256]. Further investigation is required to clarify the role of endothelium-derived ET-1 in enhanced contractility at the level of small arteries from ERβKO mice and increased blood pressure with age. We speculate that the critical event triggering hypertension in ERβKO males could be a loss of compensatory potency of β-adrenergic response to prevent enhanced α_1-adrenoceptor-mediated vasoconstriction at the level of small arteries. Indeed, the relaxation caused by β-adrenoceptor agonist ISO has been shown to be reduced during aging in different arteries and veins that could be attributable to β-adrenoceptor desensitization due to the increased levels in endogenous catecholamines during aging [238].

In conclusion, this study (Paper V) demonstrates that ERβ seems to be more important for the regulation of small artery function in males compared to females. Increased α_1-adrenergic reactivity followed by β-adrenoceptor modification (Table 3) and changes in basal release of endothelium-derived vasoactive factors (NO versus ET-1) might commence the development of hypertension in ERβKO males.

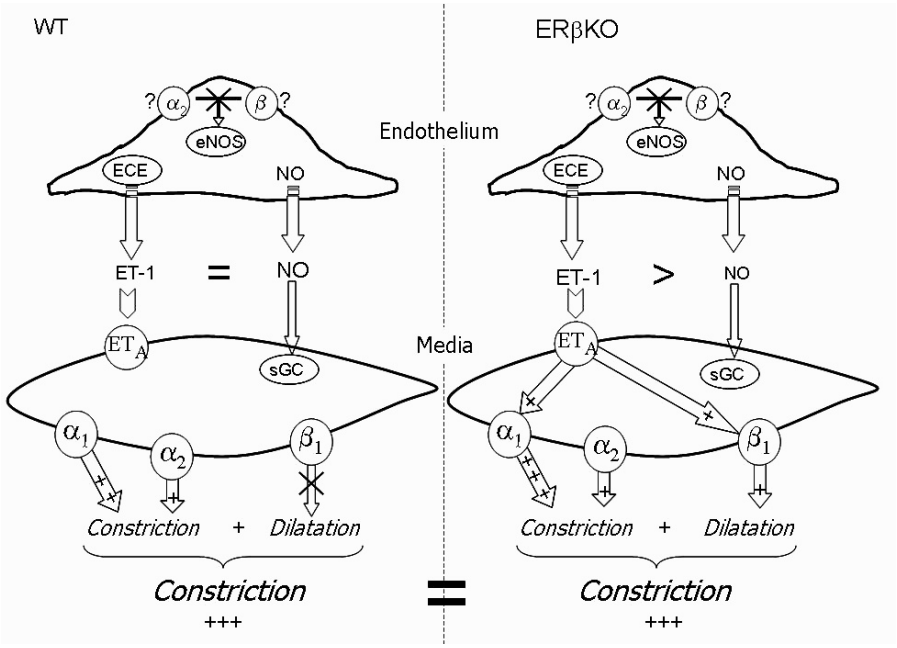

Figure 20. A schematic presentation of the differences observed in young WT and ERβKO males, the balance of adrenergic constriction/relaxation, and the contribution of endothelium-derived factors.

Table 3. Summary of the NE-induced response in arteries from WT and ERβKO male mice

Constriction/ Relaxation	WT	ERβKO
Constriction		
α_1-adrenoceptor	++	+++
α_2-adrenoceptor	+	+
Relaxation		
α_2-adrenoceptor	–	–
β-adrenoceptor	–	+
Total constriction	+++	+++

"The important thing is not to stop questioning."

Albert Einstein (1879-1955)
The Nobel Prize in Physics 1921

4.2.4 Final remarks (IV,V)

We report that the absence of ERβ in the peripheral vasculature of mice has a more significant influence on small artery function in males rather than females. Improvement of EDHF-mediated responses in ERβKO male mice compensates for the lack of gender differences in endothelium-dependent relaxation in WT animals. This observation is specific to femoral arteries with a diameter less than 200 μm (Table 4). Moreover, the absence of ERβ in the male vasculature is characterized by altered basal production of other endothelium-derived factors, presumably ET-1. In general, it is possible to treat ERβ as a steroid receptor, which primarily targets the vasculature of males by modifying vasoactive properties of the endothelium. Furthermore, the deletion of ERβ in males is also accompanied by up-regulation of α_1- and β-adrenoceptor-mediated responses.

At the level of small arteries, the absence of ERβ causes the up-regulation of α_1-AR-mediated constriction followed by β-adrenoceptor modification and changes in basal and receptor-mediated release of endothelium-derived factors (EDHF, ET-1 and NO) to maintain the peripheral vascular tone and to insure normal blood pressure. Therefore, despite increased constriction to selective agonists of α_1-adrenoceptors PhE, in young ERβKO males the compensatory "power" is sufficient to preserve the total response to endogenous adrenergic agonist NE to a level observed in WT mice. Since with age ERβKO animals become hypertensive, it is possible to suggest that the deficiency of compensatory mechanisms to prevent enhanced α_1-adrenergic constriction through desensitization of β-adrenoceptor and/or further misbalance between endothelium-derived constrictor (ET-1) and dilators (NO and EDHF, Figure 21) may serve as a trigger for this event.

Table 4. Summary of the gender differences in vascular reactivity between arteries from WT and ERβKO mice

Response	WT	ERβKO
Increase in tone after NOS and COX inhibition	♀ > ♂	♀ < ♂
Relaxation		
Endothelium-dependent in PSS	♀ > ♂	♀ = ♂
EDHF-mediated relaxation	♀ > ♂	♀ = ♂
Constriction		
adrenoceptor-mediated, NE	♀ = ♂	♀ = ♂
α_1-adrenoceptor-mediated, PhE	♀ = ♂	♀ < ♂
U46619-induced constriction	♀ = ♂	♀ = ♂

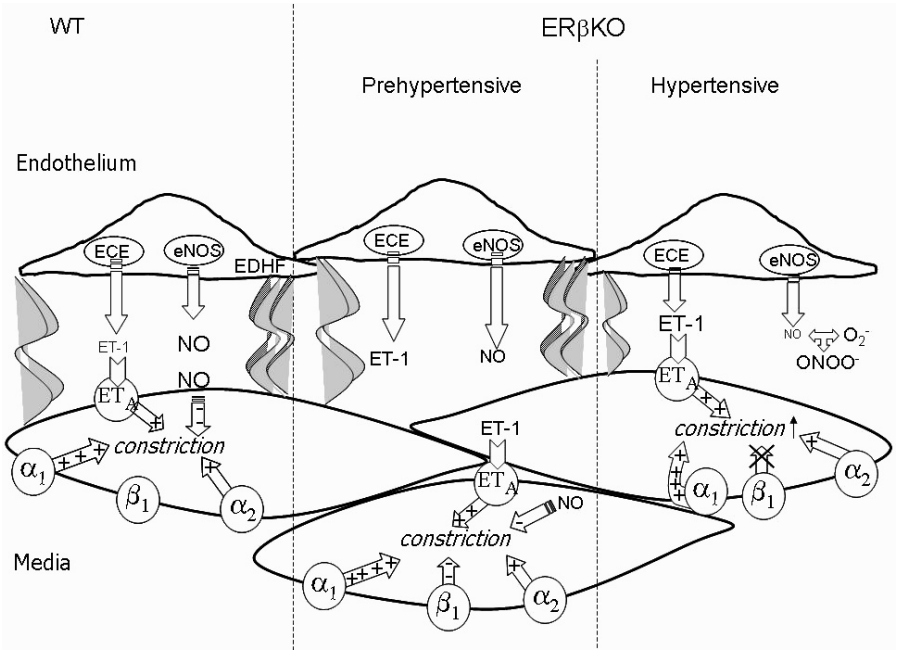

Figure 21. A schematic presentation of proposed differences in adrenergic and endothelial control of small artery tone in WT, prehypertensive and hypertensive ERβKO male mice.

In WT and young ERβKO mice there is a predominance of endothelium-derived vasodilators (NO and EDHF), although an increased level of basal ET-1 release in ERβKO mice is suggested. In hypertensive ERβKO mice the balance between endothelium-derived factors could be shifted towards ET-1, and if inactivation of NO occurs due to suggested oxidative stress [248], the basal tone of vessels will be further enhanced. The contribution of β-adrenoceptors to NE-induced constriction seems to be negligible in WT animals, whereas in pre-hypertensive ERβKO mice β-adrenoceptor-mediated relaxation is acting as ameliorator against the enhanced activation of α_1-adrenoceptor, and as result the total response to NE is similar between WT and pre-hypertensive ERβKO mice. However, age-related desensitization of β-adrenoceptor will exaggerate the rise of adrenergic constriction and possible trigger the development of hypertension peculiar for aged ERβKO mice.

Enhanced ACh-induced relaxation in arteries from ERβKO males, but not females, could hypothetically be explained by the lack of unknown ERβ-controlled pathways that limit EDHF contribution in males. The amplified contribution of EDHF to endothelium-dependent relaxation in ERβKO males could also be considered as a compensatory mechanism to offset male-specific alterations in adrenergic constriction observed in this thesis. An increased cAMP accumulation in SMC as a result of amplified β-AR stimulation may facilitate electrical coupling via gap junctions [153], which in our study have been confirmed to be involved in EDHF-mediated responses. Finally, as some indications exist to suggest synergistic or antagonistic effects between ERs [225, 247], it is possible that ERβ acts as a repressor or modulator of ERα biological activity with respect to EDHF contribution in males. Our group has recently

provided support for a compatible event to occur in femoral arteries from the same animal model. Indeed, ERβ down-regulated ERα mediated NO contribution to the acute dilatory action of 17β-E_2 but in both male and female mice [86]. It is possible to suggest, that deletion of ERβ in males results in enhanced signaling through ERα, the pharmacological stimulation of which has been shown to be sufficient to prevent endothelial dysfunction elicited by estrogen deficiency [396].

The predominant physiological role of ERβ in the male vasculature may appear surprising, although the compelling evidence from literature about the role of estrogen and ERs in males strengthens our suggestion. Estrogen deficiency in males has been associated with increased cardiovascular risk [202, 346]. The male mice lacking a functional aromatase enzyme demonstrate blunted responses to ACh in aortic rings [198]. It has been shown that aromatase is also expressed in SMCs [161] and local changes in estrogen concentrations within the vasculature may also play a role in the modification of vascular tone in response to vasoactive substances.

Estrogen can affect the male cardiovascular system in a manner that may have a potential clinical benefit. Acute administration of estradiol to young men, at concentrations comparable to that obtained in pre-menopausal women, induces enhancement of endothelium-dependent dilatation to ACh in the skin circulation [201]. Longer-term estrogen supplementation in male-to-female transsexuals enhances flow-mediated responses [272]. Our group has also recently reported that phytoestrogens *ex vivo* at concentrations achievable *in vivo* with moderate red wine or soy-derived products consumption in daily diet evoke an acute relaxation in small subcutaneous arteries from men with coronary heart disease or healthy controls [87].

In contrast to males, female small artery function (constriction and EDHF-mediated responses) was unaffected by ERβ deficiency, and the reasons behind this are open to speculation. It might be that in females both ER subtypes are important to a similar extent, and some support exists for such a suggestion. For example, our group has recently shown the predominant role of ERβ in estrogen-induced up-regulation of flow responses in mesenteric arteries from male mice, whereas in females, ERα is responsible [86]. ERβ played an important role in 17β-E_2-mediated relaxation of the rat aorta from males [17], whereas aortic rings isolated from 17β-E_2-treated OVX rats are more sensitive to ERα than to ER-β agonists [32]. It has also been shown by our group that acute responses to genistein [87] (considered as natural ligand for ERβ [72, 207]) are more pronounced in arteries from healthy men *vs* women, and this is also accompanied by a predominant expression of ERβ in arteries from men [85].

The cross talk between ERα and ERβ in the female arterial wall could not be underestimated, and one receptor is probably capable of compensating for the absence of the other. Such an event is relatively feasible, as female gender is naturally provided with a more stable sensatory system to adapt to the constant fluctuations in endogenous estrogen concentrations.

"A conclusion is simply the place where someone got tired of thinking."

Albert Einstein (1879-1955)
The Nobel Prize in Physics 1921

5 GENERAL CONCLUSIONS

1. Endothelium-derived nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) almost equally account for endothelium-dependent relaxation to bradykinin in small subcutaneous arteries from normal pregnant women. The arachidonic acid metabolites through the cytochrome P450 pathway and endogenous H_2O_2 do not explain EDHF-mediated response. Gap junction communication and the connexin 43 subtype play a principal role in EDHF-mediated relaxation of small subcutaneous arteries from normal pregnant women.

2. In preeclampsia, the overall endothelium-dependent relaxation to bradykinin in small subcutaneous arteries is similar to that obtained in normal pregnant women, however the EDHF-mediated contribution is reduced. Preeclampsia is characterized by heterogeneity in the relative contribution of NO versus EDHF to bradykinin-mediated relaxation and in the mechanisms that confer EDHF-mediated component. Gap junctions alone or in combination with either H_2O_2 or CYP450 epoxygenase metabolites of AA comprise the EDHF-mediated response in this disorder. The morphological abnormalities are present within the small artery wall in PE, and those might severely aggravate the establishment of tight interactions between the endothelial and smooth muscle cells.

3. Estrogen receptor β confers gender differences in endothelium-dependent relaxation in small femoral arteries by tonically reducing EDHF mediated relaxation through gap junction communications in male arteries, but only in vessels less than 200μm in internal diameter. Estrogen receptor β apparently has no influence on expression of main connexin subtypes within the vascular wall, or on vascular ultrastructure and morphology of the endothelium. The up-regulation of the EDHF pathway in ERβKO males with no change in female arteries does not support the suggestion that alteration in endothelial function may predispose the development of hypertension in these animals as they age.

4. The absence of ERβ causes the up-regulation of α_1-adrenoceptor-mediated constriction followed by β-adrenoceptor modification and changes in basal and receptor-mediated release of endothelium-derived factors to maintain the vascular tone in peripheral vasculature in males (14-22 weeks old) and insure maintenance of normal blood pressure.

