VASCULAR ADAPTATION TO PREGNANCY AND RELAXIN

JORIS VAN DRONGELEN

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VASCULAR ADAPTATION TO PREGNANCY AND RELAXIN

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CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

This thesis aims to improve our understanding of the mechanisms of action involved in pregnancy-induced vasodilation.

Pregnancy is one of the most unique experiences in a woman's life and affects human physiology in many ways. The cardiovascular system is one of the most strongly affected because it provides the fetus with oxygen and nutrients needed for growth and development.

The cardiovascular adaptations to the increased metabolic needs of the various organs develop early in the first half of pregnancy. The main changes are a generalized reduction in systemic vascular resistance and consequently an increase in blood flow. By mid-pregnancy in humans vascular resistance has decreased by about 50% and cardiac output has increased by 40% [1;2]. As a result, blood pressure at mid-pregnancy is about 10% lower than in nonpregnant women. The increase in flow varies between organs and is quite pronounced in the mesenteric vascular bed, that provides flow to the bowels, and the renal vascular bed, that provides flow to the kidneys [1-4]. These changes are the physiological response to profound vasodilation, the mechanisms of which are not completely understood. Pregnancy-induced vasodilation is not limited to humans and occurs to a variable extent in all mammals. Most studies on vascular adaptations in pregnancy have been performed in rodents that show more or less comparable vascular changes [3;5;6].

Vascular resistance is determined by the smallest arteries and arterioles throughout the body and not so much by the large arteries like the aorta. The small vessels have three layers, each of which can contribute to vascular tone. These layers are: 1. The tunica intima, consisting of endothelium cells (ECs) and a supporting extra-cellular matrix (ECM), 2. The tunica media, consisting of smooth muscle cells (SMCs) and ECM, and 3. The tunica adventitia, consisting of fibroblast, autonomic nerves and ECM. From studies in nonpregnant rodents we know that vascular tone results from several pathways that affect the EC, SMC or ECM. These pathways are graphically depicted by Figure 1 in Chapter 2 and include: 1. Flow mediated vasodilation, the vasodilator response to a certain flow increase (representing the nitric oxide (NO) dependent vasodilation), 2. Reactivity to vasodilator agents (including acetylcholine, bradykinin, adrenomedullin and isoproterenol), 3.) Reactivity to vasoconstrictor agents (such as phenylephrine, norepinephrine and angiotensin-II), 4. Myogenic reactivity, the vasoconstrictor response to a certain pressure increase in the presence of intact SMC (representing SMC reactivity to pressure changes), and 5. Compliance, the vasodilator response to a certain pressure increase in the absence of intact SMC (representing the elasticity of the ECM). However, it is not well-defined how pregnancy affects these pathways.

The factors that are responsible for changing the EC, SMC and ECM-dependent pathways during pregnancy are largely unknown. The pregnancy-related hormone relaxin is thought to be an important factor. This can be concluded from several observations. In nonpregnant rats, relaxin administration induces systemic vasodilator responses as observed in healthy mid-pregnancy, including an increase in cardiac output, heart rate, stroke volume, and renal plasma flow, and a decrease in systemic vascular resistance [7-9]. In pregnant rats, blockade of relaxin (by selective antibodies completely) normalizes pregnancy-induced

vasodilator changes to nonpregnant values [10]. These observations suggest the involvement of relaxin in the gestational vascular adaptations during pregnancy.

For this thesis we questioned which mechanisms are responsible for adaptation of the different pathways during pregnancy and during exposure to the hormone relaxin in nonpregnant rats.

OVERVIEW OF THE THESIS

Literature review

Previous studies have suggested that pregnancy may affect multiple pathways, including upregulation of the endothelium-dependent NO pathway [11], reduction of the responsiveness to vasoconstrictor stimuli [12], decrease in myogenic reactivity [13], and increase in arteriolar compliance [13]. However, one may question to what extent these responses are universally valid across species, strains, gestational age and vascular beds, given the many differences in methodology and results between the various studies.

In order to study the validity of the responses involved in pregnancy-induced vasodilation under various experimental conditions, we took an approach novel to physiological research. We performed systematic reviews and meta-analyses on animal data, analogous to the systematic reviews that are commonly used in human medicine. We hypothesized that the extent of vascular adaptation to pregnancy is similar across species, strains and the course of pregnancy in mesenteric and renal vasculature (Chapters 2 and 3).

Effect of pregnancy on vasomotor control

Systemic vascular adaptations in humans and rats are virtually complete at mid-pregnancy, yet the mechanisms have not been studied extensively. Most research on the underlying pathways has been performed only in late pregnancy and it is by no means certain that the pathways involved are identical in mid- and late pregnancy. We hypothesized that midgestational vasodilation is mediated by upregulation of endothelium-dependent mechanisms and vascular compliance, and downregulation of vasoconstrictor agent sensitivity and myogenic reactivity (Chapter 4).

Effect of relaxin exposure on vasomotor control in normal, overweight, aged and hypertensive rats

Relaxin has been implied as a major factor in normal vascular adaptations to pregnancy. It is an insulin-like growth factor hormone produced by the ovaries and the placenta. Relaxin induces vasodilator responses comparable to those observed during pregnancy (increased cardiac output, heart rate, stroke volume, and renal plasma flow, and decreased systemic vascular resistance) [7-9]. Blockade of this hormone by antibodies completely reverses pregnancy-induced vascular adaptations [10]. Impaired vascular adaptations in pregnancy are common in overweight, aged and chronic hypertensive subjects. We hypothesized that resistance to relaxin in overweight, aged and hypertensive rats may negatively affect vascular adaptations to relaxin (Chapters 5, 6 and 7).

Research almost always leads to new insights and ideas for future studies. This also applies to our studies. Therefore, in the final chapter we put our findings into a broader perspective (Chapter 8).

In summary, this thesis aims to explore the mechanisms of action of adaptation of the different pathways during pregnancy and during exposure to the hormone relaxin in nonpregnant rats. To this end, we posed three research questions: 1. To what extent are the pregnancy-induced changes in the renal and mesenteric vasculature similar among species, strains and gestational periods (Chapter 2 and 3)? 2. To what extent is midgestational vasodilation mediated by upregulation of endothelium-dependent mechanisms and vascular compliance, and downregulation of vasoconstrictor agent sensitivity and myogenic reactivity (Chapter 4)? and 3. To what extent are local vascular responses to relaxin impaired in overweight, aged and hypertensive female rats (Chapter 5, 6 and 7)?

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CHAPTER 2

ADAPTIVE CHANGES OF MESENTERIC ARTERIES IN PREGNANCY: A META-ANALYSIS

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ABSTRACT

The vascular response to pregnancy has been frequently studied in mesenteric artery models by investigating endothelium cell (EC) and smooth muscle cell (SMC)-dependent responses to mechanical (flow-mediated vasodilation, myogenic reactivity, and vascular compliance) and pharmacological stimuli (G protein-coupled receptor responses: Gq_{EC}, Gs_{SMC}, Gq_{SMC}). It is unclear to what extent these pathways contribute to normal pregnancy-induced vasodilation across species, strains, and/or gestational age, and at which receptor level pregnancy affects the pathways. We performed a meta-analysis on responses to mechanical and pharmacological stimuli associated with pregnancy-induced vasodilation of mesenteric arteries and included 55 (188 responses) out of 398 studies. Most included studies (84%) were performed in Wistar and Sprague Dawley rats (SDR) and compared late gestation versus nonpregnant controls (80%). Pregnancy promotes flow-mediated vasodilation in all investigated species. Only in SDR, pregnancy additionally stimulates both vasodilator Gq_{EC} sensitivity (EC_{50} reduced by -0.76 [-0.92, - 0.60] log[M]) and Gs_{SMC} sensitivity (EC_{50} reduced by -0.51 [-0.82, -0.20] log[M]), depresses vasopressor Gq_{SMC} sensitivity (EC_{50} increase in SDR by 0.23 [0.16, 0.31] log[M]) and enhances arterial compliance. We conclude that: 1. Pregnancy facilitates flow-mediated vasodilation at term, among all investigated species, and the contribution of additional vascular responses is species and strain specific, and 2. Late pregnancy mediates vasodilation through changes at the receptor level for the substances tested. The initial steps of vasodilation in early pregnancy remain to be elucidated.

INTRODUCTION

Vascular adaptation to pregnancy is characterized by marked vasodilation. Vasodilation is reflected by a reduction in systemic vascular resistance. Systemic vascular resistance drops early in gestation, is at its lowest at midgestation, and gradually increases toward term. This is true for both humans [1;2] and animals [3].

The local vascular mechanisms underlying adaptation to pregnancy have been studied extensively, mainly in animal experiments. They involve both endothelium cell- (EC) and smooth muscle cell- (SMC) dependent changes. The underlying mechanisms are complex and probably gestational age-specific and species- or strain-specific. As a result of such confounding factors, conclusions from various studies may seem inconsistent. Systematic review is a tool to determine qualitative and quantitative responses objectively, while taking into account methodological quality, study design and various confounders. It has been used to evaluate human research on a variety of topics, as collected in the Cochrane database [4]. The same technique is applicable to animal experimental work [2].

Adaptation to pregnancy has been studied in several vascular beds and by a variety of stimuli. The mesenteric circulation has been commonly used because this vascular bed is quantitatively important, since it receives one-third of cardiac output in pregnancy [2;5], and because it is easily accessible for study. The local vascular responses can be arranged by the stimuli imposed or the pathways involved. Mechanical stimuli include flow-mediated vasodilation, myogenic reactivity, and arterial compliance. Electric stimuli concern experiments on electric field stimulation (EFS). Pharmacological stimuli consist of responses to agents such as acetylcholine, norepinephrine, and phenylephrine. As most of these pharmacological stimuli involve G protein-coupled receptors, the response to the agents can be arranged according to their receptor pathways. These include the EC (Gq_{EC}-) pathway and two SMC (Gq_{SMC}- and Gs_{SMC}-) pathways [6-12], as shown in Figure 1.

Many authors have generalized the conclusions of their vascular studies from species, and/or strain, and/or gestational age period, to pregnancy in mammals in general. The aim of our study was to evaluate to what extent vascular adaptation to pregnancy is indeed similar across species, strains and the course of pregnancy. To study this effect, we applied the methods of meta-analysis to experimental data, limiting ourselves for practical reasons to one important vascular bed, that of the mesenteric circulation. The questions to be answered were the following: 1. To what extent do mechanical stimuli and/or pharmacological receptor pathways contribute to normal vasodilation of the mesenteric circulation in pregnancy across species, strains, and/or gestational age? and 2. At which receptor level does pregnancy affect the different pathways in this circulation across species, strains, and/or gestational age?

MATERIALS AND METHODS

Literature search

We searched the PubMed and Embase databases for (all) original studies on adaptive responses of mesenteric arteries to a first pregnancy, from 1948 (PubMed) and 1980 (Embase) until April 2012. No restriction was used for language or species (studies on

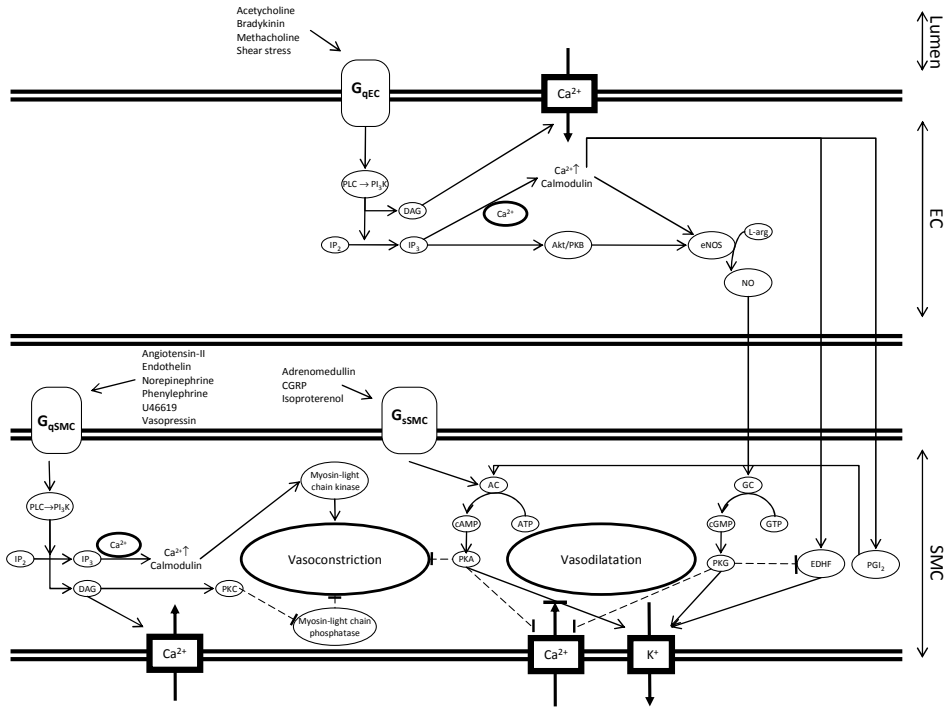


Figure 1. The three common types of G protein coupled receptors, the involved agents and the simplified endothelium cell (EC) and smooth muscle cell (SMC) pathways.

1. The Gq_{EC}-pathway: acetylcholine, bradykinin and methacholine enhance EC phospholipase C activity and the formation of phosphatidylinositol 3-kinase (PI_3K), which enhances inositol 1,4,5-triphospate (IP_3) and diaglycerol (DAG) production. This leads to Ca^{2+}-infux in the EC and Ca^{2+}-release from the sarcoplasmic reticulum. This in turn enhances the availability of Ca^{++}/Calmodulin and activates Akt/phospokinase B (Akt/PKB), leading to endothelial nitric oxide synthase (eNOS) activation and the production of nitric oxide (NO), prostacyclin (PGI_2) and endothelium-dependent hyperpolarizing factor (EDHF). These substances diffuse to the SMC, where they activate protein kinase G (PKG) through guanyl cyclase (GC) and protein kinase A (PKA) through adenyl cyclase (AC). These substances induce vasodilation by blockade of the Ca^{2+}-channel influx and stimulation of the K^+-channel efflux.
2. The Gq_{SMC}-pathway: angiotensin-II, endothelin, norepinephrine, phenylephrine, thromboxane (U46619), and vasopressin activate SMC phospolipase C, leading to the formation of inositol 1,4,5-triphospate (IP_3) and diaglycerol (DAG). This leads to Ca^{2+}-influx in the SMC and Ca^{2+}-release from the sarcoplasmic reticulum, which results in vasoconstriction.
3. The Gs_{SMC}-pathway: adrenomedullin, calcitonin gene-related peptide (CGRP), human chorionic gonadotrophin (hCG) and isoproterenol activate SMC adenylate cyclase (AC), which enhances protein kinase A (PKA) production. This stimulates K^+-influx and inhibits Ca^{2+}-influx, which results in vasodilation.

humans were included). The search strategies focused on pregnancy, mesenteric arteries, and vasoconstrictor and/or vasodilator responses, as detailed in Tables 1 and 2.

Selection of studies

From the original studies identified by the literature search, we selected studies qualified for inclusion in the meta-analysis, through the three-phase selection process shown in Figure 2.

In phase 1, all potential studies were screened for inclusion criteria on the basis of title and abstract. The screening was performed independently by two investigators (first and second author). In case of any doubt, the full publication was evaluated. Differences between investigators were resolved by mutual agreement or, in case of remaining disagreement, by a third investigator (M. Leenaars, Central Animal Laboratory, SYRCLE). Inclusion criteria were: 1. Original data, 2. Healthy subjects, 3. First pregnancy, 4. Nulliparous controls, 5. Age-matched subjects, 6. Mesenteric artery vasoconstrictor and/or vasodilator responses, and 7. Standard medium used in the experiments. As some studies did not literally describe information concerning inclusion criteria 3, 4, and 5, studies were included if it was very assumable that they complied.

In phase 2, articles that passed the initial screening were analyzed in full, by the same two investigators for the same inclusion criteria.

In phase 3, full articles that passed the inclusion criteria were evaluated for the assessability of the responses, based on the presence of greater of equal to five measurements for each response. From these, we included all responses in the absence of any blockade, in the presence of denuded endothelium or blockade of well-known, commonly studied, EC vasodilator mechanisms [blockade of nitric oxide (NO) and/or prostacyclin (PGI_2)].

Data extraction

From each article with assessable responses, data were extracted. We recorded primary data on species, strain, gestation age (early, mid-, and late gestation, defined by trimester), estrous/cycle stage, and type of myograph. From each vascular response we recorded the number of subjects, effect-size, and standard deviation or standard error of the mean. In case of missing, incomplete, or indeterminate data, an effort was made to complete the data by approaching the authors by e-mail. The response rate to these requests was 46%. In case of duplicate reporting, we used only the results of the most recent publication.

From the pharmacological and electrical responses, we extracted the sensitivity (EC_{50} = effective concentration, needed to obtain 50% of maximum effect) and maximum effect (E_{max} = not responding to $\geq$2 further increases in concentration or intensity). From the mechanical responses, we determined only the direction of the effect (favoring vasodilation, vasoconstriction, or no effect), because quantitative comparison was not feasible. This was caused by the highly variable outcome measures reported in these studies and the use of statistics (often ANOVA) that did not allow calculation of overall effect-sizes.

Quality assessment

The quality of each study was assessed by two independent investigators (first and second author), based on randomization, blinding of outcome assessment, age or weight of subjects, and number of animals used at study entry and analysis. Subsequently, each response was ranked on three points: 1. Clear number of subjects used for analyses, 2. Presence of a response graph (based on at least 5 measurements), and 3. Achievement of E_{max}. The overall quality of the responses was expressed as the percentage of responses that fulfilled the criteria compared with the total number of responses in each study.

Table 1. Literature search-strategy for Pubmed.

Component	Description
Pregnancy	"pregnancy"[MeSH Terms] OR "pregnancy"[tiab] OR "pregnancies"[tiab] OR "gestation"[tiab] OR "pregnant"[tiab] OR "maternal-fetal relations"[tiab]
Mesenteric arteries	"mesenteric arteries"[MeSH Terms] OR "Mesentery/blood supply"[Mesh] OR "mesenteric"[tiab] OR "mesentery artery"[tiab] OR "mesentery arteries"[tiab] OR "mesenterial artery"[tiab] OR "mesenterial arteries"[tiab] OR "arteria mesenterica"[tiab] OR "omental microvessels"[tiab] OR "omental arteries"[tiab] OR "omental artery"[tiab]
Vasoconstrictor and vasodilator responses	"vasoconstriction"[MeSH Terms] OR "vasoconstriction"[tiab] OR "vasoconstrictions"[tiab] OR "vasoconstrictor agents"[MeSH Terms] OR "vasoconstrictor agents"[Pharmacological Action] OR "vascular resistance"[MeSH Terms] OR "vascular resistance"[tiab] OR "vascular capacitance"[MeSH Terms] OR ("vascular"[tiab] AND "capacitance"[tiab]) OR "vasoconstrictor"[tiab] OR "vasoconstrictors"[tiab] OR "vasopressor"[tiab] OR "vasoactive agonist"[tiab] OR "vasoactive agonists"[tiab] OR "vasopressors"[tiab] OR "vasomotor system"[MeSH Terms] OR "vasomotor system"[tiab] OR "peripheral resistance"[tiab] OR "artery constriction"[tiab] OR "vessel constriction"[tiab] OR "vasoconstrictive"[tiab] OR "vasoconstricting"[tiab] OR "vasoconstricted"[tiab] OR "vasodilation"[MeSH Terms] OR "vasodilation"[tiab] OR "vasodilatation"[tiab] OR "vasodilatating"[tiab] OR "vasodilating"[tiab] OR "vasodilative"[tiab] OR "vasodilatative"[tiab] OR "artery dilation"[tiab] OR "vessel dilation"[tiab] OR "artery dilatation"[tiab] OR "vessel dilatation"[tiab] OR "vasodilator agents"[MeSH Terms] OR "vasodilator agents"[Pharmacological Action] OR "vasodilator"[tiab] OR "vasodilators"[tiab] OR "vasorelaxation"[tiab] OR "Vascular Endothelium Dependent Relaxation"[tiab] OR "Endothelium Dependent Relaxation"[tiab] OR "Vascular Endothelium-Dependent Relaxation"[tiab] OR "Endothelium-Dependent-Relaxation"[tiab] OR "hemodynamics"[MeSH Terms] OR "hemodynamics"[tiab]OR "hemodynamic"[tiab] OR "vasodilated"[tiab] OR "vasoactive agent"[tiab] OR "vasoactive drug"[tiab] OR "vasoactive drugs"[tiab] OR "dilation"[tiab] OR "dilatation"[tiab] OR "contraction"[tiab] OR "relaxation"[tiab]

Data analysis

Values of EC_{50} and E_{max} for pharmacological and electrical stimuli were analyzed and displayed in forest plots (Review Manager 5, The Cochrane Collaboration, 2008).

Responses to pharmacological stimuli were grouped together by the type of stimulus and type of G protein-coupled pathway (Gq_{EC}, Gq_{SMC}, and Gs_{SMC}) involved. If they could not be attributed to such pathway, they were arranged by type of stimulus (NO donors, EFS, $MgSO_4$, and potassium). Subgroup analysis was performed to assess species and strain differences and to explore possible causes for heterogeneity. The responses were calculated as weighed mean differences (pregnant minus nonpregnant) for EC_{50} or as standardized mean differences (pregnant minus nonpregnant) for E_{max}, depending on whether the variable was uniformly expressed among studies or not. These data were presented as means ± 95% confidence interval. $P < 0.05$ was considered statistically significant. Responses to mechanical stimuli were analyzed qualitatively and the direction of combined responses was determined relative to the number of animals.

Table 2. Literature search-strategy for Embase.