5. The absence of ERβ in peripheral vasculature has an important influence on small artery function in males rather than in females. In general, it is possible to consider ERβ as a steroid receptor, which modifies vasoactive properties of the endothelium and adrenergic control of small femoral arteries primarily in males in the mouse model.

6 ACKNOWLEDGEMENTS

First and foremost, I would like to say that I had a fantastic time here in Stockholm, one of the most beautiful cities I have ever visited, and I would like to thank all of you who made this time so incredibly pleasant and scientifically fruitful. I am very grateful and obliged to all of you.

In particular, I wish to express my deep gratitude to:

Doctor Karolina Kublickiene, my principle supervisor, who welcomed me to Sweden with outstretched arms. You showed me different ways to approach a research problem and the need to be persistent to accomplish any goal. Without your encouragement and constant guidance, I could not have finished this dissertation. Thank you for all the helpful discussions we had, for your constructive ideas on how to improve our research, and that you had always time for the details. Thank you for your everyday support, for helping me so often and for always been ready to help, regardless of what kind of problems I had. Thank you very much for all your time and energy that you invested to improve not only the research but my "Swedish" life as well.

Docent Henry Nisell, my co-supervisor, for his continuous support in the PhD program, to have confidence in me, for encouragement, hard questions and insightful comments about our manuscripts.

I would like to express my best gratitude to **Prof. Lucilla Poston** from Maternal and Fetal Research Unit, St Thomas's Hospital, Kings College London, United Kingdom, **Prof. Jan-Åke Gustafsson** from the Department of Medical Nutrition, Novum, Stockholm, **Prof. David E Newby** from Centre for Cardiovascular Science, University of Edinburgh, United Kingdom for the good encouragement and support during our fruitful collaborations.

Professors at Department of Obstetrics and Gynecology, Karolinska University Hospital, Huddinge **Britt-Marie Landgren, Outi Hovatta, Lennart Nordström,** the Head of our Department, and particularly **Magnus Westgren** for giving me an opportunity to work at the Department, facilities and motivation for the work I am able to do. Thank you for creating a comfortable research environment and for comprehensive introduction to the field of Obstetric and Gynecology by sharing your expansive knowledge. I'm deeply indebted to you for the support from the very beginning.

I would like to express my gratitude to Professor **Li Tsai** for support and possibility to do research at the Department of Clinical Science, Intervention and Technology.

The half of the studies performed within my PhD program would have been impossible without all the women who showed the interest in our work by donation of subcutaneous fat and myometrial biopsies. Thank you all those women who agreed to participate in the studies as well as to the doctors and midwifes at the Department of Obstetrics and Gynecology, Karolinska University Hospital, Huddinge for their time spending in collecting these biopsies for us and explaining for women the potential significance of our research. In particular, I would like to express my appreciation to **Karin Petersson**, **Gunilla Ajne** (particular thanks that you always remember our huge interest in women with preeclampsia), **Annelie Kaldma, Sverker Ek, Marja Liisa Swahn, Teija Taimi** and others doctors who have been always kind to take for us the biopsies good enough to find arteries there.

My co-authors in papers included in the thesis: **Dr. Ninian N Lang** from University of Edinburgh, for exciting collaboration, short but productive research, excellent revision of my English and pleasant time together in Stockholm and at meeting in Amsterdam; Docent **Kjell**

Hultenby from Department of Laboratory Medicine for sharing the knowledge and introduction to transmission electron microscopy and help with immunohistochemistry; **Dr. Lusine Aghajanova** for help with scanning electron microscopy technique and enjoyable time at SGI meetings.

Dr. Marius Kublickas I will never forget that you had always time when I needed help (despite of your extremely busy timetable) and especially thank for all the help on the whole diversity of problems in everyday life from the very beginning and for the ongoing tremendous support. Without your contribution in recruitment of the women I would not be able to finish this thesis, at least in 2007.

Many thanks also to **Lisabeth Löfstrand**, administrator at our Department, for all-round assistance.
Ingrid Lindell and especially **Eva Blomen** from Department of Laboratory Medicine for the introducing me to the immunostaining technique and for the really nice cryosections.

My companions in the lab, both past and present, **Eimantas Svedas**, for opening my eyes in many different aspects of life and research in Sweden, I missed you a lot after you left Stockholm, with all my heart I wish you all the best for your career as a Clinician; **Natalia Cruz, Gillian Douglas**, **Anton Paier**, **Judith Blaauw**, **Marlene Lindskog** for creating nice working environment, for your friendship and support.

My time in Sweden wouldn't have been so nice without support from other colleagues at Department and Novum: **Mia Lindeberg, Marina Yassenko, Pu Zhang, Signe Altmäe, Julius Hreinsson, Jose Inzunza, Anneli Stavreus-Evers, Lev Levkov, Victoria Keros, Jennifer Scott, Jessica Cedervall, Ami Stromberg, Gayane Avetisyan, Karin Gertow, Sussane Ström, Juan Jesus Carrero, Anna Berglöf, Prof. Stefan Agewall** (Dept. of Medicine, Div of Cardiology)**, Prof. Lars Arhlund-Ritcher, Prof. Moustapha Hassan** and many others.

Special acknowledgments I would like to express to **Inger Britt Carlsson** for the cheerful spirit but mostly for the revision of my English.

I am also greatly indebted to many people in Belarus. First of all, to Professor **Leonid Mihailovich Lobanok,** for getting me interested in vascular research, for introducing me to the "world of the endothelium", and teaching me as a researcher from the very beginning.
I will always remember the **Laboratory of Physiology** where my research career has begun. Thank you all who have shared with me the destiny of Belarusian researcher, for support and friendship. I'm really sorry that this Lab does not exist anymore. But I'm always happy to meet my good friends from the "Belarusian lab" and particularly **Alexander Antonenko** and **Dmitrij Shkadinskij,** thank you for sharing good time during my vacations in Belarus.

There were many very important for me teachers at the Pedagogical University in Minsk and especially docent **Lisov Nikolaj Dmitrievich** from the Department of General Biology, thank you for the support over the years and believing in me. My old friend docent **Sheverdov Vladimir** and others.
Professor **Kaminskaya Evelina Andreevna** for always being my great tutor to the end, she was the one who distinguished my inquisitive creature and sent me in the right direction.

I would like to express my gratitude to **my Parents**, for giving me life in the first place, for unconditional support and encouragement to pursue my interests and goals, for unlimited love and always being proud of what I do.

My gratitude cannot be expressed in words to my **Family** who saw me through all the enjoyments and frustrations of the research, while helping me stay focused on the live issues. My most heartfelt thanks go to my beloved wife **Natallia** for patience, love and always believing in me. My son, **Zhenya**, for giving me happiness in every day, for love and inspiration.

The studies were supported by grants from the Swedish Institute, Swedish Heart and Lung Foundation; the Centre of Gender related Medicine at Karolinska Instituetet, Karolinska Institutet, CLINTEC, FoUU, Anders Otto Swärds Stifelse, The Swedish Medical Society and The Swedish Society for Medical Research, Åke Wiberg Foundation, and Tore Nilssons Foundation.

7 REFERENCES

1. Adeagbo AS, Triggle CR (1993) Varying extracellular [K+]: a functional approach to separating EDHF- and EDNO-related mechanisms in perfused rat mesenteric arterial bed. J Cardiovasc Pharmacol 21:423-429
2. Adeagbo ASO HM (1998) Calcium-Dependent Phospholipase A2 Mediates the Production of Endothelium-Derived Hyperpolarizing Factor in Perfused Rat Mesenteric Prearteriolar Bed. J Vasc Res 35:27-35
3. Agatisa PK, Ness RB, Roberts JM, Costantino JP, Kuller LH, McLaughlin MK (2004) Impairment of endothelial function in women with a history of preeclampsia: an indicator of cardiovascular risk. Am J Physiol Heart Circ Physiol 286:H1389-1393
4. Agewall S, Henareh L, Kublickiene K (2006) Endothelial function in conduit and resistance arteries in men with coronary disease. Atherosclerosis 184:130-136
5. Ahluwalia A, Hobbs AJ (2005) Endothelium-derived C-type natriuretic peptide: more than just a hyperpolarizing factor. Trends Pharmacol Sci 26:162-167
6. Akishita M, Kozaki K, Eto M, Yoshizumi M, Ishikawa M, Toba K, Orimo H, Ouchi Y (1998) Estrogen Attenuates Endothelin-1 Production by Bovine Endothelial Cells via Estrogen Receptor. Biochemical and Biophysical Research Communications 251:17-21
7. Ali SH ODA, Mohamed S, Mousa S, Dandona P. (1999) Stable over-expression of estrogen receptor-alpha in ECV304 cells inhibits proliferation and levels of secreted endothelin-1 and vascular endothelial growth factor. Mol Cell Endocrinol. 152:1-9
8. Anderson LC, Martin DJ, Phillips DL, Killpack KJ, Bone SE, Rahimian R (2006) The influence of gender on parasympathetic vasodilatation in the submandibular gland of the rat. Exp Physiol 91:435-444
9. Andersson DA, Zygmunt PM, Movahed P, Andersson TL, Hogestatt ED (2000) Effects of inhibitors of small- and intermediate-conductance calcium-activated potassium channels, inwardly-rectifying potassium channels and Na(+)/K(+) ATPase on EDHF relaxations in the rat hepatic artery. Br J Pharmacol 129:1490-1496
10. Ang C, Hillier C, Johnston F, Cameron A, Greer I, Lumsden MA (2002) Endothelial function is preserved in pregnant women with well-controlled type 1 diabetes. Bjog 109:699-707
11. Angus JA FC, Sudhir K, Kaye DM, Jennings GL (1993) Impaired contraction and relaxation in skin resistance arteries from patients with congestive heart failure. Cardiovasc Res 27:204-210
12. Archer SL, Gragasin FS, Wu X, Wang S, McMurtry S, Kim DH, Platonov M, Koshal A, Hashimoto K, Campbell WB, Falck JR, Michelakis ED (2003) Endothelium-Derived Hyperpolarizing Factor in Human Internal Mammary Artery Is 11,12-Epoxyeicosatrienoic Acid and Causes Relaxation by Activating Smooth Muscle BKCa Channels. Circulation 107:769-776
13. Asano M MK, Matsuda T. (1998) Evidence for reduced beta-adrenoceptor coupling to adenylate cyclase in femoral arteries from spontaneously hypertensive rats. Br J Pharmacol 94:73-86
14. Ashworth JR, Warren AY, Baker PN, Johnson IR (1997) Loss of endothelium-dependent relaxation in myometrial resistance arteries in pre-eclampsia. Br J Obstet Gynaecol 104:1152-1158
15. August P, Oparil S (1999) Hypertension in women. J Clin Endocrinol Metab 84:1862-1866
16. Austin CE (2000) Chronic and acute effects of oestrogens on vascular contractility. J Hypertens 18:1365-1378

17. Ba ZF, Lu A, Shimizu T, Szalay L, Schwacha MG, Rue LW, III, Bland KI, Chaudry IH (2007) 17beta-Estradiol modulates vasoconstriction induced by endothelin-1 following trauma-hemorrhage. Am J Physiol Heart Circ Physiol 292:H245-250
18. Barber DA, Miller VM (1997) Gender differences in endothelium-dependent relaxations do not involve NO in porcine coronary arteries. Am J Physiol 273:H2325-2332
19. Baron A, Frieden M, Beny JL (1997) Epoxyeicosatrienoic acids activate a high-conductance, Ca(2+)-dependent K + channel on pig coronary artery endothelial cells. J Physiol 504 (Pt 3):537-543
20. Barton M, Beny JL, d'Uscio LV, Wyss T, Noll G, Luscher TF (1998) Endothelium-independent relaxation and hyperpolarization to C-type natriuretic peptide in porcine coronary arteries. J Cardiovasc Pharmacol 31:377-383
21. Bauersachs J, Popp R, Hecker M, Sauer E, Fleming I, Busse R (1996) Nitric oxide attenuates the release of endothelium-derived hyperpolarizing factor. Circulation 94:3341-3347
22. Bell DR, Rensberger HJ, Koritnik DR, Koshy A (1995) Noradrenergic vasorelaxation of porcine coronary arteries is enhanced by direct, acute exposure to 17[beta]-estradiol. General Pharmacology: The Vascular System 26:1289-1294
23. Bengt Johansson AE, Ismo Virtanen, Lars-Eric Thornell, (1997) Intermediate filament proteins in adult human arteries. The Anatomical Record 247:439-448
24. Beny JL, Koenigsberger M, Sauser R (2006) Role of myoendothelial communication on arterial vasomotion. Am J Physiol Heart Circ Physiol 291:H2036-2038
25. Beny J-L, von der Weid P-Y (1991) Hydrogen peroxide: An endogenous smooth muscle cell hyperpolarizing factor. Biochemical and Biophysical Research Communications 176:378-384
26. Berman RS, Martin PE, Evans WH, Griffith TM (2002) Relative contributions of NO and gap junctional communication to endothelium-dependent relaxations of rabbit resistance arteries vary with vessel size. Microvasc Res 63:115-128
27. Best PJM, Berger PB, Miller VM, Lerman A (1998) The Effect of Estrogen Replacement Therapy on Plasma Nitric Oxide and Endothelin-1 Levels in Postmenopausal Women. Ann Intern Med 128:285-288
28. Bian Z, Nilsson S, Gustafsson J-A (2001) Selective Estrogen Receptor Modulators and Coronary Heart Disease. Trends in Cardiovascular Medicine 11:196-202
29. Bird IM, Zhang L, Magness RR (2003) Possible mechanisms underlying pregnancy-induced changes in uterine artery endothelial function. Am J Physiol Regul Integr Comp Physiol 284:R245-258
30. Blaauw J, van Pampus MG, Van Doormaal JJ, Fokkema MR, Fidler V, Smit AJ, Aarnoudse JG (2006) Increased Intima-Media Thickness After Early-Onset Preeclampsia. Obstet Gynecol 107:1345-1351
31. Boer C SG, de Lange JJ, Westerhof N, Sipkema P (1999) Alpha-1-Adrenoceptor Stimulation Induces Nitric Oxide Release in Rat Pulmonary Arteries. J Vasc Res 36:79-61
32. Bolego C, Cignarella A, Sanvito P, Pelosi V, Pellegatta F, Puglisi L, Pinna C (2005) The Acute Estrogenic Dilation of Rat Aorta Is Mediated Solely by Selective Estrogen Receptor-{alpha} Agonists and Is Abolished by Estrogen Deprivation. J Pharmacol Exp Ther 313:1203-1208
33. Bolton TB, Lang RJ, Takewaki T (1984) Mechanisms of action of noradrenaline and carbachol on smooth muscle of guinea-pig anterior mesenteric artery. J Physiol 351:549-572