Component	Description
Pregnancy	exp pregnancy/ OR (pregnancy or pregnant).ti,ab. OR (pregnacies or gestation). ti,ab. OR (pregnancy or pregnant or pregnacies or gestation).ti,ab.
Mesenteric arteries	exp mesenteric artery/ OR mesenteric.ti,ab. OR mesentery artery.ti,ab. OR mesentery arteries.ti,ab. OR mesenterial artery.ti,ab. OR mesenterial arteries. ti,ab. OR arteria mesenterica.ti,ab. OR (mesenterium adj3 circulation).ti,ab. OR (mesentery adj3 circulation).ti,ab. OR (mesenterial adj3 circulation).ti,ab. OR (mesenterium adj3 blood flow).ti,ab. OR (mesentery adj3 blood flow).ti,ab. OR (mesenterial adj3 blood flow).ti,ab. OR mesentery vessel.ti,ab. OR mesentery vessels.ti,ab. OR mesentery blood vessel.ti,ab. mesentery blood vessels.ti,ab. OR mesenterial vessel.ti,ab. OR mesenterial vessels.ti,ab. OR mesenterial blood vessel. ti,ab. OR mesenterial blood vessels.ti,ab. OR (omental arteries or omental artery or omental microvessels or omental microvessel).ti,ab.
Vasoconstrictor and vasodilator responses	exp vasoconstriction/ OR exp vasoconstrictor agent/ OR vasoconstrict*.ti,ab. OR exp vascular resistance/ OR blood flow resistance.ti,ab. OR blood vessel resistance. ti,ab. OR (peripheral adj1 resistance).ti,ab. OR vascular vessel resistance.ti,ab. OR artery resistance.ti,ab. OR exp hemodynamics/ OR exp blood vessel capacitance/ OR blood vessel capacitance.ti,ab. OR vascular capacitance.ti,ab. OR (hemodynamic or hemodynamics).ti,ab. OR (haemodynamic or haemodynamics).ti,ab. OR vasopressor. ti,ab. OR exp vasoactive agent/ OR (vasoactive agonist OR vasoactive agonists or vasoactive agent or vasoactive agents or vasoactive drug or vasoactive drugs). ti,ab. OR (vessel contriction or vessel contrictions or artery contriction or artery constrictions).ti,ab. OR exp vasodilatation/ OR (vasodilatation or vasodilation or vasodilator or vasodilatator or vasodilating or vasodilative or vasodilatative).ti,ab. OR (vaso dilatation or vaso dilation or vaso dilator or vaso dilatator or vaso dilating or vaso dilative or vaso dilatative).ti,ab. OR vaso relaxation.ti,ab. OR vascular endothelium dependent relaxation.ti,ab. OR vascular endothelium-dependent relaxation.ti,ab. OR endothelium dependent relaxation.ti,ab. OR endothelium-dependent relaxation.ti,ab. OR (vessel dilatation* or vessel dilation* or vascular dilation* or vascular dilatation* or vasorelaxation* or artery dilatation* or artery dilation*).ti,ab. OR (dilatation* or dilation* or relaxation* or contraction*).ti,ab.

Variation of the combined responses was expressed as heterogeneity, calculated as I^2, under the assumption of a random effects model (Review Manager 5). A value of I^2 <60% represents moderate heterogeneity, and I^2 >60% is substantial to considerable heterogeneity [13].

Publication bias was assessed by the method of Egger et al. (for 10 or more studies) [14] or by the amount of funnel plot asymmetry (for less than 10 studies).

The robustness of results from meta-analysis was assessed by sensitivity-analysis [13]. To this effect we repeated all analyses in the presence and absence of the excluded studies to determine the extent to which decisions made during in- and exclusion based on parity or age-matching had any major effect on our results.

References used for analysis included the following: [15-20;23;26;35-79].

RESULTS

Our search identified 398 studies on vascular adaptation to pregnancy, involving the mesenteric vascular bed. From these, 345 studies were not included for the reasons shown in Figure 2, leaving 55 studies that met the criteria for inclusion in the meta-analysis. Human studies did not meet the criteria for inclusion. In one case double reporting was suspected [15;16].

The characteristics of the included studies are shown in Table 3. Most studies concerned rodents [rats=46 (Spraque-Dawley rats (SDR) and Wistar rats (WR), mice=5 (C57BL/6J), and guinea pigs=2 (Hartley)], one study used sheep and one used pigs. Most of the studies had methodological shortcomings (Table 4). Only 25% (14/55) reported the number of animals used in the study, 53% (29/55) parity; only 4% (2/55) performed randomization, and 0% blinded outcome.

From the 55 included studies with 266 vascular responses, 188 responses fully met the criteria for inclusion in the meta-analysis, as shown in Figure 2. Stimuli were pharmacological

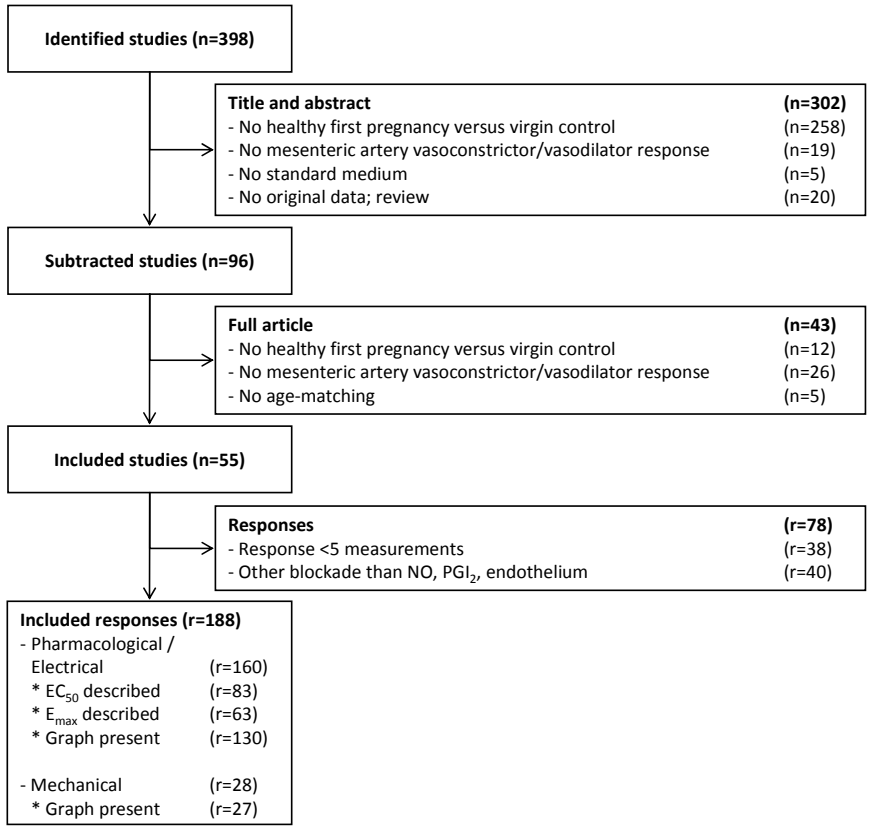


Figure 2. Flow chart for selection, inclusion and exclusion of studies and responses on vascular adaptation to pregnancy in mesenteric arteries.

n= number of studies; r= number of responses.

(82%, 154/188), electrical (3%, 6/188), and mechanical (15%, 28/188) and were performed in early (2%, 3/188), mid- (12%, 23/188), and late (86%, 161/188) pregnancy (one undefined pregnancy stage). For pharmacological and electrical responses, EC_{50} was detectable in 52% (83/160) of cases and E_{max} in 39% (63/160), and a graph was present in 81% (130/160). For mechanical responses the effect size was quantified in 7% (2/28) and a graph was present in 96% (27/28). Responses were obtained by wire-myography (49%, 93/188), pressure myography (18%, 33/188), pressure-perfusion myography (9%, 16/188), and whole organ perfusion (24%, 46/188).

Pharmacological responses

Vascular adaptation to pregnancy, assessed in mesenteric arteries through pharmacological stimuli related to the Gq_{EC}-coupled pathway, is shown in Figure 3. Responses were available for mid- and late gestation, mainly in rats. In midgestation, two studies in SDR showed no significant effect of acetylcholine or bradykinin on $EC_{50,}$ E_{max}, or the overall (acetylcholine plus bradykinin) Gq_{EC} response. In late gestation, responses differed for SDR and WR. In WR, data from four studies showed no significant effect of pregnancy on EC_{50} or E_{max} for any stimulus. In SDR, the responses from three studies showed considerable heterogeneity for EC_{50} and $E_{max.}$ This was largely caused by one whole organ perfusion myography response (Parent et al. [17]), as exclusion of this response markedly reduced heterogeneity. The two other wire-myograph studies in SDR showed a reduction in EC_{50} (by -0.76 [-0.92, -0.60] log[M]) in late gestation. In guinea pigs, pregnancy slightly reduced EC_{50} (by -0.34 [-0.47, -0.20] log[M]). This suggests in SDR and to a lesser amount in guinea pigs, but not WR, that Gq_{EC}-mediated vasodilation is activated in late pregnancy.

The effect of pregnancy on the Gq_{EC}-pathway in mesenteric arteries under blockade is shown in Figure 4. Responses were available for late gestation only, in WR, SDR, mice and guinea pigs. In both WR, SDR, and mice, blockade of NO and/or PGI_2 abolished any Gq_{EC}-mediated vasodilation, if present. In guinea pigs, however, the reduction in EC_{50} in pregnancy persisted under blockade of NO and PGI_2. Apparently, in WR the Gq_{EC}-pathway is not activated, in SDR and mice it is activated and NO and PGI_2 dependent, and in guinea pigs the pathway is activated and independent of NO and PGI_2.

Vasoconstriction through pharmacological stimuli related to the Gq_{SMC}-coupled pathway in mesenteric arteries in pregnancy is shown in Figures 5 and 6. The responses to the various agents showed considerable heterogeneity. The overall Gq_{SMC} response at midgestation showed no effect of pregnancy. Responses for late gestation were available in SDR, WR, mice and guinea pigs. The overall Gq_{SMC} response suggested downregulation of vasoconstriction, but the responses differed markedly between species and strains. In WR and guinea pigs, pregnancy did not affect EC_{50} or E_{max} for all agents studied, except for one response [18] in WR of increased sensitivity to norepinephrine. This response caused considerable heterogeneity. Additionally, the data showed funnel plot asymmetry, which may suggest publication bias. SDR and mice showed reduced vasoconstrictor sensitivity, as demonstrated by the increase in EC_{50} and no change in E_{max} for all agents tested.

Table 3. Characteristics of included studies, arranged by author and year.

Study	Species (strain)	Estrous stage control group	Gestation period	Myograph type[a]	Number of described responses
Ballejo et al., 2002	rat (WR)	estrous	late	PPM	10
Chauhan et al., 2007	rat (SDR)	di-estrous	-	WM	1
Chu et al., 1993	rat (WKY)	-	late	WOP	14
Chu et al., 1994	rat (WKY)	-	late	WOP	6
Chu et al., 1998	rat (WKY)	-	late	WOP	2
Cockell et al., 1996	rat (WR)	-	late	PM / PPM	4
Coelho et al., 1997	rat (WR)	estrous	late	WOP	4
Cook et al., 2001	mouse (C57BL/6J)	-	late	PM	4
Cooke et al., 2003	mouse (C57BL/6J)	all stages	late	WM	6
Crandall et al., 1990	rat (SDR)	-	mid/late	WM	5
Dalle Lucca et al., 2000	rat (SDR)	estrous	mid	PPM	4
D'Angelo et al., 1993	rat (SDR)	all stages	late	PM	2
Davidge et al., 1992	rat (SDR)	-	late	WM	10
Euser et al., 2005	rat (SDR)	-	late	PM	1
Gangula et al., 2004	rat (SDR)	all stages	late	WM	1
Gerber et al., 1998	rat (SDR)	-	late	WM	7
Gerber et al., 1999	rat (WR)	-	late	WM	6
Goulopoulou et al., 2012	rat (SDR)	all stages	late	WM	6
Hagedorn et al., 2007	mouse (C57BL/6J)	-	late	WM	2
Hart et al., 1986	rat (SDR)	estrous	late	WM	4
Hermsteiner et al., 1999	rat (SDR)	-	mid/late	PM	8
Hermsteiner et al., 2001	rat (SDR)	di-estrous	mid/late	PM	6
Hines et al., 1992	rat (SDR)	-	late	WOP	2
Kim et al., 1994	guinea pig (Hartley)	-	late	WM	7
Koumentaki et al., 2002	rat (WR)	-	late	WM	5
Lanlua et al., 2002	rat (SDR)	all stages	late	WM	1
Le Marquer-Domagala et al., 1997	rat (-)	-	late	WOP	6
Learmont et al., 1996	rat (WR)	-	late	WM / PM	6
Mackey et al., 1992	rat (SDR)	-	late	PM	1
Mandelbaum et al., 1999	rat (WR)	-	late	WOP	4
Massicotte et al., 1987	rat (SDR/WKY)	-	late	WOP	4
McLaughlin et al., 1986	rat (SDR)	all stages	all	WM	6
Meyer et al., 1993	rat (SDR)	-	late	PM	2
Meyer et al., 1997	rat (SDR)	-	late	PM / PPM	2
Meziani et al., 2005	rat (WR)	-	late	WM	4

Table 3. *Continued.*

Study	Species (strain)	Estrous stage control group	Gestation period	Myograph type[a]	Number of described responses
Miller et al., 2002	rat (SDR)	-	late	WM	2
Neves et al., 2003	rat (SDR)	all stages	late	PM	2
Nuwayhid et al., 1975	sheep (-)	-	late	WOP	1
Ognibene et al., 2012	rat (WR)	di-estrous	late	WOP	4
Parent et al., 1990	rat (SDR)	-	late	WM	3
Parent et al., 1989	rat (SDR)	-	mid/late	WOP	4
Pascoal et al., 1995	rat (SDR)	-	late	WM	4
Powers et al., 2004	mouse (-)	-	late	WM	6
Ralevic et al., 1996	rat (SDR)	-	late	WOP	22
Resende et al., 2004	rat (WR)	estrous	late	WOP	5
Ross et al., 2007	rat (SDR)	di-estrous	late	WM	3
Stice et al., 1987	pig (Yorkshire gilt)	all stages	early	PPM	2
St-Louis et al., 1995	rat (SDR)	all stages	late	WM	5
Van Drongelen et al., 2011	rat (WR)	all stages	mid	PPM/WM	6
Van Eijndhoven et al., 2007	rat (-)	-	mid	WM	6
Van Eijndhoven et al., 2008	rat (WR)	-	mid	WM	4
Van Eijndhoven et al., 2003	rat (WR)	-	mid	WM	2
Veerareddy et al., 2002	mouse (C57BL/6J)	-	late	PM	6
White et al., 1998	guinea pig (Hartley)	-	late	WM	2

[a] WM = wire myograph, PM = pressure myograph, PPM = pressure-perfusion myograph, WOP = whole organ perfusion

Apparently, in WR and guinea pigs the Gq_{SMC}-mediated vasoconstrictor sensitivity is not affected, whereas in SDR and mice the pathway is reduced in late pregnancy.

The effect of late pregnancy on the Gq_{SMC}-pathway in mesenteric arteries under blockade is shown in Figure 7. Responses were available for late gestation only in SDR, mice, and guinea pigs, but not in WR. In these species, blockade of NO, PGI_2, or absence of endothelium did not affect EC_{50} or E_{max}, except for the responses in SDR in which EC_{50} remained elevated [19]. This suggests that the downregulation in late pregnancy of the vasoconstrictor Gq_{SMC}-mediated pathway is dependent on endothelium-related changes in mice and guinea pigs, but not convincingly in SDR.

The effect of pregnancy on the SMC relaxing Gs_{SMC}-pathway in mesenteric arteries is shown in Figure 8. Responses were available for mid- and late gestation mainly in SDR, to a limited extent in WR, and not in other species. Human chorion gonadothrophin (hCG) responses were qualitatively different from all other Gs_{SMC}-coupled responses. In SDR, hCG increased EC_{50} in the presence of endothelium, and reduced EC_{50} in the absence of

Table 4. Quality assessment of the included studies and subsequent responses.

	Study quality						Response quality			
Study	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**	**Score**	**(6)**	**(7)**	**(8)**	**Score**
Ballejo et al., 2002	-	-	+	+	-	2	0.4	0.6	0.2	1.2
Chauhan et al., 2007	-	-	?	-	-	0	1.0	1.0	0.0	2.0
Chu et al., 1993	-	-	?	+	-	1	1.0	0.0	0.0	1.0
Chu et al., 1994	-	-	?	-	-	0	0.0	0.0	0.0	0.0
Chu et al., 1998	-	-	?	-	-	0	1.0	0.0	0.0	1.0
Cockell et al., 1996	-	-	+	+	-	2	1.0	1.0	1.0	3.0
Coelho et al., 1997	-	-	+	+	-	2	1.0	1.0	0.5	2.5
Cook et al., 2001	-	-	?	+	-	1	0.0	0.8	0.0	0.8
Cooke et al., 2003	-	-	?	+	+	2	0.8	0.8	0.0	1.7
Crandall et al., 1990	-	-	+	-	-	1	1.0	0.6	0.0	1.6
Dalle Lucca et al., 2000	-	-	+	+	-	2	1.0	1.0	0.3	2.3
D'Angelo et al., 1993	-	-	+	+	-	2	1.0	1.0	1.0	3.0
Davidge et al., 1992	-	-	+	+	+	3	0.2	0.5	0.5	1.2
Euser et al., 2005	-	-	+	+	+	3	1.0	1.0	1.0	3.0
Gangula et al., 2004	-	-	?	+	-	1	1.0	1.0	1.0	3.0
Gerber et al., 1998	-	-	?	-	+	1	0.9	1.0	0.6	2.4
Gerber et al., 1999	-	-	+	+	-	2	1.0	0.8	0.5	2.3
Goulopoulou et al., 2012	-	-	+	+	-	2	1.0	1.0	0.0	2.0
Hagedorn et al., 2007	-	-	?	+	-	1	1.0	1.0	1.0	3.0
Hart et al., 1986	-	-	?	+	-	1	1.0	0.5	0.0	1.5
Hermsteiner et al., 1999	-	-	+	+	-	2	0.9	0.4	0.0	1.3
Hermsteiner et al., 2001	-	-	+	+	-	2	1.0	1.0	0.3	2.3
Hines et al., 1992	-	-	+	-	-	1	1.0	0.0	0.0	1.0
Kim et al., 1994	-	-	?	-	-	0	1.0	1.0	1.0	3.0
Koumentaki et al., 2002	-	-	+	+	-	2	1.0	0.4	0.4	1.8
Lanlua et al., 2002	-	-	?	+	-	1	1.0	0.0	0.0	1.0
Le Marquer-Domagala et al., 1997	-	-	+	+	-	2	0.5	0.7	0.2	1.3
Learmont et al., 1996	-	-	+	+	-	2	1.0	0.8	0.4	2.2
Mackey et al., 1992	-	-	+	-	+	2	1.0	1.0	1.0	3.0
Mandelbaum et al., 1999	-	-	+	+	-	2	1.0	1.0	0.0	2.0
Massicotte et al., 1987	-	-	?	+	-	1	1.0	1.0	0.0	2.0
McLaughlin et al., 1986	-	-	+	-	-	1	1.0	1.0	0.5	2.5
Meyer et al., 1993	-	-	+	+	-	2	1.0	1.0	0.0	2.0
Meyer et al., 1997	-	-	+	-	+	2	0.0	0.0	0.0	0.0
Meziani et al., 2005	-	-	?	+	-	1	1.0	0.8	0.0	1.8
Miller et al., 2002	-	-	?	+	-	1	0.5	0.5	0.0	1.0

Table 4. *Continued*

	Study quality						Response quality			
Study	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**	**Score**	**(6)**	**(7)**	**(8)**	**Score**
Mitchell et al., 2007	-	-	?	+	-	1	0.8	0.8	0.8	2.3
Neves et al., 2003	-	-	+	-	-	1	0.0	1.0	0.0	1.0
Nuwayhid et al., 1975	-	-	?	-	-	0	0.0	0.0	0.0	0.0
Ognibene et al., 2012	-	-	+	+	+	3	0.5	0.0	0.0	0.5
Parent et al., 1990	-	-	?	+	-	1	1.0	1.0	0.7	2.7
Parent et al., 1989	-	-	?	+	-	1	1.0	1.0	0.5	2.5
Pascoal et al., 1995	+	-	+	+	-	3	1.0	1.0	0.0	2.0
Powers et al., 2004	-	-	?	+	-	1	0.3	0.3	0.0	0.7
Ralevic et al., 1996	-	-	+	+	-	2	0.0	0.1	0.0	0.2
Resende et al., 2004	-	-	+	-	-	1	1.0	1.0	0.2	2.2
Ross et al., 2007	-	-	?	+	-	1	1.0	1.0	0.0	2.0
Stice et al., 1987	+	-	?	+	+	3	1.0	0.5	0.0	1.5
St-Louis et al., 1995	-	-	+	+	+	3	0.8	0.8	0.8	2.4
Van Drongelen et al., 2011	-	-	+	+	+	3	1.0	1.0	1.0	3.0
Van Eijndhoven et al., 2007	-	-	+	+	+	3	0.2	0.3	0.0	0.5
Van Eijndhoven et al., 2008	-	-	?	+	+	2	0.0	0.5	0.0	0.5
Van Eijndhoven et al., 2003	-	-	?	+	+	2	1.0	1.0	0.0	2.0
Veerareddy et al., 2002	-	-	?	+	-	1	1.0	1.0	0.0	2.0
White et al., 1998	-	-	?	-	+	1	1.0	1.0	1.0	3.0

(1) randomization, (2) blinding of outcome assessor, (3) virgin/nulliparous at entry study, (4) age or weight of animals described, (5) number of used animals clear from methods, (6) fraction of responses with clear number of animals used for statistical analyses (as a percentage of the number of described responses), (7) fraction of responses with dose-response curve containing ≥5 measurements, (8) fraction of responses with accomplished E_{max}.

it. It seems likely that hCG activates both EC- and SMC-receptors. For this reason hCG-responses were not included in further analysis of the Gs_{SMC}-pathway. In WR, vasodilation was upregulated in midgestation, as CGRP was associated with a marked reduction in EC_{50} and an increase in E_{max}. Late in pregnancy, isoproterenol increased EC_{50} and did not affect E_{max}, indicating less vasodilation compared with controls. In late pregnant SDR, adrenomedullin and CGRP reduced EC_{50}, whereas isoproterenol did not. Overall, in late pregnancy, SDR were more sensitive to Gs_{SMC}-coupled vasodilation than nonpregnant controls, whereas WR were not.