34. Bolz S-S, Fisslthaler B, Pieperhoff S, De Wit C, Fleming I, Busse R, Pohl U (2000) Antisense oligonucleotides against cytochrome P450 2C8 attenuate EDHF-mediated Ca2+ changes and dilation in isolated resistance arteries. FASEB J. 14:255-260
35. Brandes RP, Schmitz-Winnenthal FH, Feletou M, Godecke A, Huang PL, Vanhoutte PM, Fleming I, Busse R (2000) An endothelium-derived hyperpolarizing factor distinct from NO and prostacyclin is a major endothelium-dependent vasodilator in resistance vessels of wild-type and endothelial NO synthase knockout mice. Proc Natl Acad Sci U S A 97:9747-9752
36. Brandin L, Bergstrom G, Manhem K, Gustafsson H (2003) Oestrogen modulates vascular adrenergic reactivity of the spontaneously hypertensive rat. J Hypertens 21:1695-1702
37. Brehm BR HH, Risler T, Wolf SC. (2000) Chronically elevated endothelin-1 concentrations modulate the beta-adrenergic receptor system in vitro and in vivo. J Cardiovasc Pharmacol. 36:S157-159
38. Brehm BR ZM, Bernhard R, Hoffmeister HM, Wolf SC, Karsch KR. (1998) Dynamic regulation of beta-adrenergic receptors by endothelin-1 in smooth-muscle cells. J Cardiovasc Pharmacol. 31 Suppl 1:S77-80
39. Brink PR (1998) Gap junctions in vascular smooth muscle. Acta Physiol Scand 164:349-356
40. Bryan RMJY, J; Golding, EM.; Marrelli, SP (2005) Endothelium-derived Hyperpolarizing Factor: A Cousin to Nitric Oxide and Prostacyclin. Anesthesiology. 102:1261-1277
41. Buckley C, Hadoke PWF, Henry E, O'Brien C (2002) Systemic vascular endothelial cell dysfunction in normal pressure glaucoma. Br J Ophthalmol 86:227-232
42. Burnstock G (1999) Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. J Anat 194 (Pt 3):335-342
43. Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH (2002) EDHF: bringing the concepts together. Trends Pharmacol Sci 23:374-380
44. Bussemaker E, Popp R, Binder J, Busse R, Fleming I (2003) Characterization of the endothelium-derived hyperpolarizing factor (EDHF) response in the human interlobar artery. 63:1749-1755
45. Buus NH, Simonsen U, Pilegaard HK, Mulvany MJ (2000) Nitric oxide, prostanoid and non-NO, non-prostanoid involvement in acetylcholine relaxation of isolated human small arteries. Br J Pharmacol 129:184-192
46. C. Punyadeera HD, J. Klomp, G. Dunselman, R. Kamps, F. Dijcks, A. Ederveen, A. de Goeij, P. Groothuis, (2005) Oestrogen-modulated gene expression in the human endometrium. Cellular and Molecular Life Sciences (CMLS) 62:239 - 250
47. Carroll MA, Doumad AB, Li J, Cheng MK, Falck J, McGiff JC (2006) Adenosine 2A receptor vasodilatation of rat preglomerular microvessels is mediated by epoxyecosatrienoic acids that activate the cAMP/pritein kinase A pathway. Am J Physiol Renal Physiol.291(1):F155-161
48. Case J, Davison CA (1999) Estrogen alters relative contributions of nitric oxide and cyclooxygenase products to endothelium-dependent vasodilation. J Pharmacol Exp Ther 291:524-530
49. Catella F, Lawson JA, Fitzgerald DJ, FitzGerald GA (1990) Endogenous biosynthesis of arachidonic acid epoxides in humans: increased formation in pregnancy-induced hypertension. Proc Natl Acad Sci U S A 87:5893-5897
50. Chambliss KL, Yuhanna IS, Anderson RGW, Mendelsohn ME, Shaul PW (2002) ER{beta} Has Nongenomic Action in Caveolae. Mol Endocrinol 16:938-946

51. Chan EC, Woodman OL (1999) Enhanced role for the opening of potassium channels in relaxant responses to acetylcholine after myocardial ischaemia and reperfusion in dog coronary arteries. Br J Pharmacol 126:925-932
52. Chan HY, Yao X, Tsang SY, Bourreau J-P, Chan FL, Huang Y (2002) Isoproterenol amplifies 17beta-estradiol-mediated vasorelaxation: role of endothelium/nitric oxide and cyclic AMP. Cardiovascular Research 53:627-633
53. Chataigneau T, Feletou M, Huang PL, Fishman MC, Duhault J, Vanhoutte PM (1999) Acetylcholine-induced relaxation in blood vessels from endothelial nitric oxide synthase knockout mice. 126:219-226
54. Chauhan SD, Hobbs AJ, Ahluwalia A (2004) C-type natriuretic peptide: new candidate for endothelium-derived hyperpolarising factor. Int J Biochem Cell Biol 36:1878-1881
55. Chauhan SD, Nilsson H, Ahluwalia A, Hobbs AJ (2003) Release of C-type natriuretic peptide accounts for the biological activity of endothelium-derived hyperpolarizing factor. Proc Natl Acad Sci U S A 100:1426-1431
56. Chaytor AT, Bakker LM, Edwards DH, Griffith TM (2005) Connexin-mimetic peptides dissociate electrotonic EDHF-type signalling via myoendothelial and smooth muscle gap junctions in the rabbit iliac artery. Br J Pharmacol 144:108-114
57. Chaytor AT, Edwards DH, Bakker LM, Griffith TM (2003) Distinct hyperpolarizing and relaxant roles for gap junctions and endothelium-derived H2O2 in NO-independent relaxations of rabbit arteries. Proc Natl Acad Sci U S A 100:15212-15217
58. Chaytor AT, Evans WH, Griffith TM (1998) Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries. J Physiol 508 (Pt 2):561-573
59. Chaytor AT, Martin PE, Edwards DH, Griffith TM (2001) Gap junctional communication underpins EDHF-type relaxations evoked by ACh in the rat hepatic artery. Am J Physiol Heart Circ Physiol 280:H2441-2450
60. Chen G, Suzuki H, Weston AH (1988) Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. Br J Pharmacol 95:1165-1174
61. Chen GF, Suzuki H (1990) Calcium dependency of the endothelium-dependent hyperpolarization in smooth muscle cells of the rabbit carotid artery. J Physiol 421:521-534
62. Chen HH, Burnett JC, Jr. (1998) C-type natriuretic peptide: the endothelial component of the natriuretic peptide system. J Cardiovasc Pharmacol 32 Suppl 3:S22-28
63. Chen YF MQ (1991) Sexual dimorphism of blood pressure in spontaneously hypertensive rats is androgen dependent. Life Sci. 48:85-96
64. Chen Y-Y, Doggrell SA (2002) Responsiveness, affinity constants and beta-adrenoceptor reserves for isoprenaline on aortae from normo-, pre- and hypertensive rats. Journal of Pharmacy and Pharmacology 54:515-522
65. Cheng G, Weihua Z, Warner M, Gustafsson J-A (2004) Inaugural Article: Estrogen receptors ERalpha and ERbeta in proliferation in the rodent mammary gland. PNAS 101:3739-3746
66. Chiba M, Hensleigh M, Nishime J, Balani S, Lin J (1996) Role of cytochrome P450 3A4 in human metabolism of MK-639, a potent human immunodeficiency virus protease inhibitor. Drug Metab Dispos 24:307-314
67. Chotani MA, Mitra S, Su BY, Flavahan S, Eid AH, Clark KR, Montague CR, Paris H, Handy DE, Flavahan NA (2004) Regulation of alpha2-adrenoceptors in human vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 286:H59-67

68. Chrissobolis S, Sobey CG (2004) Influence of gender on K+-induced cerebral vasodilatation. Stroke 35:747-752
69. Christensen KL, Mulvany MJ (2001) Location of resistance arteries. J Vasc Res 38:1-12
70. Chruscinski A, Brede ME, Meinel L, Lohse MJ, Kobilka BK, Hein L (2001) Differential distribution of beta-adrenergic receptor subtypes in blood vessels of knockout mice lacking beta(1)- or beta(2)-adrenergic receptors. Mol Pharmacol 60:955-962
71. Clark SG, Fuchs LC (1997) Role of nitric oxide and Ca++-dependent K+ channels in mediating heterogeneous microvascular responses to acetylcholine in different vascular beds. J Pharmacol Exp Ther 282:1473-1479
72. Clarkson TB (2002) Soy, soy phytoestrogens and cardiovascular disease. J Nutr 132:566S-569S
73. Cleary C, Buckley CH, Henry E, McLoughlin P, O'Brien C, Hadoke PWF (2005) Enhanced endothelium derived hyperpolarising factor activity in resistance arteries from normal pressure glaucoma patients: implications for vascular function in the eye. Br J Ophthalmol 89:223-228
74. Coats P, Johnston F, MacDonald J, McMurray JJ, Hillier C (2001) Endothelium-derived hyperpolarizing factor : identification and mechanisms of action in human subcutaneous resistance arteries. Circulation 103:1702-1708
75. Cockell AP, Poston L (1997) Flow-Mediated Vasodilatation Is Enhanced in Normal Pregnancy but Reduced in Preeclampsia. Hypertension 30:247-251
76. Cohen RA, Plane F, Najibi S, Huk I, Malinski T, Garland CJ (1997) Nitric oxide is the mediator of both endothelium-dependent relaxation and hyperpolarization of the rabbit carotid artery. PNAS 94:4193-4198
77. Coleman HA, Tare M, Parkington HC (2001) K+ currents underlying the action of endothelium-derived hyperpolarizing factor in guinea-pig, rat and human blood vessels. J Physiol (Lond) 531:359-373
78. Coleman HA, Tare M, Parkington HC (2002) Myoendothelial Electrical Coupling In Arteries And Arterioles And Its Implications For Endothelium-Derived Hyperpolarizing Factor. Clinical and Experimental Pharmacology and Physiology 29:630-637
79. Conde MV, Marin J, Fernandez-Criado C, Balfagon G (2000) Regulation of beta-adrenoceptor-mediated relaxation of the rat aorta is modulated by endogenous ovarian hormones. Clin Sci (Lond) 98:381-387
80. Cooke CL, Davidge ST (2003) Pregnancy-induced alterations of vascular function in mouse mesenteric and uterine arteries. Biol Reprod 68:1072-1077
81. Corriu C, Feletou M, Canet E, Vanhoutte PM (1996) Endothelium-derived factors and hyperpolarization of the carotid artery of the guinea-pig. Br J Pharmacol 119:959-964
82. Cosentino F, Barker JE, Brand MP, Heales SJ, Werner ER, Tippins JR, West N, Channon KM, Volpe M, Luscher TF (2001) Reactive Oxygen Species Mediate Endothelium-Dependent Relaxations in Tetrahydrobiopterin-Deficient Mice. Arterioscler Thromb Vasc Biol 21:496-502
83. Cosentino F, Katusic ZS (1995) Tetrahydrobiopterin and Dysfunction of Endothelial Nitric Oxide Synthase in Coronary Arteries. Circulation 91:139-144
84. Crauwels HM, Van Hove CE, Herman AG, Bult H (2000) Heterogeneity in relaxation mechanisms in the carotid and the femoral artery of the mouse. European Journal of Pharmacology 404:341-351
85. Cruz MN (2006) Gender-related small artery function: implications for estrogenic compounds. In: CLINTEC, Dep. Ob&Gyn. Karolinska Institutet, Stockholm, p 65

86. Cruz MN, Douglas G, Gustafsson JA, Poston L, Kublickiene K (2006) Dilatory responses to estrogenic compounds in small femoral arteries of male and female estrogen receptor-beta knockout mice. Am J Physiol Heart Circ Physiol 290:H823-829
87. Cruz MN, Luksha L, Logman H, Poston L, Agewall S, Kublickiene K (2006) Acute responses to phytoestrogens in small arteries from men with coronary heart disease. Am J Physiol Heart Circ Physiol 290:H1969-1975
88. Daly CJ, Deighan C, McGee A, Mennie D, Ali Z, McBride M, McGrath JC (2002) A knockout approach indicates a minor vasoconstrictor role for vascular alpha1B-adrenoceptors in mouse. Physiol Genomics 9:85-91
89. Dantas MF, Urban M, Spray D, Catelli De Carvalho MH, Passaglia RD (1999) Increased acetylcholine-induced vasodilation in pregnant rats: A role for gap junctional communication. Hypertension 34:937-942
90. Darblade B, Pendaries C, Krust A, Dupont S, Fouque M-J, Rami J, Chambon P, Bayard F, Arnal J-F (2002) Estradiol Alters Nitric Oxide Production in the Mouse Aorta Through the alpha-, but not beta-, Estrogen Receptor. Circ Res 90:413-419
91. Davidson J, Baumgarten I (1988) Glycyrrhetinic acid derivatives: a novel class of inhibitors of gap- junctional intercellular communication. Structure-activity relationships. J Pharmacol Exp Ther 246:1104-1107
92. De Clerck I, Pannier JL, Van de Voorde J (2006) K+ potentiates hyperosmolarity-induced vasorelaxations in rat skeletal muscle arterioles. Eur J Appl Physiol 96:679-685
93. de Luca Brunori I, Battini L, Brunori E, Lenzi P, Paparelli A, Simonelli M, Valentino V, Genazzani AR (2005) Placental barrier breakage in preeclampsia: ultrastructural evidence. European Journal of Obstetrics & Gynecology and Reproductive Biology 118:182-189
94. De Vriese AS, Van de Voorde J, Lameire NH (2002) Effects of connexin-mimetic peptides on nitric oxide synthase- and cyclooxygenase-independent renal vasodilation. Kidney Int 61:177-185
95. de Wit C HB, Wolfle SE. (2006) Endothelial mediators and communication through vascular gap junctions. Biol Chem 387:3-9
96. de Wit C, Roos F, Bolz SS, Kirchhoff S, Kruger O, Willecke K, Pohl U (2000) Impaired conduction of vasodilation along arterioles in connexin40-deficient mice. Circ Res 86:649-655
97. de Wit C, Roos F, Bolz S-S, Pohl U (2003) Lack of vascular connexin 40 is associated with hypertension and irregular arteriolar vasomotion. Physiol. Genomics 13:169-177
98. DeFily DV, Nishikawa Y, Chilian WM (1999) Endothelin antagonists block alpha 1-adrenergic constriction of coronary arterioles. Am J Physiol Heart Circ Physiol 276:H1028-1034
99. Dhein S (2004) Pharmacology of gap junctions in the cardiovascular system. Cardiovasc Res 62:287-298
100. Ding H, Hashem M, Wiehler WB, Lau W, Martin J, Reid J, Triggle C (2005) Endothelial dysfunction in the streptozotocin-induced diabetic apoE-deficient mouse. 146:1110-1118
101. Dora KA, Doyle MP, Duling BR (1997) Elevation of intracellular calcium in smooth muscle causes endothelial cell generation of NO in arterioles. PNAS 94:6529-6534
102. Dora KA, Hinton JM, Walker SD, Garland CJ (2000) An indirect influence of phenylephrine on the release of endothelium-derived vasodilators in rat small mesenteric artery. 129:381-387
103. Dora KA, Sandow SL, Gallagher NT, Takano H, Rummery NM, Hill CE, Garland CJ (2003) Myoendothelial gap junctions may provide the pathway for EDHF in mouse mesenteric artery. J Vasc Res 40:480-490