The effect of pregnancy on non-G protein-coupled responses in mesenteric arteries is presented in Figures 9 en 10. Responses were available for SDR in mid- and late gestation, and for WR and mice in late gestation. Independent of gestational age, species, or strain, pregnancy did not affect EC_{50} or E_{max} to any type of NO donor or potassium. This suggests

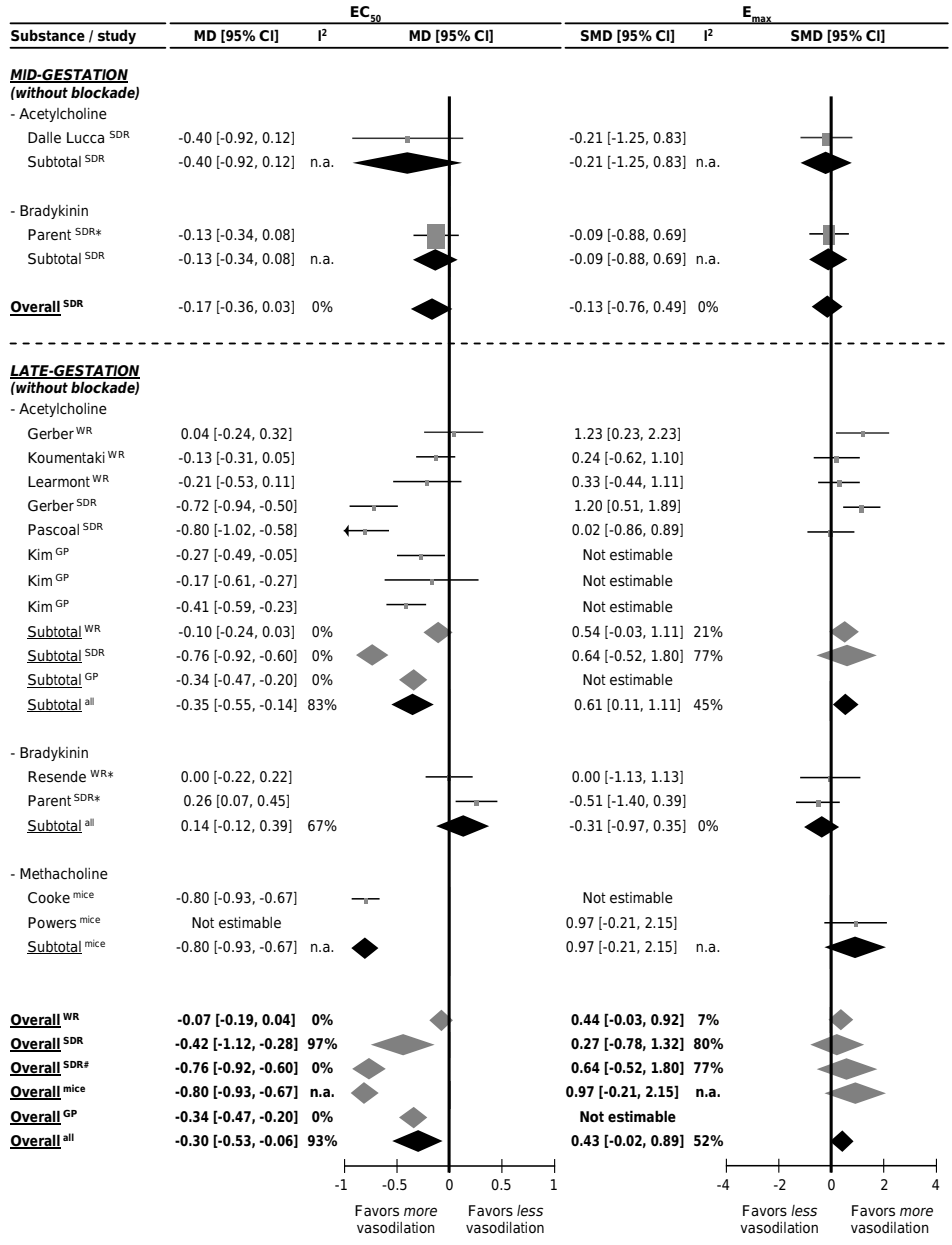


Figure 3. Effect of mid and late pregnancy on mesenteric artery responses to stimuli involved in vasodilation through the Gq_{EC}-coupled pathway.

The effect of pregnancy on EC_{50} (the dose of stimulus inducing 50% response) is depicted as mean difference (MD) and its 95% confidence interval (95% CI). E_{max} (maximum response) is presented as standardized mean difference (SMD) and its 95% CI. Studies and totals based on Sprague Dawley rats (SDR), Wistar rats (WR), mice and guinea pigs (GP). * Whole Organ Perfusion Myograph. # Whole Organ Perfusion Myograph excluded. I^2 represents the amount of heterogeneity. n.a. = not applicable.

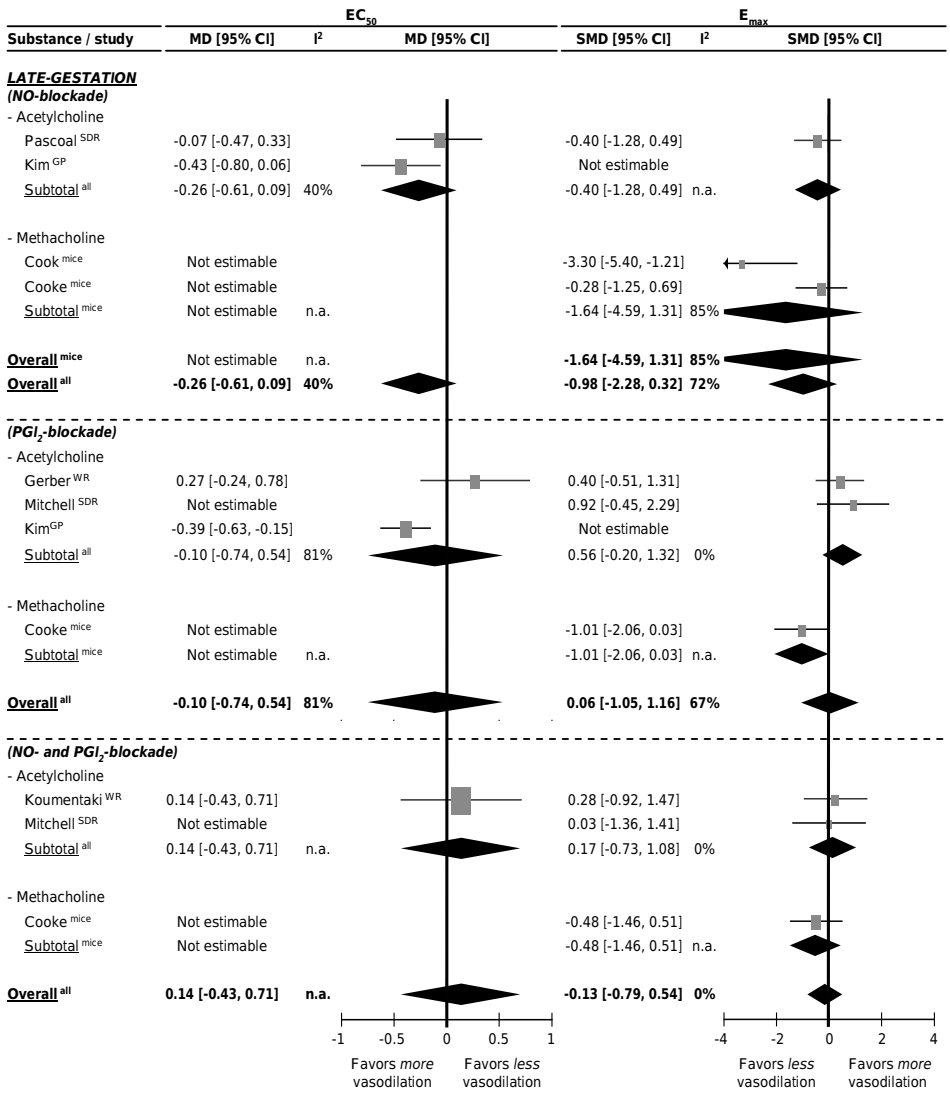


Figure 4. Effect of late pregnancy on mesenteric artery responses to stimuli involved in vasodilation through the Gq_{EC}-coupled pathway after blockade of the nitric oxide- (NO), prostacyclin- (PGI2) pathway or both.

The effect of pregnancy on EC_{50} (the dose of stimulus inducing 50% response) is depicted as mean difference (MD) and its 95% confidence interval (95% CI). E_{max} (maximum response) is presented as standardized mean difference (SMD) and its 95% CI. Studies and totals based on Sprague Dawley rats (SDR), Wistar rats (WR), mice and guinea pigs (GP). I^2 represents the amount of heterogeneity. n.a. = not applicable.

that pregnancy does not affect SMC-sensitivity to EC-dependent stimuli. Single responses showed that vasoconstrictor response to electrical field stimulation (EFS) in late pregnant WR reduced E_{max} [19;20]; in SDR MgSO4 reduced EC_{50}. This suggests that late pregnancy may affect vascular tone also through non-G protein-coupled responses.

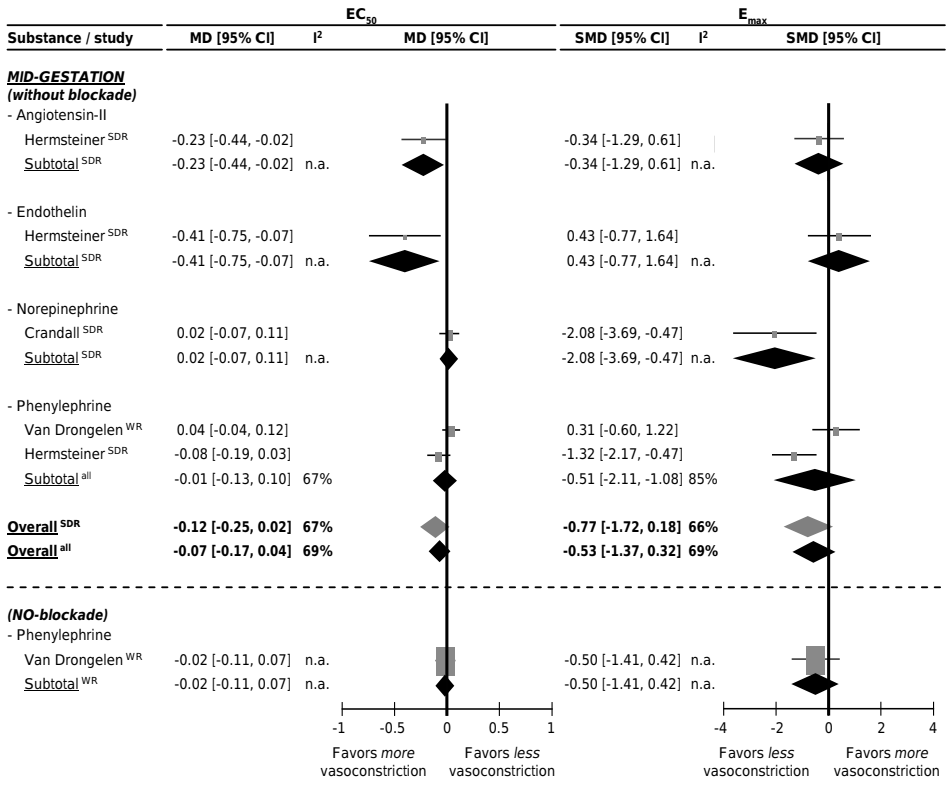


Figure 5. Effect of mid pregnancy on mesenteric artery responses to stimuli involved in vasoconstriction through the Gq_{SMC}-coupled pathway in presence and absence of nitric oxide (NO) blockade.

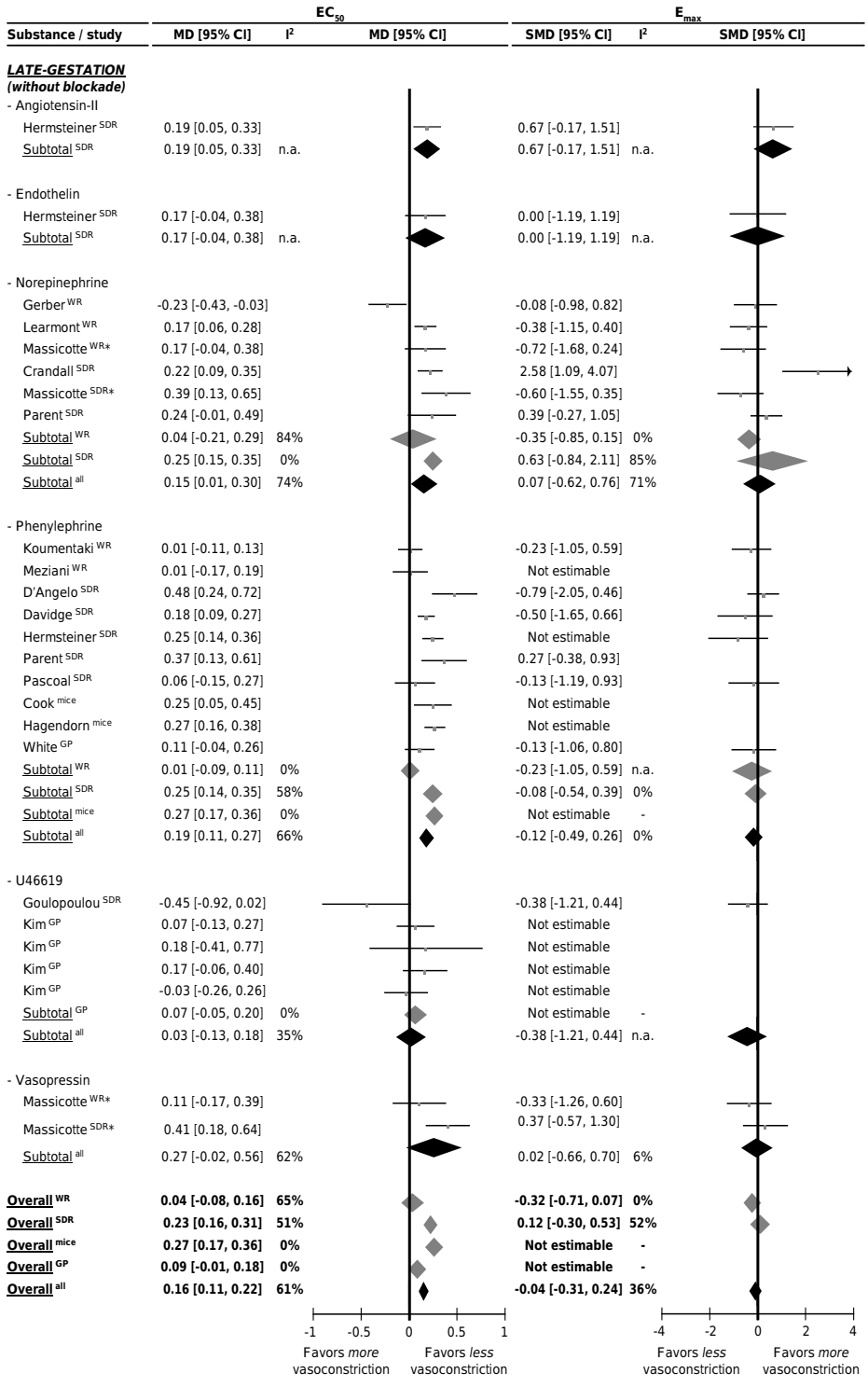
The effect of pregnancy on EC_{50} (the dose of stimulus inducing 50% response) is depicted as mean difference (MD) and its 95% confidence interval (95% CI). E_{max} (maximum response) is presented as standardized mean difference (SMD) and its 95% CI. Studies and totals based on Sprague Dawley rats (SDR) and Wistar rats (WR). I^2 represents the amount of heterogeneity. n.a. = not applicable.

The effect of pregnancy on vascular responses to mechanical stimuli was assessed qualitatively, as shown in Figure 11. Responses on early-gestation were available only in SDR, responses on midgestation in both WR and SDR, whereas qualitative different responses on late gestation were available in WR, SDR, and mice. In mid- and late pregnant WR, only flow-mediated vasodilation was upregulated. In SDR, compliance

Figure 6. Effect of late pregnancy on mesenteric artery responses to stimuli involved in vasoconstriction through the Gq_{SMC}-coupled pathway.

The effect of pregnancy on EC_{50} (the dose of stimulus inducing 50% response) is depicted as mean difference (MD) and its 95% confidence interval (95% CI). E_{max} (maximum response) is presented as standardized mean difference (SMD) and its 95% CI. Studies and totals based on Sprague Dawley rats (SDR), Wistar rats (WR), mice and guinea pigs (GP). * Whole Organ Perfusion Myograph. I^2 represents the amount of heterogeneity. n.a. = not applicable.

►


EC50
Emax
Substance / study
MD [95% CI]
I²
MD [95% CI]
SMD [95% CI]
I²
SMD [95% CI]
LATE-GESTATION (without blockade)
- Angiotensin-II
Hermsteiner SDR 0.19 [0.05, 0.33] 0.67 [-0.17, 1.51]
Subtotal SDR 0.19 [0.05, 0.33] n.a. 0.67 [-0.17, 1.51] n.a.
- Endothelin
Hermsteiner SDR 0.17 [-0.04, 0.38] 0.00 [-1.19, 1.19]
Subtotal SDR 0.17 [-0.04, 0.38] n.a. 0.00 [-1.19, 1.19] n.a.
- Norepinephrine
Gerber WR -0.23 [-0.43, -0.03] -0.08 [-0.98, 0.82]
Learmont WR 0.17 [0.06, 0.28] -0.38 [-1.15, 0.40]
Massicotte WR* 0.17 [-0.04, 0.38] -0.72 [-1.68, 0.24]
Crandall SDR 0.22 [0.09, 0.35] 2.58 [1.09, 4.07]
Massicotte SDR* 0.39 [0.13, 0.65] -0.60 [-1.55, 0.35]
Parent SDR 0.24 [-0.01, 0.49] 0.39 [-0.27, 1.05]
Subtotal WR 0.04 [-0.21, 0.29] 84% -0.35 [-0.85, 0.15] 0%
Subtotal SDR 0.25 [0.15, 0.35] 0% 0.63 [-0.84, 2.11] 85%
Subtotal all 0.15 [0.01, 0.30] 74% 0.07 [-0.62, 0.76] 71%
- Phenylephrine
Koumentaki WR 0.01 [-0.11, 0.13] -0.23 [-1.05, 0.59]
Meziani WR 0.01 [-0.17, 0.19] Not estimable
D'Angelo SDR 0.48 [0.24, 0.72] -0.79 [-2.05, 0.46]
Davidge SDR 0.18 [0.09, 0.27] -0.50 [-1.65, 0.66]
Hermsteiner SDR 0.25 [0.14, 0.36] Not estimable
Parent SDR 0.37 [0.13, 0.61] 0.27 [-0.38, 0.93]
Pascoal SDR 0.06 [-0.15, 0.27] -0.13 [-1.19, 0.93]
Cook mice 0.25 [0.05, 0.45] Not estimable
Hagendorn mice 0.27 [0.16, 0.38] Not estimable
White GP 0.11 [-0.04, 0.26] -0.13 [-1.06, 0.80]
Subtotal WR 0.01 [-0.09, 0.11] 0% -0.23 [-1.05, 0.59] n.a.
Subtotal SDR 0.25 [0.14, 0.35] 58% -0.08 [-0.54, 0.39] 0%
Subtotal mice 0.27 [0.17, 0.36] 0% Not estimable -
Subtotal all 0.19 [0.11, 0.27] 66% -0.12 [-0.49, 0.26] 0%
- U46619
Goulopoulou SDR -0.45 [-0.92, 0.02] -0.38 [-1.21, 0.44]
Kim GP 0.07 [-0.13, 0.27] Not estimable
Kim GP 0.18 [-0.41, 0.77] Not estimable
Kim GP 0.17 [-0.06, 0.40] Not estimable
Kim GP -0.03 [-0.26, 0.26] Not estimable
Subtotal GP 0.07 [-0.05, 0.20] 0% Not estimable -
Subtotal all 0.03 [-0.13, 0.18] 35% -0.38 [-1.21, 0.44] n.a.
- Vasopressin
Massicotte WR* 0.11 [-0.17, 0.39] -0.33 [-1.26, 0.60]
Massicotte SDR* 0.41 [0.18, 0.64] 0.37 [-0.57, 1.30]
Subtotal all 0.27 [-0.02, 0.56] 62% 0.02 [-0.66, 0.70] 6%
Overall WR 0.04 [-0.08, 0.16] 65% -0.32 [-0.71, 0.07] 0%
Overall SDR 0.23 [0.16, 0.31] 51% 0.12 [-0.30, 0.53] 52%
Overall mice 0.27 [0.17, 0.36] 0% Not estimable -
Overall GP 0.09 [-0.01, 0.18] 0% Not estimable -
Overall all 0.16 [0.11, 0.22] 61% -0.04 [-0.31, 0.24] 36%
-1 -0.5 0 0.5 1
Favors more vasoconstriction
Favors less vasoconstriction
-4 -2 0 2 4
Favors less vasoconstriction
Favors more vasoconstriction