104. Drouin A, Thorin-Trescases N, Hamel E, Falck JR, Thorin E (2007) Endothelial nitric oxide synthase activation leads to dilatory H2O2 production in mouse cerebral arteries. Cardiovascular Research 73:73-81
105. Drummond GR, Cai H, Davis ME, Ramasamy S, Harrison DG (2000) Transcriptional and Posttranscriptional Regulation of Endothelial Nitric Oxide Synthase Expression by Hydrogen Peroxide. Circ Res 86:347-354
106. Duckitt K, Harrington D (2005) Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. Bmj 330:565
107. Duley L (1992) Maternal mortality associated with hypertensive disorders of pregnancy in Africa, Asia, Latin America and the Caribbean. Br J Obstet Gynaecol 99:547-553
108. Dusting GJ, Moncada S, Vane JR (1977) Prostacyclin is the endogenous metabolite responsible for relaxation of coronary arteries induced by arachindonic acid. Prostaglandins 13:3-15
109. Eckman DM HN, McBride C, Keef KD. (1998) Endothelium-dependent relaxation and hyperpolarization in guinea-pig coronary artery: role of epoxyeicosatrienoic acid. Br J Pharmacol 124:181-189
110. Edwards G, Dora KA, Gardener MJ, Garland CJ, Weston AH (1998) K+ is an endothelium-derived hyperpolarizing factor in rat arteries. Nature 396:269-272
111. Edwards G, Thollon C, Gardener MJ, Feletou M, Vilaine J, Vanhoutte PM, Weston AH (2000) Role of gap junctions and EETs in endothelium-dependent hyperpolarization of porcine coronary artery. Br J Pharmacol 129:1145-1154
112. Edwards G, Weston AH (1998) Endothelium-derived hyperpolarizing factor--a critical appraisal. Prog Drug Res 50:107-133
113. Edwards G, Zygmunt PM, Hogestatt ED, Weston AH (1996) Effects of cytochrome P450 inhibitors on potassium currents and mechanical activity in rat portal vein. Br J Pharmacol 119:691-701.
114. Ellis A, Pannirselvam M, Anderson TJ, Triggle CR (2003) Catalase has negligible inhibitory effects on endothelium-dependent relaxations in mouse isolated aorta and small mesenteric artery. 140:1193-1200
115. Ellis A TC (2003) Endothelium-derived reactive oxygen species: their relationship to endothelium-dependent hyperpolarization and vascular tone. Can J Physiol Pharmacol. 81:1013-1028
116. Evans WH, De Vuyst E, Leybaert L (2006) The gap junction cellular internet: connexin hemichannels enter the signalling limelight. Biochem J 397:1-14
117. Feletou M, Vanhoutte PM (1999) The alternative: EDHF. J Mol Cell Cardiol 31:15-22
118. Feletou M, Vanhoutte PM (2004) EDHF: new therapeutic targets? Pharmacol Res 49:565-580
119. Feletou M, Vanhoutte PM (2006) Endothelium-Derived Hyperpolarizing Factor: Where Are We Now? Arterioscler Thromb Vasc Biol 26:1215-1225
120. Ferrer M, Meyer M, Osol G (1996) Estrogen replacement increases beta-adrenoceptor-mediated relaxation of rat mesenteric arteries. J Vasc Res 33:124-131
121. Ferrer M OG (1998) Estrogen replacement modulates resistance artery smooth muscle and endothelial alpha2-adrenoceptor reactivity. Endothelium. 6:133-141
122. Figueroa XF, Isakson BE, Duling BR (2004) Connexins: Gaps in Our Knowledge of Vascular Function. Physiology 19:277-284

123. Figueroa XF, Paul DL, Simon AM, Goodenough DA, Day KH, Damon DN, Duling BR (2003) Central role of connexin40 in the propagation of electrically activated vasodilation in mouse cremasteric arterioles in vivo. Circ Res 92:793-800
124. Fisslthaler B FI, Busse R. (2000) EDHF: a cytochrome P450 metabolite in coronary arteries. Semin Perinatol. 24:15-19
125. Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I, Busse R (1999) Cytochrome P450 2C is an EDHF synthase in coronary arteries. Nature 401:493-497
126. Fisslthaler B, Popp R, Michaelis UR, Kiss L, Fleming I, Busse R (2001) Cyclic Stretch Enhances the Expression and Activity of Coronary Endothelium-Derived Hyperpolarizing Factor Synthase. Hypertension 38:1427-1432
127. Fleming I (2000) Cytochrome P450 2C is an EDHF synthase in coronary arteries. Trends Cardiovasc Med 10:166-170
128. Fleming I (2004) Cytochrome P450 epoxygenases as EDHF synthase(s). Pharmacol Res 49:525-533
129. Forster C, Kietz S, Hultenby K, Warner M, Gustafsson J-A (2004) Characterization of the ERbeta-/-mouse heart. PNAS 101:14234-14239
130. Freitas MR, Schott C, Corriu C, Sassard J, Stoclet JC, Andriantsitohaina R (2003) Heterogeneity of endothelium-dependent vasorelaxation in conductance and resistance arteries from Lyon normotensive and hypertensive rats. J Hypertens 21:1505-1512
131. Fujimoto S, Fujimoto KS, Moriyama A (2001) Diminished beta-adrenoceptor-mediated relaxation of femoral arteries from young spontaneously hypertensive rats. Auton Neurosci 87:178-186
132. Fukao M, Hattori Y, Kanno M, Sakuma I, Kitabatake A (1997) Sources of Ca2+ in relation to generation of acetylcholine-induced endothelium-dependent hyperpolarization in rat mesenteric artery. Br J Pharmacol 120:1328-1334
133. Fulton D, McGiff JC, Wolin MS, Kaminski P, Quilley J (1997) Evidence against a cytochrome P450-derived reactive oxygen species as the mediator of the nitric oxide-independent vasodilator effect of bradykinin in the perfused heart of the rat. J Pharmacol Exp Ther 280:702-709
134. Furchgott RF ZJ (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288:373-376
135. Gabriels JE, Paul DL (1998) Connexin43 is highly localized to sites of disturbed flow in rat aortic endothelium but connexin37 and connexin40 are more uniformly distributed. Circ Res 83:636-643
136. Gauthier KM, Deeter C, Krishna UM, Reddy YK, Bondlela M, Falck JR, Campbell WB (2002) 14,15-Epoxyeicosa-5(Z)-enoic Acid: A Selective Epoxyeicosatrienoic Acid Antagonist That Inhibits Endothelium-Dependent Hyperpolarization and Relaxation in Coronary Arteries. Circ Res 90:1028-1036
137. Gauthier KM, Liu C, Popovic A, Albarwani S, Rusch NJ (2002) Freshly isolated bovine coronary endothelial cells do not express the BKCa channel gene. J Physiol (Lond) 545:829-836
138. Gerber RT, Anwar MA, Poston L (1998) Enhanced acetylcholine induced relaxation in small mesenteric arteries from pregnant rats: an important role for endothelium-derived hyperpolarizing factor (EDHF). Br J Pharmacol 125:455-460

139. Germain AM, Romanik MC, Guerra I, Solari S, Reyes MS, Johnson RJ, Price K, Karumanchi SA, Valdes G (2007) Endothelial Dysfunction. A Link Among Preeclampsia, Recurrent Pregnancy Loss, and Future Cardiovascular Events? Hypertension: 49:15-16
140. Ghanam K, Ea-Kim L, Javellaud J, Oudart N (2000) Involvement of potassium channels in the protective effect of 17beta-estradiol on hypercholesterolemic rabbit carotid artery. Atherosclerosis 152:59-67
141. Gilbert P TE (2003) Endothelin-1 limits vascular smooth muscle beta-adrenergic receptor sensitivity by a PKC-dependent pathway. J Cardiovasc Pharmacol. 42:534-538
142. Gilbert P, Tremblay J, Thorin E (2001) Endothelium-Derived Endothelin-1 Reduces Cerebral Artery Sensitivity to Nitric Oxide by a Protein Kinase C-Independent Pathway. Stroke 32:2351-2355
143. Gillham JC, Kenny LC, Baker PN (2003) An overview of endothelium-derived hyperpolarising factor (EDHF) in normal and compromised pregnancies. Eur J Obstet Gynecol Reprod Biol 109:2-7
144. Golding EM, Kepler TE (2001) Role of estrogen in modulating EDHF-mediated dilations in the female rat middle cerebral artery. Am J Physiol Heart Circ Physiol 280:H2417-2423
145. Goode GK, Garcia S, Heagerty AM (1997) Dietary Supplementation With Marine Fish Oil Improves In Vitro Small Artery Endothelial Function in Hypercholesterolemic Patients : A Double-Blind Placebo-Controlled Study. Circulation 96:2802-2807
146. Goto K, Fujii K, Abe I (2001) Impaired beta-Adrenergic Hyperpolarization in Arteries From Prehypertensive Spontaneously Hypertensive Rats. Hypertension 37:609-613
147. Goto K, Fujii K, Kansui Y, Abe I, Iida M (2002) Critical Role Of Gap Junctions In Endothelium-Dependent Hyperpolarization In Rat Mesenteric Arteries. Clinical and Experimental Pharmacology and Physiology 29:595-602
148. Grady D, Rubin SM, Petitti DB, Fox CS, Black D, Ettinger B, Ernster VL, Cummings SR (1992) Hormone therapy to prevent disease and prolong life in postmenopausal women. Ann Intern Med 117:1016-1037
149. Granger JP (2002) Maternal and fetal adaptations during pregnancy: lessons in regulatory and integrative physiology. Am J Physiol Regul Integr Comp Physiol 283:R1289-1292
150. Granger JP, Alexander BT, Llinas MT, Bennett WA, Khalil RA (2002) Pathophysiology of preeclampsia: linking placental ischemia/hypoxia with microvascular dysfunction. Microcirculation 9:147-160
151. Griffith TM (2004) Endothelium-dependent smooth muscle hyperpolarization: do gap junctions provide a unifying hypothesis? Br J Pharmacol 141:881-903
152. Griffith TM, Chaytor AT, Bakker LM, Edwards DH (2005) 5-Methyltetrahydrofolate and tetrahydrobiopterin can modulate electrotonically mediated endothelium-dependent vascular relaxation. Proc Natl Acad Sci U S A 102:7008-7013
153. Griffith TM, Chaytor AT, Taylor HJ, Giddings BD, Edwards DH (2002) cAMP facilitates EDHF-type relaxations in conduit arteries by enhancing electrotonic conduction via gap junctions. Proc Natl Acad Sci U S A 99:6392-6397
154. Griffith TM, Taylor HJ (1999) Cyclic AMP mediates EDHF-type relaxations of rabbit jugular vein. Biochem Biophys Res Commun 263:52-57
155. Guimaraes S, Moura D (2001) Vascular Adrenoceptors: An Update. Pharmacol Rev 53:319-356