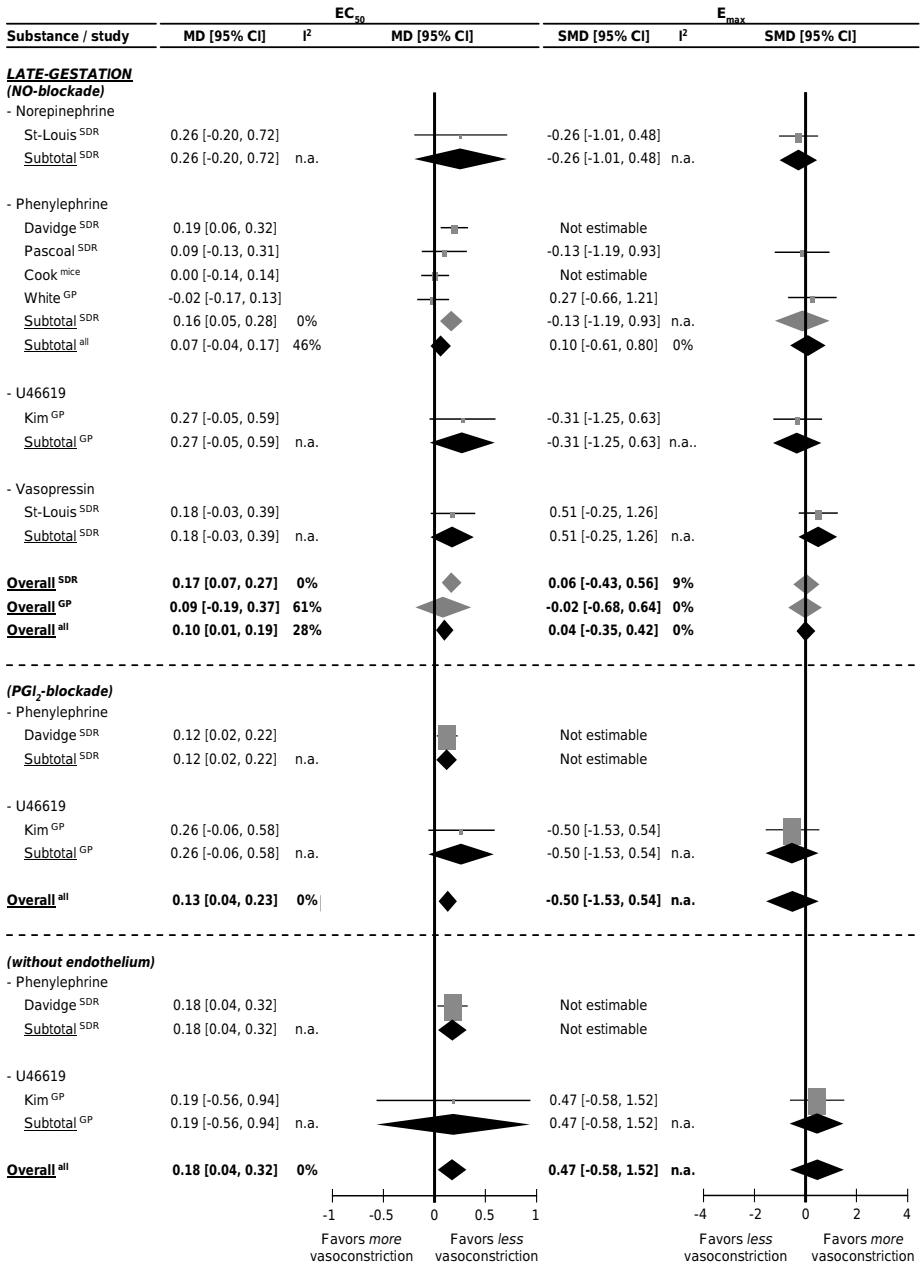


Figure 7. Effect of late pregnancy on mesenteric artery responses to stimuli involved in vasoconstriction through the Gq_{SMC}-coupled pathway after blockade of the nitric oxide- (NO), prostacyclin- (PGI2) pathway or endothelium denudation.

The effect of pregnancy on EC_{50} (the dose of stimulus inducing 50% response) is depicted as mean difference (MD) and its 95% confidence interval (95% CI). E_{max} (maximum response) is presented as standardized mean difference (SMD) and its 95% CI. Studies and totals based on Sprague Dawley rats (SDR), Wistar rats (WR), mice and guinea pigs (GP). I^2 represents the amount of heterogeneity. n.a. = not applicable.

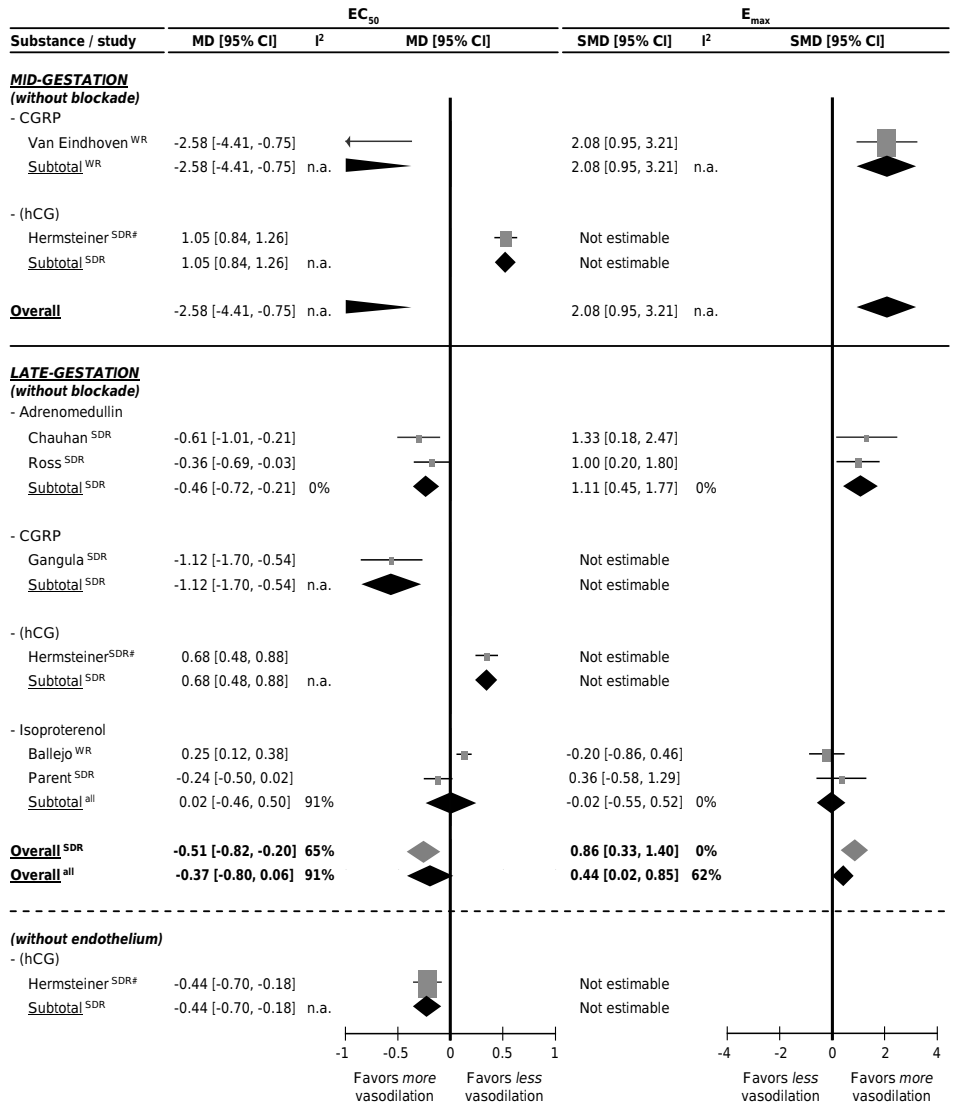


Figure 8. Effect of mid and late pregnancy on mesenteric artery responses to stimuli involved in vasodilation through the Gs_{SMC}-coupled pathway in presence and absence of endothelium.

The effect of pregnancy on EC_{50} (the dose of stimulus inducing 50% response) is depicted as mean difference (MD) and its 95% confidence interval (95% CI). E_{max} (maximum response) is presented as standardized mean difference (SMD) and its 95% CI. Studies and totals based on Sprague Dawley rats (SDR) and Wistar rats (WR). I^2 represents the amount of heterogeneity. n.a. = not applicable. # excluded study-response because of both EC and SMC involvement.

was upregulated in early and midgestation without change in myogenic reactivity. In late pregnant SDR, flow-mediated vasodilation and compliance were upregulated, and two out of three studies showed a reduction in myogenic reactivity. One study showed similar

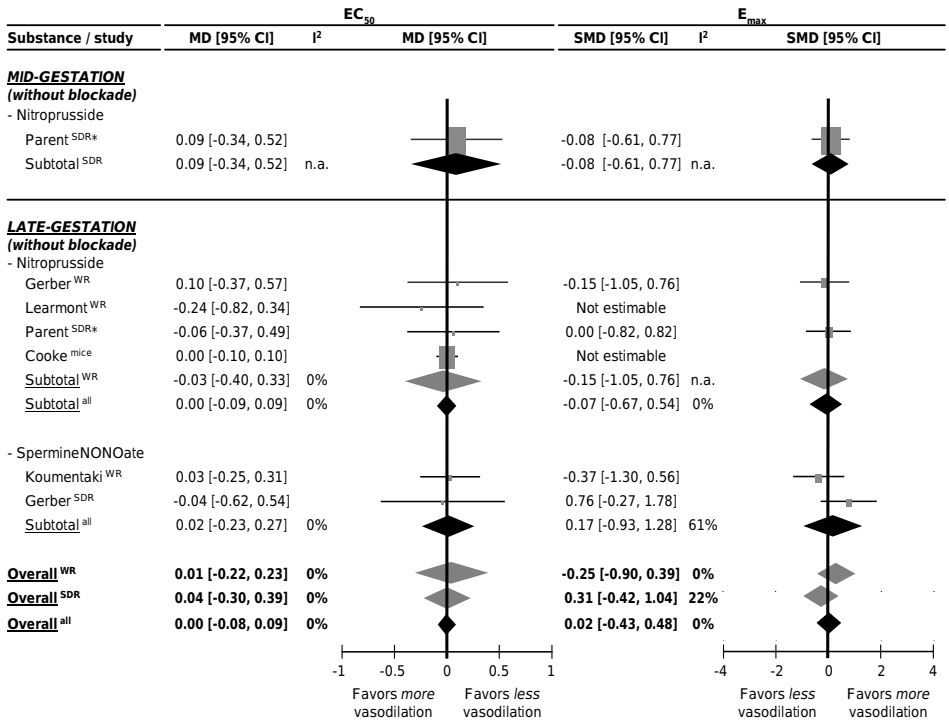


Figure 9. Effect of mid and late pregnancy on mesenteric artery responses to nitric oxide (NO).

The effect of pregnancy on EC_{50} (the dose of stimulus inducing 50% response) is depicted as mean difference (MD) and its 95% confidence interval (95% CI). E_{max} (maximum response) is presented as standardized mean difference (SMD) and its 95% CI. Studies and totals based on Sprague Dawley rats (SDR), Wistar rats (WR) and mice. * Whole Organ Perfusion Myograph. I^2 represents the amount of heterogeneity. n.a. = not applicable.

Table 5. Summary of qualitative changes in mesenteric artery adaptation to pregnancy.