156. Gustafsson F MH, Arensbak B, Thuneberg L, Neve S, Jensen LJ, Holstein-Rathlou NH. (2003) Expression of connexin 37, 40 and 43 in rat mesenteric arterioles and resistance arteries. Histochem Cell Biol. 119:139-148
157. H C Parkington MAT, H A Coleman, and M Tare (1995) Role of membrane potential in endothelium-dependent relaxation of guinea-pig coronary arterial smooth muscle. J Physiol 484:469-480
158. Haefliger J-A, Nicod P, Meda P (2004) Contribution of connexins to the function of the vascular wall. Cardiovascular Research 62:345-356
159. Halcox JPJ, Narayanan S, Cramer-Joyce L, Mincemoyer R, Quyyumi AA (2001) Characterization of endothelium-derived hyperpolarizing factor in the human forearm microcirculation. Am J Physiol Heart Circ Physiol 280:H2470-2477
160. Hamilton CA, McPhaden AR, Berg G, Pathi V, Dominiczak AF (2001) Is hydrogen peroxide an EDHF in human radial arteries? Am J Physiol Heart Circ Physiol 280:H2451-2455
161. Harada N, Sasano H, Murakami H, Ohkuma T, Nagura H, Takagi Y (1999) Localized Expression of Aromatase in Human Vascular Tissues. Circ Res 84:1285-1291
162. Haynes MP, Russell KS, Bender JR (2000) Molecular mechanisms of estrogen actions on the vasculature. Journal of Nuclear Cardiology 7:500-508
163. Haynes WG WD (1994) Contribution of endogenous generation of endothelin-1 to basal vascular tone. Lancet 344:852-854
164. Hayward CS, Kelly RP, Collins P (2000) The roles of gender, the menopause and hormone replacement on cardiovascular function. Cardiovasc Res 46:28-49
165. Hecker M (2000) Endothelium-Derived Hyperpolarizing Factor--Fact or Fiction? News Physiol Sci 15:1-5
166. Heinemann A, Wachter CH, Holzer P (1998) Differential regulation of mesenteric and femoral blood flow in the rat as revealed by computerized data acquisition and evaluation. Journal of Autonomic Pharmacology 18:39-48
167. Hill CE, Hickey H, Sandow SL (2000) Role of gap junctions in acetylcholine-induced vasodilation of proximal and distal arteries of the rat mesentery. Journal of the Autonomic Nervous System 81:122-127
168. Hill CE, Rummery N, Hickey H, Sandow SL (2002) Heterogeneity in the distribution of vascular gap junctions and connexins: implications for function. Clin Exp Pharmacol Physiol 29:620-625
169. Hillig T, Krustrup P, Fleming I, Osada T, Saltin B, Hellsten Y (2003) Cytochrome P450 2C9 plays an important role in the regulation of exercise-induced skeletal muscle blood flow and oxygen uptake in humans. J Physiol (Lond) 546:307-314
170. Hinojosa-Laborde C, Chapa I, Lange D, Haywood JR (1999) Gender differences in sympathetic nervous system regulation. Clinical and Experimental Pharmacology and Physiology 26:122-126
171. Hobbs A, Foster P, Prescott C, Scotland R, Ahluwalia A (2004) Natriuretic peptide receptor-C regulates coronary blood flow and prevents myocardial ischemia/reperfusion injury: novel cardioprotective role for endothelium-derived C-type natriuretic peptide. Circulation 110:1231-1235
172. Hodges YK, Tung L, Yan X-D, Graham JD, Horwitz KB, Horwitz LD (2000) Estrogen Receptors alpha and beta : Prevalence of Estrogen Receptor beta mRNA in Human Vascular Smooth Muscle and Transcriptional Effects. Circulation 101:1792-1798

173. Hodgin JB, Knowles JW, Kim HS, Smithies O, Maeda N (2002) Interactions between endothelial nitric oxide synthase and sex hormones in vascular protection in mice. J Clin Invest 109:541-548
174. Hong T HC (1998) Restricted expression of the gap junctional protein connexin 43 in the arterial system of the rat. J Anat. 192 (Pt 4):583-593
175. Hortovanyi E VS, Tokes AM, Illyes G, Szekacs B, Paku S, Kerenyi T, Kadar A. (2001) Connexin 43 expression in rat aortic smooth muscle after ovariectomy and hormonal replacement. Pathol Res Pract. 197:109-112
176. Huang A, Kaley G (2004) Gender-specific regulation of cardiovascular function: estrogen as key player. Microcirculation 11:9-38
177. Huang A, Sun D, Jacobson A, Carroll MA, Falck JR, Kaley G (2005) Epoxyeicosatrienoic Acids Are Released to Mediate Shear Stress-Dependent Hyperpolarization of Arteriolar Smooth Muscle. Circ Res 96:376-383
178. Huang A, Wu Y, Sun D, Koller A, Kaley G (2001) Effect of estrogen on flow-induced dilation in NO deficiency: role of prostaglandins and EDHF. J Appl Physiol 91:2561-2566
179. Huang AH, Busse R, Bassenge E (1988) Endothelium-dependent hyperpolarization of smooth muscle cells in rabbit femoral arteries is not mediated by EDRF (nitric oxide). Naunyn Schmiedebergs Arch Pharmacol 338:438-442
180. Hubel C (1999) Oxidative stress in the pathogenesis of preeclampsia. Proc Soc Exp Biol Med 222:222-235
181. Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, Vittinghoff E, for the Heart and Estrogen/progestin Replacement Study Research Group (1998) Randomized Trial of Estrogen Plus Progestin for Secondary Prevention of Coronary Heart Disease in Postmenopausal Women. JAMA 280:605-613
182. Ibarra M, Pardo JP, Lopez-Guerrero JJ, Villalobos-Molina R (2000) Differential response to chloroethylclonidine in blood vessels of normotensive and spontaneously hypertensive rats: role of alpha 1D- and alpha 1A-adrenoceptors in contraction. Br J Pharmacol 129:653-660
183. Iglarz M LB, Henrion D. (1999) Prolonged blockade of endothelin ET(A) receptors decreases vascular reactivity in the aorta of spontaneously hypertensive rats in vitro. J Cardiovasc Pharmacol. 34:354-358
184. Isakson BE, Duling BR (2005) Heterocellular contact at the myoendothelial junction influences gap junction organization. Circ Res 97:44-51
185. Jauniaux E, Watson AL, Hempstock J, Bao Y-P, Skepper JN, Burton GJ (2000) Onset of Maternal Arterial Blood Flow and Placental Oxidative Stress : A Possible Factor in Human Early Pregnancy Failure. Am J Pathol 157:2111-2122
186. Kamei M, Yoneda Y, Suzuki H (2000) Endothelial factors involved in the bradykinin-induced relaxation of the guinea-pig aorta. J Smooth Muscle Res 36:127-135
187. Kansui Y, Fujii K, Nakamura K, Goto K, Oniki H, Abe I, Shibata Y, Iida M (2004) Angiotensin II receptor blockade corrects altered expression of gap junctions in vascular endothelial cells from hypertensive rats. Am J Physiol Heart Circ Physiol 287:H216-224
188. Karumanchi SA, Bdolah Y (2004) Hypoxia and sFlt-1 in preeclampsia: the "chicken-and-egg" question. Endocrinology 145:4835-4837
189. Katz SD, Krum H (2001) Acetylcholine-mediated vasodilation in the forearm circulation of patients with heart failure: indirect evidence for the role of endothelium-derived hyperpolarizing factor. Am J Cardiol 87:1089-1092

190. Kauser K, Rubanyi GM (1994) Gender difference in bioassayable endothelium-derived nitric oxide from isolated rat aortae. Am J Physiol 267:H2311-2317
191. Kauser K, Rubanyi GM (1995) Gender Difference in Endothelial Dysfunction in the Aorta of Spontaneously Hypertensive Rats. Hypertension 25:517-523
192. Kawashima K, Watanabe N, Oohata H, Fujimoto K, Suzuki T, Ishizaki Y, Morita I, Murota S (1990) Synthesis and release of acetylcholine by cultured bovine arterial endothelial cells. Neurosci Lett 119:156-158
193. Kemp BK CT (1997) Evidence that mechanisms dependent and independent of nitric oxide mediate endothelium-dependent relaxation to bradykinin in human small resistance-like coronary arteries. Br J Pharmacol 120:757-762
194. Kenny LC, Baker PN, Kendall DA, Randall MD, Dunn WR (2002) Differential mechanisms of endothelium-dependent vasodilator responses in human myometrial small arteries in normal pregnancy and pre-eclampsia. Clin Sci (Lond) 103:67-73
195. Kenny LC, Baker PN, Kendall DA, Randall MD, Dunn WR (2002) The role of gap junctions in mediating endothelium-dependent responses to bradykinin in myometrial small arteries isolated from pregnant women. Br J Pharmacol 136:1085-1088
196. Kestenbaum B, Seliger SL, Easterling TR, Gillen DL, Critchlow CW, Stehman-Breen CO, Schwartz SM (2003) Cardiovascular and thromboembolic events following hypertensive pregnancy. Am J Kidney Dis 42:982-989
197. Kim ND, Kang, K.W., Kang, S.Y. and Vanhoutte, P.M. (1999) Alpha2-adrenoceptor antagonists evoke endothelium-dependent and -independent relaxations in the isolated rat aorta. J. Cardiovasc. Pharmacol. 34:148-152
198. Kimura M, Sudhir K, Jones M, Simpson E, Jefferis A-M, Chin-Dusting JPF (2003) Impaired Acetylcholine-Induced Release of Nitric Oxide in the Aorta of Male Aromatase-Knockout Mice: Regulation of Nitric Oxide Production by Endogenous Sex Hormones in Males. Circ Res 93:1267-1271
199. Knock GA, Poston L (1996) Bradykinin-mediated relaxation of isolated maternal resistance arteries in normal pregnancy and preeclampsia. Am J Obstet Gynecol 175:1668-1674
200. Kohno M MK, Yokokawa K, Yasunari K, Horio T, Kurihara N, Takeda T. (1989) Production of endothelin by cultured porcine endothelial cells: modulation by adrenaline. J Hypertens Suppl. 7:S130-131
201. Komesaroff PA, Black CVS, Westerman RA (1998) A Novel, Nongenomic Action of Estrogen on the Cardiovascular System. J Clin Endocrinol Metab 83:2313-2316
202. Komesaroff PA SK (2001) Estrogens and human cardiovascular physiology. Reproduction, Fertility and Development 13:261-272
203. Komori K, Lorenz RR, Vanhoutte PM (1988) Nitric oxide, ACh, and electrical and mechanical properties of canine arterial smooth muscle. Am J Physiol 255:H207-212
204. Komori K, Vanhoutte PM (1990) Endothelium-derived hyperpolarizing factor. Blood Vessels 27:238-245
205. Koop EA, Gebbink MFBG, Sweeney TE, Mathy M-J, Heijnen HFG, Spaan JAE, Voest EE, VanBavel E, Peters SLM (2005) Impaired flow-induced dilation in mesenteric resistance arteries from receptor protein tyrosine phosphatase-{micro}-deficient mice. Am J Physiol Heart Circ Physiol 288:H1218-1223
206. Kublickiene K, Svedas E, Landgren B-M, Crisby M, Nahar N, Nisell H, Poston L (2005) Small Artery Endothelial Dysfunction in Postmenopausal Women: In Vitro Function, Morphology,

and Modification by Estrogen and Selective Estrogen Receptor Modulators. J Clin Endocrinol Metab 90:6113-6122

207. Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139:4252-4263
208. Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-A (1996) Cloning of a novel estrogen receptor expressed in rat prostate and ovary. PNAS 93:5925-5930
209. Kuriyama H, Suzuki H (1978) The effects of acetylcholine on the membrane and contractile properties of smooth muscle cells of the rabbit superior mesenteric artery. Br J Pharmacol 64:493-501
210. Kwak BR, Veillard N, Pelli G, Mulhaupt F, James RW, Chanson M, Mach F (2003) Reduced Connexin43 Expression Inhibits Atherosclerotic Lesion Formation in Low-Density Lipoprotein Receptor-Deficient Mice. Circulation 107:1033-1039
211. Kwon SC, Pyun WB, Park GY, Choi HK, Paik KS, Kang BS (1999) The involvement of K+ channels and the possible pathway of EDHF in the rabbit femoral artery. Yonsei Med J 40:331-338
212. Lacza Z, Puskar M, Kis B, Perciaccante JV, Miller AW, Busija DW (2002) Hydrogen peroxide acts as an EDHF in the piglet pial vasculature in response to bradykinin. Am J Physiol Heart Circ Physiol 283:H406-411
213. Lamping KG, Nuno DW (1996) Effects of 17 beta-estradiol on coronary microvascular responses to endothelin-1. Am J Physiol Heart Circ Physiol 271:H1117-1124
214. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG (2003) Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J. Clin. Invest. 111:1201-1209
215. Lang NN, Luksha L, Newby D, Kublickiene K (2007) Connexin 43 Mediates Endothelium-Derived Hyperpolarising Factor Induced Vasodilatation in Subcutaneous Resistance Arteries from Healthy Pregnant Women. Am J Physiol Heart Circ Physiol.292:H1026-H1032
216. Leung HS, Leung FP, Yao X, Ko WH, Chen Z-Y, Vanhoutte PM, Huang Y (2006) Endothelial mediators of the acetylcholine-induced relaxation of the rat femoral artery. Vascular Pharmacology 44:299-308
217. Levin ER (2002) Estrogen receptor-beta and the cardiovascular system. Trends Endocrinol Metab 13:184-185
218. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, Schisterman EF, Thadhani R, Sachs BP, Epstein FH, Sibai BM, Sukhatme VP, Karumanchi SA (2004) Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med 350:672-683
219. Lewis TV, Cooper BA, Dart AM, Chin-Dusting JPF (1998) Responses to endothelium-dependent agonists in subcutaneous arteries excised from hypercholesterolaemic men. 124:222-228
220. Li PL, Campbell WB (1997) Epoxyeicosatrienoic acids activate K+ channels in coronary smooth muscle through a guanine nucleotide binding protein. Circ Res 80:877-884
221. Li WEI, Ochalski PAY, Hertzberg EL, Nagy JI (1998) Immunorecognition, ultrastructure and phosphorylation status of astrocytic gap junctions and connexin43 in rat brain after cerebral focal ischaemia. European Journal of Neuroscience 10:2444-2463
222. Liao Y, Day KH, Damon DN, Duling BR (2001) Endothelial cell-specific knockout of connexin 43 causes hypotension and bradycardia in mice. Proc Natl Acad Sci U S A 98:9989-9994

223. Lieberman EH, Gerhard MD, Uehata A, Walsh BW, Selwyn AP, Ganz P, Yeung AC, Creager MA (1994) Estrogen improves endothelium-dependent, flow-mediated vasodilation in postmenopausal women. Ann Intern Med 121:936-941
224. Lind H EL (2002) Enhanced vasodilator responses to calcitonin gene-related peptide (CGRP) in subcutaneous arteries in human hypertension. J Hum Hypertens 16:53-59
225. Lindberg MK, Moverare S, Skrtic S, Gao H, Dahlman-Wright K, Gustafsson JA, Ohlsson C (2003) Estrogen receptor (ER)-beta reduces ERalpha-regulated gene transcription, supporting a "ying yang" relationship between ERalpha and ERbeta in mice. Mol Endocrinol 17:203-208
226. Lindner V, Kim SK, Karas RH, Kuiper GGJM, Gustafsson J-A, Mendelsohn ME (1998) Increased Expression of Estrogen Receptor-ß mRNA in Male Blood Vessels After Vascular Injury. Circ Res 83:224-229
227. Liu MY, Hattori Y, Fukao M, Sato A, Sakuma I, Kanno M (2001) Alterations in EDHF-mediated hyperpolarization and relaxation in mesenteric arteries of female rats in long-term deficiency of oestrogen and during oestrus cycle. Br J Pharmacol 132:1035-1046
228. Liu M-Y, Hattori Y, Fukao M, Sato A, Sakuma I, Kanno M (2001) Alterations in EDHF-mediated hyperpolarization and relaxation in mesenteric arteries of female rats in long-term deficiency of oestrogen and during oestrus cycle. British Journal of Pharmacology 132:1035-1046
229. Liu MY, Hattori Y, Sato A, Ichikawa R, Zhang XH, Sakuma I (2002) Ovariectomy attenuates hyperpolarization and relaxation mediated by endothelium-derived hyperpolarizing factor in female rat mesenteric artery: a concomitant decrease in connexin-43 expression. J Cardiovasc Pharmacol 40:938-948
230. Looft-Wilson RC, Payne GW, Segal SS (2004) Connexin expression and conducted vasodilation along arteriolar endothelium in mouse skeletal muscle. J Appl Physiol 97:1152-1158
231. Lucca JJD, Adeagbo ASO, Alsip NL (2000) Oestrous cycle and pregnancy alter the reactivity of the rat uterine vasculature. Hum. Reprod. 15:2496-2503
232. Lui AH, McManus BM, Laher I (2000) Endothelial and myogenic regulation of coronary artery tone in the mouse. Eur J Pharmacol 410:25-31
233. Luksha L, Nisell H, Kublickiene K (2004) The mechanism of EDHF-mediated responses in subcutaneous small arteries from healthy pregnant women. Am J Physiol Regul Integr Comp Physiol 286:R1102-1109
234. Lye SJ, Nicholson BJ, Mascarenhas M, MacKenzie L, Petrocelli T (1993) Increased expression of connexin-43 in the rat myometrium during labor is associated with an increase in the plasma estrogen:progesterone ratio. Endocrinology 132:2380-2386
235. Maliqueo M, Clementi M, Gabler F, Johnson MC, Palomino A, Sir-Petermann T, Vega M (2003) Expression of steroid receptors and proteins related to apoptosis in endometria of women with polycystic ovary syndrome*1. Fertility and Sterility 80:812-819
236. Mangos GJ (2006) Cardiovascular disease following pre-eclampsia: understanding the mechanisms. J Hypertens 24:639-641
237. Marcondes S, Antunes E (2005) The plasma and tissue kininogen-kallikrein-kinin system: role in the cardiovascular system. Curr Med Chem Cardiovasc Hematol Agents 3:33-44
238. Marin J, Rodriguez-Martinez MA (1999) Age-related changes in vascular responses. Experimental Gerontology 34:503-512

239. Marrelli SP (2001) Mechanisms of endothelial P2Y(1)- and P2Y(2)-mediated vasodilatation involve differential [Ca2+]i responses. Am J Physiol Heart Circ Physiol 281:H1759-1766
240. Matchkov VV, Rahman A, Bakker LM, Griffith TM, Nilsson H, Aalkjaer C (2006) Analysis of effects of connexin-mimetic peptides in rat mesenteric small arteries. Am J Physiol Heart Circ Physiol 291:H357-367
241. Matchkov VV, Rahman A, Peng H, Nilsson H, Aalkjaer C (2004) Junctional and nonjunctional effects of heptanol and glycyrrhetinic acid derivates in rat mesenteric small arteries. 142:961-972
242. Matoba T, Shimokawa H, Kubota H, Morikawa K, Fujiki T, Kunihiro I, Mukai Y, Hirakawa Y, Takeshita A (2002) Hydrogen Peroxide Is an Endothelium-Derived Hyperpolarizing Factor in Human Mesenteric Arteries. Biochemical and Biophysical Research Communications 290:909-913
243. Matoba T, Shimokawa H, Morikawa K, Kubota H, Kunihiro I, Urakami-Harasawa L, Mukai Y, Hirakawa Y, Akaike T, Takeshita A (2003) Electron spin resonance detection of hydrogen peroxide as an endothelium-derived hyperpolarizing factor in porcine coronary microvessels. Arterioscler Thromb Vasc Biol 23:1224-1230
244. Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kanaide H, Takeshita A (2000) Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. J Clin Invest 106:1521-1530
245. Matsumoto T, Kobayashi T, Kamata K (2006) Mechanisms underlying the impaired EDHF-type relaxation response in mesenteric arteries from Otsuka Long-Evans Tokushima Fatty (OLETF) rats. European Journal of Pharmacology 538:132-140
246. Matsumoto T, Wakabayashi K, Kobayashi T, Kamata K (2004) Alterations in vascular endothelial function in the aorta and mesenteric artery in type II diabetic rats. Can J Physiol Pharmacol 82:175-182
247. Matthews J, Gustafsson JA (2003) Estrogen signaling: a subtle balance between ER alpha and ER beta. Mol Interv 3:281-292
248. Matz RL SC, Stoclet JC, Andriantsitohaina R. (2000) Age-related endothelial dysfunction with respect to nitric oxide, endothelium-derived hyperpolarizing factor and cyclooxygenase products. Physiol Res 49:11-18
249. McCarthy AL, Taylor P, Graves J, Raju SK, Poston L (1994) Endothelium-dependent relaxation of human resistance arteries in pregnancy. American Journal Of Obstetrics And Gynecology 171:1309-1315
250. McCarthy AL, Woolfson RG, Raju SK, Poston L (1993) Abnormal endothelial cell function of resistance arteries from women with preeclampsia. Am J Obstet Gynecol 168:1323-1330
251. McCulloch AI, Randall MD (1998) Sex differences in the relative contributions of nitric oxide and EDHF to agonist-stimulated endothelium-dependent relaxations in the rat isolated mesenteric arterial bed. Br J Pharmacol 123:1700-1706
252. McGuire JJ, Ding H, Triggle CR (2001) Endothelium-derived relaxing factors: a focus on endothelium-derived hyperpolarizing factor(s). Can J Physiol Pharmacol 79:443-470
253. McIntyre C-A, Buckley CH, Jones GC, Sandeep TC, Andrews RC, Elliott AI, Gray GA, Williams BC, McKnight JA, Walker BR, Hadoke PWF (2001) Endothelium-derived hyperpolarizing factor and potassium use different mechanisms to induce relaxation of human subcutaneous resistance arteries. Br. J. Pharmacol. 133:902-908

254. McNeish AJ, Dora KA, Garland CJ (2005) Possible role for K+ in endothelium-derived hyperpolarizing factor-linked dilatation in rat middle cerebral artery. Stroke 36:1526-1532
255. Mendelsohn ME, Karas RH (1999) The protective effects of estrogen on the cardiovascular system. N Engl J Med 340:1801-1811
256. Mercier I, Pham-Dang M, Clement R, Gosselin H, Colombo F, Rouleau J-L, Calderone A (2002) Elevated mean arterial pressure in the ovariectomized rat was normalized by ETA receptor antagonist therapy: absence of cardiac hypertrophy and fibrosis. Br J Pharmacol 136:685-692
257. Miller VM (1999) Gender and vascular reactivity. Lupus 8:409-415
258. Milner P, Kirkpatrick KA, Ralevic V, Toothill V, Pearson J, Burnstock G (1990) Endothelial cells cultured from human umbilical vein release ATP, substance P and acetylcholine in response to increased flow. Proc Biol Sci 241:245-248
259. Minamiyama Y, Takemura S, Imaoka S, Funae Y, Tanimoto Y, Inoue M (1997) Irreversible Inhibition of Cytochrome P450 by Nitric Oxide. J Pharmacol Exp Ther 283:1479-1485
260. Miura H, Bosnjak JJ, Ning G, Saito T, Miura M, Gutterman DD (2003) Role for hydrogen peroxide in flow-induced dilation of human coronary arterioles. Circ Res 92:e31-40
261. Miura H, Gutterman DD (1998) Human Coronary Arteriolar Dilation to Arachidonic Acid Depends on Cytochrome P-450 Monooxygenase and Ca2+-Activated K+ Channels. Circ Res 83:501-507
262. Momoi H, Ikomi F, Ohhashi T (2003) Estrogen-induced augmentation of endothelium-dependent nitric oxide-mediated vasodilation in isolated rat cerebral small arteries. Jpn J Physiol 53:193-203
263. Morikawa K FT, Matoba T, Kubota H, Hatanaka M, Takahashi S, Shimokawa H. (2004) Important role of superoxide dismutase in EDHF-mediated responses of human mesenteric arteries. J Cardiovasc Pharmacol. 44:552-556
264. Muller-Delp JM, Lubahn DB, Nichol KE, Philips BJ, Price EM, Curran EM, Laughlin MH (2003) Regulation of nitric oxide-dependent vasodilation in coronary arteries of estrogen receptor-{alpha}-deficient mice. Am J Physiol Heart Circ Physiol 285:H2150-2157
265. Mulvany MJ, C. A (1990) Structure and function of small arteries. Physiol Rev. 70:921-961
266. Mulvany MJ AC, Christensen J. (1980) Changes in noradrenaline sensitivity and morphology of arterial resistance vessels during development of high blood pressure in spontaneously hypertensive rats. Hypertension Sep-Oct;2:664-671
267. Myatt L, Cui X (2004) Oxidative stress in the placenta. Histochemistry and Cell Biology V122:369-382
268. Nakamura K IT, Nakamura K, Shibata Y. (1999) Distribution of gap junction protein connexin 37 in smooth muscle cells of the rat trachea and pulmonary artery. Arch Histol Cytol 62:27-37
269. Nakashima M VP (1995) Isoproterenol causes hyperpolarization through opening of ATP-sensitive potassium channels in vascular smooth muscle of the canine saphenous vein. J Pharmacol Exp Ther. 272:379-384
270. Nawate S, Fukao M, Sakuma I, Soma T, Nagai K, Takikawa O, Miwa S, Kitabatake A (2005) Reciprocal changes in endothelium-derived hyperpolarizing factor- and nitric oxide-system in the mesenteric artery of adult female rats following ovariectomy. British Journal of Pharmacology 144:178-189

271. Nelli S, Wilson WS, Laidlaw H, Llano A, Middleton S, Price AG, Martin W (2003) Evaluation of potassium ion as the endothelium-derived hyperpolarizing factor (EDHF) in the bovine coronary artery. Br J Pharmacol 139:982-988
272. New M, Gishel, Timmins BH, Katrina L., Duffy M, Stephen J., Tran BH, Binh T. (1997) Long-Term Estrogen Therapy Improves Vascular Function in Male to Female Transsexuals. Journal of the American College of Cardiology 29:1437-1444
273. Nguyen T-D, Vequaud P, Thorin E (1999) Effects of endothelin receptor antagonists and nitric oxide on myogenic tone and [alpha]-adrenergic-dependent contractions of rabbit resistance arteries. Cardiovascular Research 43:755-761
274. Nilsson B, Ekblad E, Heine T, Gustafsson J (2000) Increased magnitude of relaxation to oestrogen in aorta from oestrogen receptor beta knock-out mice. J Endocrinol 166:R5-9
275. Ohgushi M, Yasue H, Kugiyama K, Murohara T, Sakaino N (1993) Contraction and endothelium dependent relaxation via [alpha] adrenoceptors are variable in various pig arteries. Cardiovascular Research 27:779-784
276. Olsen MH, Wachtell K, Aalkjaer C, Devereux RB, Dige-Petersen H, Ibsen H (2001) Endothelial dysfunction in resistance arteries is related to high blood pressure and circulating low density lipoproteins in previously treated hypertension. American Journal of Hypertension 14:861-867
277. Orshal JM, Khalil RA (2004) Gender, sex hormones, and vascular tone. Am J Physiol Regul Integr Comp Physiol 286:R233-249
278. Ouyang P, Michos ED, Karas RH (2006) Hormone replacement therapy and the cardiovascular system lessons learned and unanswered questions. J Am Coll Cardiol 47:1741-1753
279. Pak KJ, Geary GG, Duckles SP, Krause DN (2002) Male-female differences in the relative contribution of endothelial vasodilators released by rat tail artery. Life Sci 71:1633-1642
280. Palmer RM, Ferrige AG, Moncada S (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327:524-526
281. Pannirselvam M, Ding H, Anderson TJ, Triggle CR (2006) Pharmacological characteristics of endothelium-derived hyperpolarizing factor-mediated relaxation of small mesenteric arteries from db/db mice. European Journal of Pharmacology 551:98-107
282. Parkington HC, Chow JA, Evans RG, Coleman HA, Tare M (2002) Role for endothelium-derived hyperpolarizing factor in vascular tone in rat mesenteric and hindlimb circulations in vivo. J Physiol 542:929-937
283. Parkington HC TM, Tonta MA, Coleman HA. (1993) Stretch revealed three components in the hyperpolarization of guinea-pig coronary artery in response to acetylcholine. J Physiol 465:459-476
284. Parnavelas JG, Kelly W, Burnstock G (1985) Ultrastructural localization of choline acetyltransferase in vascular endothelial cells in rat brain. Nature 316:724-725
285. Pascoal IF, Lindheimer MD, Nalbantian-Brandt C, Umans JG (1998) Preeclampsia selectively impairs endothelium-dependent relaxation and leads to oscillatory activity in small omental arteries. J Clin Invest 101:464-470
286. Pascoal IF, Umans JG (1996) Effect of pregnancy on mechanisms of relaxation in human omental microvessels. Hypertension 28:183-187
287. Pfister SL, Falck JR, Campbell WB (1991) Enhanced synthesis of epoxyeicosatrienoic acids by cholesterol-fed rabbit aorta. Am J Physiol Heart Circ Physiol 261:H843-852

288. Piascik MT, Soltis EE, Piascik MM, Macmillan Leigh B. (1996) [alpha]-Adrenoceptors and vascular regulation: Molecular, pharmacologic and clinical correlates. Pharmacology & Therapeutics 72:215-241
289. Polderman KH, Stehouwer CDA, van Kamp GJ, Dekker GA, Verheugt FWA, Gooren LJG (1993) Influence of Sex Hormones on Plasma Endothelin Levels. Ann Intern Med 118:429-432
290. Polontchouk L, Ebelt B, Jackels M, Dhein S (2001) Chronic effects of endothelin-1 and angiotensin-II on gap junctions and intercellular communication in cardiac cells. FASEB J.16:87-89
291. Pomposiello S, Rhaleb NE, Alva M, Carretero OA (1999) Reactive oxygen species: role in the relaxation induced by bradykinin or arachidonic acid via EDHF in isolated porcine coronary arteries. J Cardiovasc Pharmacol 34:567-574
292. Popp R, Brandes RP, Ott G, Busse R, Fleming I (2002) Dynamic modulation of interendothelial gap junctional communication by 11,12-epoxyeicosatrienoic acid. Circ Res 90:800-806
293. Popp R, Fleming I, Busse R (1998) Pulsatile stretch in coronary arteries elicits release of endothelium-derived hyperpolarizing factor: a modulator of arterial compliance. Circ Res 82:696-703
294. Porteri E RD, Guelfi D, De CC, Castellano M, Bettoni G, Tiberio GA, Giulini SM, Sleiman I, Agabiti-Rosei E. (2002) Role of ET(A) receptors in the vasoconstriction induced by endothelin-1 in subcutaneous small arteries of normotensive subjects and hypertensive patients. Blood Press 11:6-12
295. Potischman N, Hoover RN, Brinton LA, Siiteri P, Dorgan JF, Swanson CA, Berman ML, Mortel R, Twiggs LB, Barrett RJ, Wilbanks GD, Persky V, Lurain JR (1996) Case-control study of endogenous steroid hormones and endometrial cancer. J Natl Cancer Inst 88:1127-1135
296. Pratt PF, Li P, Hillard CJ, Kurian J, Campbell WB (2001) Endothelium-independent, ouabain-sensitive relaxation of bovine coronary arteries by EETs. Am J Physiol Heart Circ Physiol 280:H1113-1121
297. Quilley J, McGiff JC (2000) Is EDHF an epoxyeicosatrienoic acid? Trends in Pharmacological Sciences 21:121-124
298. Reckelhoff JF, Fortepiani LA (2004) Novel mechanisms responsible for postmenopausal hypertension. Hypertension 43:918-923
299. Redman CW, Sargent IL (2005) Latest advances in understanding preeclampsia. Science 308:1592-1594
300. Redman CW, Sargent IL (2000) Placental debris, oxidative stress and pre-eclampsia. Placenta 21:597-602
301. Rizzoni D PE, Guelfi D, Muiesan ML, Piccoli A, Valentini U, Cimino A, Girelli A, Salvetti M, De Ciuceis C, Tiberio GA, Giulini SM, Sleiman I, Monteduro C, Rosei EA. (2001) Endothelial dysfunction in small resistance arteries of patients with non-insulin-dependent diabetes mellitus. J Hypertens 19:913-919
302. Roberts JM, Gammill HS (2005) Preeclampsia: recent insights. Hypertension 46:1243-1249
303. Robson S (1996) Maternal respiratory and cardiovascular changes during pregnancy. Scientific essentials of reproductive medicine:443-454
304. Rossouw JE (2002) Hormones, genetic factors, and gender differences in cardiovascular disease. Cardiovasc Res 53:550-557
305. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J (2002) Risks and benefits

of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA 288:321-333

306. Rubanyi GM, Freay AD, Kauser K, Sukovich D, Burton G, Lubahn DB, Couse JF, Curtis SW, Korach KS (1997) Vascular estrogen receptors and endothelium-derived nitric oxide production in the mouse aorta. Gender difference and effect of estrogen receptor gene disruption. J Clin Invest 99:2429-2437
307. Rubanyi GM, Vanhoutte PM (1987) Nature of endothelium-derived relaxing factor: are there two relaxing mediators? Circ Res 61:II61-67
308. Rubanyi GM VP (1987) Nature of endothelium-derived relaxing factor: are there two relaxing mediators? Circ Res. 61:II61-67.
309. Rummery NM, Hill CE (2004) Vascular gap junctions and implications for hypertension. Clin Exp Pharmacol Physiol 31:659-667
310. Rusko J, Li L, van Breemen C (1995) 17-beta-Estradiol stimulation of endothelial K+ channels. Biochem Biophys Res Commun 214:367-372
311. Rzigalinski BA, Willoughby KA, Hoffman SW, Falck JR, Ellis EF (1999) Calcium influx factor, further evidence it is 5, 6-epoxyeicosatrienoic acid. J Biol Chem 274:175-182
312. Sader MA, Celermajer DS (2002) Endothelial function, vascular reactivity and gender differences in the cardiovascular system. Cardiovasc Res 53:597-604
313. Sakuma I, Liu MY, Sato A, Hayashi T, Iguchi A, Kitabatake A, Hattori Y (2002) Endothelium-dependent hyperpolarization and relaxation in mesenteric arteries of middle-aged rats: influence of oestrogen. Br J Pharmacol 135:48-54
314. Sandow SL (2004) Factors, fiction and endothelium-derived hyperpolarizing factor. Clinical and Experimental Pharmacology and Physiology 31:563-570
315. Sandow SL, Tare M (2007) C-type natriuretic peptide: a new endothelium-derived hyperpolarizing factor? Trends in Pharmacological Sciences 28:61-67
316. Sankaralingam S, Arenas IA, Lalu MM, Davidge ST (2006) Preeclampsia: current understanding of the molecular basis of vascular dysfunction. Expert Rev Mol Med 8:1-20
317. Santos RL, Abreu GR, Bissoli NS, Moyses MR (2004) Endothelial mediators of 17 beta-estradiol-induced coronary vasodilation in the isolated rat heart. Braz J Med Biol Res 37:569-575
318. Sato A, Miura H, Liu Y, Somberg LB, Otterson MF, Demeure MJ, Schulte WJ, Eberhardt LM, Loberiza FR, Sakuma I, Gutterman DD (2002) Effect of gender on endothelium-dependent dilation to bradykinin in human adipose microvessels. Am J Physiol Heart Circ Physiol 283:H845-852
319. Sattar N, Greer IA (2002) Pregnancy complications and maternal cardiovascular risk: opportunities for intervention and screening? Bmj 325:157-160
320. Schiffrin E, Turgeon A, Deng L (1997) Effect of chronic ETA-selective endothelin receptor antagonism on blood pressure in experimental and genetic hypertension in rats. Br J Pharmacol 121:935-940
321. Schiffrin EL (2001) Role of endothelin-1 in hypertension and vascular disease. American Journal of Hypertension 14:S83-S89
322. Scotland RS, Madhani M, Chauhan S, Moncada S, Andresen J, Nilsson H, Hobbs AJ, Ahluwalia A (2005) Investigation of Vascular Responses in Endothelial Nitric Oxide Synthase/ Cyclooxygenase-1 Double-Knockout Mice: Key Role for Endothelium-Derived Hyperpolarizing Factor in the Regulation of Blood Pressure in Vivo. Circulation 111:796-803

323. Segretain D, Falk MM (2004) Regulation of connexin biosynthesis, assembly, gap junction formation, and removal. Biochim Biophys Acta 1662:3-21
324. Selva DM, Tirado OM, Toran N, Suarez-Quian CA, Reventos J, Munell F (2004) Estrogen Receptor {beta} Expression and Apoptosis of Spermatocytes of Mice Overexpressing a Rat Androgen-Binding Protein Transgene. Biol Reprod 71:1461-1468
325. Severs NJ, Rothery S, Dupont E, Coppen SR, Yeh HI, Ko YS, Matsushita T, Kaba R, Halliday D (2001) Immunocytochemical analysis of connexin expression in the healthy and diseased cardiovascular system. Microsc Res Tech 52:301-322
326. Shanklin DR, Sibai BM (1989) Ultrastructural aspects of preeclampsia. I. Placental bed and uterine boundary vessels. Am J Obstet Gynecol 161:735-741
327. Shimizu E OM, Masutani M, Iwasaki T. (2002) A decrease in the amount and function of the stimulatory GTP-binding protein in the small resistance arteries of spontaneously hypertensive rats. Hypertens Res. 25:743-749
328. Shimokawa H, Flavahan N, Vanhoutte P (1989) Natural course of the impairment of endothelium-dependent relaxations after balloon endothelium removal in porcine coronary arteries. Possible dysfunction of a pertussis toxin-sensitive G protein. Circ Res 65:740-753
329. Shimokawa H, Matoba T (2004) Hydrogen peroxide as an endothelium-derived hyperpolarizing factor. Pharmacol Res 49:543-549
330. Shimokawa H, Morikawa K (2005) Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in animals and humans. J Mol Cell Cardiol 39:725-732
331. Shimokawa H, Morikawa K (2005) Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in animals and humans. Journal of Molecular and Cellular Cardiology 39:725-732
332. Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, Takayanagi T, Nagao T, Egashira K, Fujishima M, Takeshita A (1996) The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. J Cardiovasc Pharmacol 28:703-711
333. Shin W, Sasaki T, Kato M, Hara K, Seko A, Yang W, Shimamoto N, Sugimoto T, Toyo-oka T (1992) Autocrine and paracrine effects of endothelium-derived relaxing factor on intracellular Ca2+ of endothelial cells and vascular smooth muscle cells. Identification by two-dimensional image analysis in coculture. J. Biol. Chem. 267:20377-20382
334. Sibai B, Dekker G, Kupferminc M (2005) Pre-eclampsia. Lancet 365:785-799
335. Siegel G (1996) Vascular smooth muscle. In: Greger R,Windhorst U, eds. Comprehensive human physiology. Berlin: Springer-Verlag;:1917-1939
336. Simon AM, Goodenough DA, Li E, Paul DL (1997) Female infertility in mice lacking connexin 37. Nature 385:525-529
337. Simon AM, McWhorter AR (2002) Vascular abnormalities in mice lacking the endothelial gap junction proteins connexin37 and connexin40. Dev Biol 251:206-220
338. Sladek SM, Magness RR, Conrad KP (1997) Nitric oxide and pregnancy. Am J Physiol 272:R441-463
339. Smith PJW, Ferro CJ, McQueen DS, Webb DJ (1999) Impaired cholinergic dilator response of resistance arteries isolated from patients with Raynaud's disease. British Journal of Clinical Pharmacology 47:507-513

340. Sokoya EM, Burns AR, Setiawan CT, Coleman HA, Parkington HC, Tare M (2006) Evidence for the involvement of myoendothelial gap junctions in EDHF-mediated relaxation in the rat middle cerebral artery. Am J Physiol Heart Circ Physiol 291:H385-393
341. Sosinsky GE, Nicholson BJ (2005) Structural organization of gap junction channels. Biochimica et Biophysica Acta (BBA) - BiomembranesThe Connexins Part II 1711:99-125
342. Spargo BH, Lichtig C, Luger AM, Katz AI, Lindheimer MD (1976) The renal lesion in preeclampsia. Perspect Nephrol Hypertens 5:129-137
343. Stankevicius E, Lopez-Valverde V, Rivera L, Hughes AD, Mulvany MJ, Simonsen U (2006) Combination of Ca(2+)-activated K(+) channel blockers inhibits acetylcholine-evoked nitric oxide release in rat superior mesenteric artery. Br J Pharmacol.149(5):560-572
344. Stepan H, Geide A, Faber R (2004) Soluble fms-like tyrosine kinase 1. N Engl J Med 351:2241-2242
345. Sudhir K, Chou TM, Messina LM, Hutchison SJ, Korach KS, Chatterjee K, Rubanyi GM (1997) Endothelial dysfunction in a man with disruptive mutation in oestrogen-receptor gene. Lancet 349:1146-1147
346. Sudhir K, Komesaroff PA (1999) Cardiovascular Actions of Estrogens in Men. J Clin Endocrinol Metab 84:3411-3415
347. Sumi D, Ignarro LJ (2003) Estrogen-related receptor {alpha}1 up-regulates endothelial nitric oxide synthase expression. PNAS 100:14451-14456
348. Supplemental methods r, figures, and references are available on Science Online at www.sciencemag.org/cgi/content/full/295/5554/505/DC1 (2002)
349. Suvorava T, Lauer N, Kumpf S, Jacob R, Meyer W, Kojda G (2005) Endogenous Vascular Hydrogen Peroxide Regulates Arteriolar Tension In Vivo. Circulation 112:2487-2495
350. Suzuki T, Yamazaki T, Yazaki Y (2001) The role of the natriuretic peptides in the cardiovascular system. Cardiovasc Res 51:489-494
351. Suzuki Y, Hattori T, Kajikuri J, Yamamoto T, Suzumori K, Itoh T (2002) Reduced function of endothelial prostacyclin in human omental resistance arteries in pre-eclampsia. J Physiol 545:269-277
352. Suzuki Y, Kajikuri J, Suzumori K, Itoh T (2000) Mechanisms underlying the reduced endothelium-dependent relaxation in human omental resistance artery in pre-eclampsia. J Physiol 527 Pt 1:163-174
353. Suzuki Y, Yamamoto T, Mabuchi Y, Tada T, Suzumori K, Soji T, Herbert DC, Itoh T (2003) Ultrastructural changes in omental resistance artery in women with preeclampsia. American Journal of Obstetrics and Gynecology 189:216-221
354. Svedas E, Islam KB, Nisell H, Kublickiene KR (2003) Vascular endothelial growth factor induced functional and morphologic signs of endothelial dysfunction in isolated arteries from normal pregnant women. Am J Obstet Gynecol 188:168-176
355. Svedas E, Nisell H, Vanwijk MJ, Nikas Y, Kublickiene KR (2002) Endothelial dysfunction in uterine circulation in preeclampsia: can estrogens improve it? Am J Obstet Gynecol 187:1608-1616
356. Tagawa H, Shimokawa H, Tagawa T, Kuroiwa-Matsumoto M, Hirooka Y, Takeshita A (1997) Short-term estrogen augments both nitric oxide-mediated and non-nitric oxide-mediated endothelium-dependent forearm vasodilation in postmenopausal women. J Cardiovasc Pharmacol 30:481-488

357. Tare M, Coleman HA, Parkington HC (2002) Glycyrrhetinic derivatives inhibit hyperpolarization in endothelial cells of guinea pig and rat arteries. Am J Physiol Heart Circ Physiol 282:H335-341
358. Taylor A, Al-Azzawi F (2000) Immunolocalisation of oestrogen receptor beta in human tissues. J Mol Endocrinol 24:145-155
359. Taylor SG, Weston AH (1988) Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular endothelium. Trends Pharmacol Sci 9:272-274
360. Theis M, de Wit C, Schlaeger TM, Eckardt D, Kruger O, Doring B, Risau W, Deutsch U, Pohl U, Willecke K (2001) Endothelium-specific replacement of the connexin43 coding region by a lacZ reporter gene. Genesis 29:1-13
361. Thomas SR, Chen K, Keaney JF, Jr. (2002) Hydrogen Peroxide Activates Endothelial Nitric-oxide Synthase through Coordinated Phosphorylation and Dephosphorylation via a Phosphoinositide 3-Kinase-dependent Signaling Pathway. J. Biol. Chem. 277:6017-6024
362. Thorin E, Cernacek P, Dupuis J (1998) Endothelin-1 Regulates Tone of Isolated Small Arteries in the Rat : Effect of Hyperendothelinemia. Hypertension 31:1035-1041
363. Thorin E, Huang PL, Fishman MC, Bevan JA (1998) Nitric Oxide Inhibits Alpha2-Adrenoceptor–Mediated Endothelium-Dependent Vasodilation. Circ Res 82:1323-1329
364. Thorin E, Pham-Dang M, Clement R, Mercier I, Calderone A (2003) Hyper-reactivity of cerebral arteries from ovariectomized rats: therapeutic benefit of tamoxifen. Br J Pharmacol 140:1187-1192
365. Thorin E, Shreeve SM, Thorin-Trescases N, Bevan JA (1997) Reversal of Endothelin-1 Release by Stimulation of Endothelial alpha2-Adrenoceptor Contributes to Cerebral Vasorelaxation. Hypertension 30:830-836
366. Todt I, Ngezahayo A, Ernst A, Kolb HA (2001) Hydrogen peroxide inhibits gap junctional coupling and modulates intracellular free calcium in cochlear Hensen cells. J Membr Biol 181:107-114
367. Tomioka H, Hattori Y, Fukao M, Sato A, Liu M, Sakuma I, Kitabatake A, Kanno M (1999) Relaxation in different-sized rat blood vessels mediated by endothelium-derived hyperpolarizing factor: importance of processes mediating precontractions. J Vasc Res 36:311-320
368. Tomioka H, Hattori Y, Fukao M, Watanabe H, Akaishi Y, Sato A, Kim TQ, Sakuma I, Kitabatake A, Kanno M (2001) Role of endothelial Ni(2+)-sensitive Ca(2+) entry pathway in regulation of EDHF in porcine coronary artery. Am J Physiol Heart Circ Physiol 280:H730-737
369. Torondel B, Vila JM, Segarra G, Lluch P, Medina P, Martinez-Leon J, Ortega J, Lluch S (2004) Endothelium-dependent responses in human isolated thyroid arteries from donors. J Endocrinol 181:379-384
370. Triggle CR, Hollenberg M, Anderson TJ, Ding H, Jiang Y, Ceroni L, Wiehler WB, Ng ES, Ellis A, Andrews K, McGuire JJ, Pannirselvam M (2003) The endothelium in health and disease--a target for therapeutic intervention. J Smooth Muscle Res 39:249-267
371. Tuttle JL, Falcone JC (2001) Nitric oxide release during alpha1-adrenoceptor-mediated constriction of arterioles. Am J Physiol Heart Circ Physiol 281:H873-881
372. Ujiie H, Chaytor AT, Bakker LM, Griffith TM (2003) Essential role of Gap junctions in NO- and prostanoid-independent relaxations evoked by acetylcholine in rabbit intracerebral arteries. Stroke 34:544-550

373. Urakami-Harasawa L, Shimokawa H, Nakashima M, Egashira K, Takeshita A (1997) Importance of endothelium-derived hyperpolarizing factor in human arteries. J Clin Invest 100:2793-2799
374. van Kempen MJ, Jongsma HJ (1999) Distribution of connexin37, connexin40 and connexin43 in the aorta and coronary artery of several mammals. Histochem Cell Biol 112:479-486
375. Vandeputte C, Docherty JR (2002) Modulation of contraction by [alpha]2A/D-adrenoceptors in mouse aorta: evidence employing knockout technology. 135:1209-1212
376. Vanheel B, Van de Voorde J (2000) EDHF and residual NO: different factors. Cardiovascular Research 46:370-375
377. Vanhoutte J-VMaPM (1997) Endothelium-derived hyperpolarizing factor(s): updating the unknown. Trends in Pharmacological Sciences 18:252-256
378. Vanhoutte PM (2001) Endothelial Adrenoceptors. Journal of Cardiovascular Pharmacology 38:796-808
379. Vanhoutte PM (2001) Endothelium-derived free radicals: for worse and for better. J Clin Invest. 107:23-25
380. VanWijk MJ, Boer K, van der Meulen ET, Bleker OP, Spaan JAE, VanBavel E (2002) Resistance artery smooth muscle function in pregnancy and preeclampsia. American Journal of Obstetrics and Gynecology 186:148-154
381. VanWijk MJ, Kublickiene K, Boer K, VanBavel E (2000) Vascular function in preeclampsia. Cardiovasc Res 47:38-48
382. Vazquez-Perez S, Navarro-Cid J, de las Heras N, Cediel E, Sanz-Rosa D, Ruilope LM, Cachofeiro V, Lahera V (2001) Relevance of endothelium-derived hyperpolarizing factor in the effects of hypertension on rat coronary relaxations. J Hypertens 19:539-545
383. Venkatesha S, Toporsian M, Lam C, Hanai J-i, Mammoto T, Kim YM, Bdolah Y, Lim K-H, Yuan H-T, Libermann TA, Stillman IE, Roberts D, D'Amore PA, Epstein FH, Sellke FW, Romero R, Sukhatme VP, Letarte M, Karumanchi SA (2006) Soluble endoglin contributes to the pathogenesis of preeclampsia. 12:642-649
384. Villalobos-Molina R, Lopez-Guerrero JJ, Ibarra M (1999) Functional evidence of {alpha}1D-adrenoceptors in the vasculature of young and adult spontaneously hypertensive rats. Br J Pharmacol 126:1534-1536
385. von Wowern F, Bengtsson K, Lindblad U, Rastam L, Melander O (2004) Functional Variant in the alpha2B Adrenoceptor Gene, a Positional Candidate on Chromosome 2, Associates With Hypertension. Hypertension 43:592-597
386. Waldron GJ, Cole WC (1999) Activation of vascular smooth muscle K+ channels by endothelium-derived relaxing factors. Clin Exp Pharmacol Physiol 26:180-184
387. Waldron GJ, Ding H, Lovren F, Kubes P, Triggle CR (1999) Acetylcholine-induced relaxation of peripheral arteries isolated from mice lacking endothelial nitric oxide synthase. 128:653-658
388. Walker A, Naderali E, Chattington P, Buckingham R, Williams G (1998) Differential vasoactive effects of the insulin sensitizers rosiglitazone (BRL 49653) and troglitazone on human small arteries in vitro. Diabetes 47:810-814
389. Walker DL, Vacha SJ, Kirby ML, Lo CW (2005) Connexin43 deficiency causes dysregulation of coronary vasculogenesis. Developmental Biology 284:479-498
390. Walker SD, Dora KA, Ings NT, Crane GJ, Garland CJ (2001) Activation of endothelial cell IK(Ca) with 1-ethyl-2-benzimidazolinone evokes smooth muscle hyperpolarization in rat isolated mesenteric artery. Br J Pharmacol 134:1548-1554

391. Wang X, Barber DA, Lewis DA, McGregor CGA, Sieck GC, Fitzpatrick LA, Miller VM (1997) Gender and transcriptional regulation of NO synthase and ET-1 in porcine aortic endothelial cells. Am J Physiol Heart Circ Physiol 273:H1962-1967
392. Wangensteen R, Moreno JM, Sainz J, Rodriguez-Gomez I, Chamorro V, Luna Jde D, Osuna A, Vargas F (2004) Gender difference in the role of endothelium-derived relaxing factors modulating renal vascular reactivity. Eur J Pharmacol 486:281-288
393. Wei C. M., Hu S. L., Miller V. M., Burnett J. C. (1994) Vascular Actions of C-Type Natriuretic Peptide in Isolated Porcine Coronary Arteries and Coronary Vascular Smooth Muscle Cells. Biochemical and Biophysical Research Communications 205:765-771
394. White RE, Han G, Maunz M, Dimitropoulou C, El-Mowafy AM, Barlow RS, Catravas JD, Snead C, Carrier GO, Zhu S, Yu X (2002) Endothelium-independent effect of estrogen on Ca(2+)-activated K(+) channels in human coronary artery smooth muscle cells. Cardiovasc Res 53:650-661
395. White RM, Rivera CO, Davison CA (2000) Nitric oxide-dependent and -independent mechanisms account for gender differences in vasodilation to acetylcholine. J Pharmacol Exp Ther 292:375-380
396. Widder J, Pelzer T, von Poser-Klein C, Hu K, Jazbutyte V, Fritzemeier KH, Hegele-Hartung C, Neyses L, Bauersachs J (2003) Improvement of endothelial dysfunction by selective estrogen receptor-alpha stimulation in ovariectomized SHR. Hypertension 42:991-996
397. Wisdom SJ WR, McKillop JH, Walker JJ. (1991) Antioxidant systems in normal pregnancy and in pregnancy-induced hypertension. Am J Obstet Gynecol. 165:1701-1704
398. Woodman OL, Boujaoude M (2004) Chronic treatment of male rats with daidzein and 17beta-oestradiol induces the contribution of EDHF to endothelium-dependent relaxation. Br J Pharmacol 141:322-328
399. Xu K, Lu Z, Wei H, Zhang Y, Han C (1998) Alteration of alpha1-adrenoceptor subtypes in aortas of 12-month-old spontaneously hypertensive rats. Eur J Pharmacol 344:31-36
400. Yada T, Shimokawa H, Hiramatsu O, Haruna Y, Morita Y, Kashihara N, Shinozaki Y, Mori H, Goto M, Ogasawara Y, Kajiya F (2006) Cardioprotective role of endogenous hydrogen peroxide during ischemia-reperfusion injury in canine coronary microcirculation in vivo. Am J Physiol Heart Circ Physiol 291:H1138-1146
401. Yada T, Shimokawa H, Hiramatsu O, Kajita T, Shigeto F, Goto M, Ogasawara Y, Kajiya F (2003) Hydrogen peroxide, an endogenous endothelium-derived hyperpolarizing factor, plays an important role in coronary autoregulation in vivo. Circulation 107:1040-1045
402. Yager JD, Davidson NE (2006) Estrogen Carcinogenesis in Breast Cancer. N Engl J Med 354:270-282
403. Yajima K, Nishiyama M, Yamamoto Y, Suzuki H (1999) Inhibition of endothelium-dependent hyperpolarization by endothelial prostanoids in guinea-pig coronary artery. 126:1-10
404. Yanes LL, Romero DG, Cucchiarelli VE, Fortepiani LA, Gomez-Sanchez CE, Santacruz F, Reckelhoff JF (2005) Role of endothelin in mediating postmenopausal hypertension in a rat model. Am J Physiol Regul Integr Comp Physiol 288:R229-233
405. Yang H, Shi M, VanRemmen H, Chen X, Vijg J, Richardson A, Guo Z (2003) Reduction of pressor response to vasoconstrictor agents by overexpression of catalase in mice. Am J Hypertens 16:1-5
406. Yang S, Bae L, Zhang L (2000) Estrogen increases eNOS and NOx release in human coronary artery endothelium. J Cardiovasc Pharmacol 36:242-247

407. You J, Marrelli SP, Bryan RM, Jr (2002) Role of Cytoplasmic Phospholipase A2 in Endothelium-Derived Hyperpolarizing Factor Dilations of Rat Middle Cerebral Arteries. 22:1239-1247

408. Yu W DG, Werner R. (1994) The connexin43 gene is responsive to oestrogen. Proc. Roy Soc. London Biol. Sci. 255:125-132

409. Yulug E, Yenilmez E, Unsal M, Aydin S, Tekelioglu Y, Arvas H (2006) Apoptotic and morphological features of the umbilical artery endothelium in mild and severe pre-eclampsia. Acta Obstetricia et Gynecologica Scandinavica 85:1038-1045

410. Zhang C WY, Duan J. (2000) Effects of estrogen on serum lipids, plasma endothelin-1, atherosclerosis in ovariectomized rabbits. Zhonghua Fu Chan Ke Za Zhi. 35:413-416

411. Zhang J, Hill CE (2005) Differential connexin expression in preglomerular and postglomerular vasculature: Accentuation during diabetes. 68:1171-1185

412. Zhu Y, Bian Z, Lu P, Karas RH, Bao L, Cox D, Hodgin J, Shaul PW, Thoren P, Smithies O, Gustafsson J-A, Mendelsohn ME (2002) Abnormal Vascular Function and Hypertension in Mice Deficient in Estrogen Receptor beta. Science 295:505-508

413. Zygmunt PM, Edwards G, Weston AH, Larsson B, Hogestatt ED (1997) Involvement of voltage-dependent potassium channels in the EDHF-mediated relaxation of rat hepatic artery. Br J Pharmacol 121:141-149

414. Zygmunt PM, Hogestatt ED (1996) Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. Br J Pharmacol 117:1600-1606

415. Zygmunt PM, Sorgard M, Petersson J, Johansson R, Hogestatt ED (2000) Differential actions of anandamide, potassium ions and endothelium-derived hyperpolarizing factor in guinea-pig basilar artery. Naunyn Schmiedebergs Arch Pharmacol 361:535-542