	Early-gestation		Midgestation		Late-gestation	
	WR	**SDR**	**WR**	**SDR**	**WR**	**SDR**
Vasodilator						
- Gq_{EC}	.	.	.	$=^2$	$=^4$	$\uparrow^3$
- Flow-mediated vasodilation	.	.	$\uparrow^1$	.	$\uparrow^1$	$\uparrow^1$
- Vascular compliance	.	$\uparrow^1$	$=^1$	$\uparrow^1$	$=^1$	$\uparrow^2$
- Gs_{SMC}	.	.	$\uparrow^1$	.	$\downarrow^1$	$\uparrow^4$
Vasoconstrictor						
- Gq_{SMC}	.	.	$=^1$	$=^4$	$=^6$	$\downarrow^{12}$
- Myogenic reactivity	.	$=^1$	$=^1$	$=^1$	$=^2$	$?^3$

Pregnancy-induced vascular function: increase (↑), decrease (↓), no change (=), inconsistent effects (?), no effects reported (.), Superscripted values are number of responses on which effect is based. EC, endothelial cell; SMC, smooth muscle cell

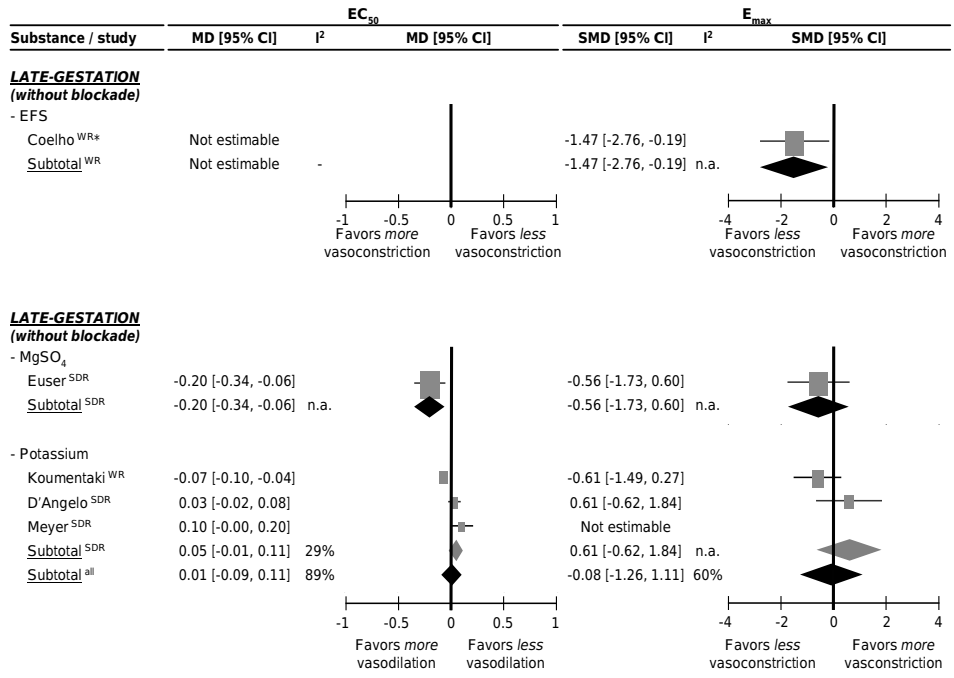


Figure 10. Effect of late pregnancy on mesenteric artery responses to electrical field stimulation (EFS), MgSO4 and potassium.

The effect of pregnancy on EC_{50} (the dose of stimulus inducing 50% response) is depicted as mean difference (MD) and its 95% confidence interval (95% CI). E_{max} (maximum response) is presented as standardized mean difference (SMD) and its 95% CI. Studies and totals based on Sprague Dawley rats (SDR) and Wistar rats (WR). * Whole Organ Perfusion Myograph. I^2 represents the amount of heterogeneity. n.a. = not applicable.

results in mice. Single studies on NO blockade showed that flow-mediated vasodilation was inconsistently NO dependent in WR, and not NO dependent in SDR. In mice, the reduced myogenic reactivity was dependent on NO and independent of PGI_2. The data suggest that pregnancy upregulates flow-mediated vasodilation in both WR and SDR. SDR, but not WR, employ reduced myogenic reactivity and increased compliance.

Robustness of estimates

Sensitivity-analysis was used to assess the robustness of our findings. It showed that the results were not affected by extending the inclusion for studies that did not match the criteria "nulliparity" (n=11) or "age-matching" (n=18) (data not shown).

Overall effect-size estimates, across species and strains, showed considerable heterogeneity (I^2 >60%). For most responses, this could be reduced to an appropriate effect-size (I^2 <60%) only by stratification for stimulus, and/or species, and/or strain. Stratification by strain was applicable only to rats (SDR vs. WR).

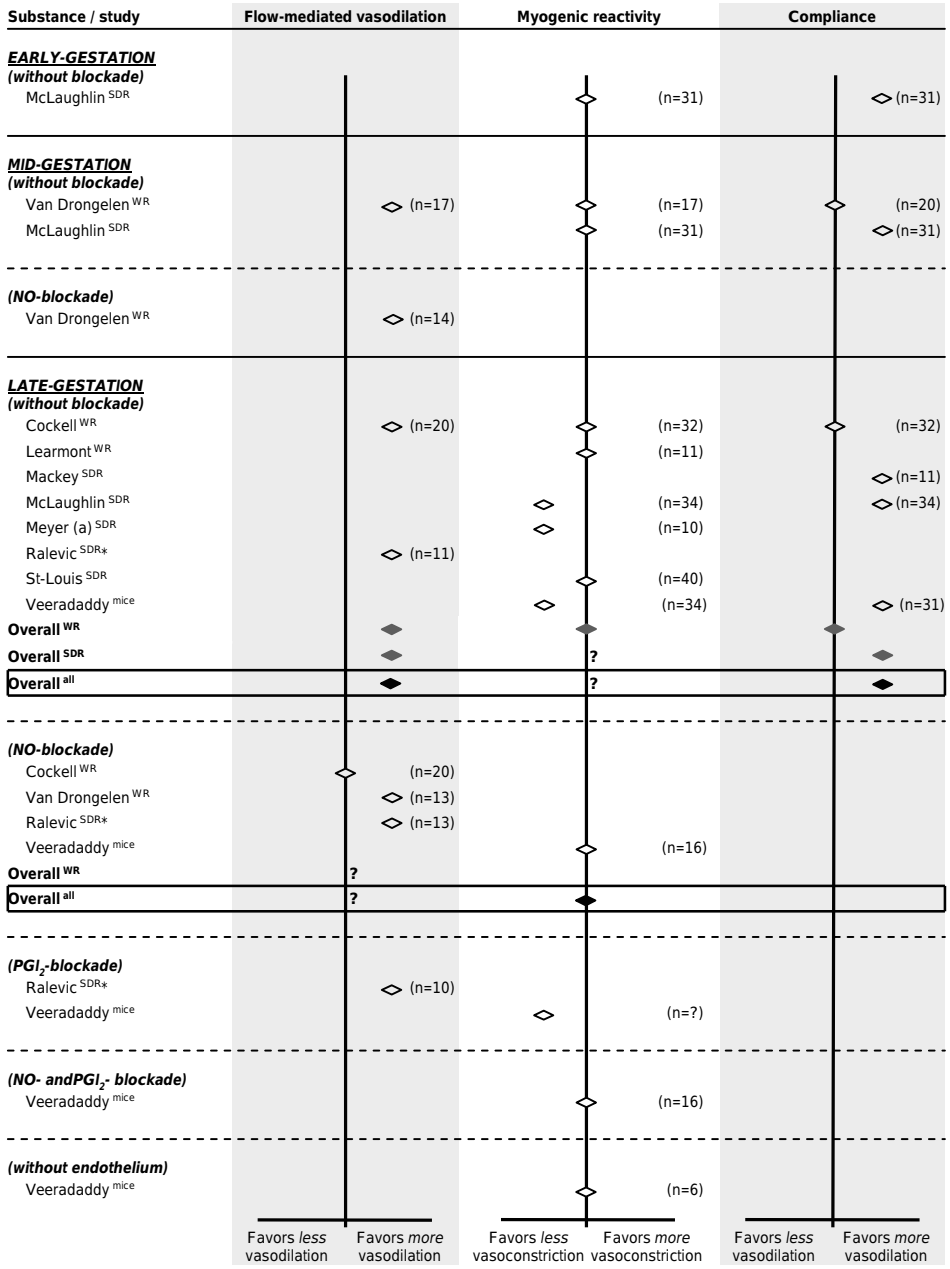


Figure 11. Effect of early, mid and late pregnancy on mesenteric artery responses to flow-mediated vasodilation, myogenic response and vascular compliance.

The effect of pregnancy on the response to stimuli, depicted as the difference in direction. ? = unknown or non-conclusive data. Studies and totals based on Sprague Dawley rats (SDR), Wistar rats (WR) and mice. * Whole Organ Perfusion Myograph. n = number of subjects.

DISCUSSION

Pregnancy has a profound vasodilator effect, yet the mechanisms underlying the altered vessel function in various vascular beds are complex and only partially understood [1;3;20-22].

Pathways involved in pregnancy-induced vasodilation in mesenteric arteries differ between species and even strains (Table 5). Our meta-analysis showed that the considerable heterogeneity for several overall responses could be reduced to acceptable levels by stratification for species and strain. This was most obvious for the differences between SDR and WR. Pregnancy increases flow-mediated vasodilation in both SDR and WR. However, whereas WR do not use any other mechanism for vasodilation, SDR additionally increase arterial compliance, activate Gq_{EC} and Gs_{SMC} receptor-coupled vasodilation pathways, and reduce Gq_{SMC}-coupled receptor-mediated vasoconstriction. The differences between the two strains may reflect their vascular health, as SDR, but not WR, are known to develop severe vascular dysfunction, including chronic progressive nephropathy, peri-/vasculitis, and chronic cardiomyopathy, later in life (internal study by K. Weber, The Researcher, no 28, March 2009, Harlan). Teleologically, one might argue that SDR must employ the additional mechanisms to cope with the vasodilatory demands of pregnancy, whereas flow-mediated vasodilation suffices in WR. If that is the case, WR would be the better model to investigate healthy pregnancy vasodilation, whereas the SDR is the more appropriate model to examine vascular maladaptation.

Vasodilation and the involved mechanisms are gestational age dependent. Data on mechanisms are absent for early pregnancy and limited for midgestation. In mesenteric arteries in midgestation WR, flow-mediated vasodilation is upregulated, whereas data on the other responses are lacking. In SDR, Gq_{EC}- and Gq_{SMC}-coupled pathways and myogenic reactivity are virtually unaffected, while Gs_{SMC}-coupled vasoconstriction is reduced and arterial compliance is increased. In late gestation WR flow-mediated vasodilation remains increased, while the other responses are not affected. In SDR, Gq_{EC}- and Gs_{SMC}-coupled vasodilation, flow-mediated vasodilation, and vascular compliance are increased, whereas the Gq_{SMC}-coupled vasoconstrictor pathway is downregulated. This suggests that, dependent on the strain, additional vascular responses are elicited with advancing gestation.

Pregnancy enhances relaxation by adaptation of the EC (endothelium-dependent pathways) and the SMC itself (endothelium-independent pathways), depending on species/strain. Endothelium-dependent vasodilator responses are studied through flow and Gq_{EC}-related stimuli, which share a common down-stream pathway [12;23]. Our meta-analysis showed that endothelium-mediated vasodilation in pregnancy depends on increased production of NO and PGI_2 by the EC, as proposed by Carbillon et al. [24], whereas SMC sensitivity to these substances remains unaltered. The effect of pregnancy on SMC itself is studied by deactivation of the EC. Under NO, PGI_2 blockade, or endothelium denudation, pregnancy reduces the vasoconstrictor Gq_{SMC}-coupled pathway and activates the vasodilator Gs_{SMC}-coupled pathway, whereas myogenic reactivity is not consistently affected. These data suggest that pregnancy affects the endothelium-dependent pathways and, dependent on the species/strain, also the endothelium-independent pathways.

It is unknown how pregnancy influences the endothelium-dependent and -independent responses. For several vasoactive substances, the prereceptor level is unaffected [24].

Our meta-analysis shows that pregnancy stimulates the Gq_{EC} pathway and blunts the Gq_{SMC} pathway, whereas they share similar downstream pathways. This suggests that, for the substances tested, adaptation takes place at the receptor level and not at the pre- or postreceptor level. However, this does not exclude the possibility that other substances known to be related to pregnancy-induced vasodilation, for example relaxin or progesterone [25;26], might be involved at the prereceptor by acting as a facilitator or inhibitor for the reported pathways.

Our meta-analysis aimed to provide insight in the complexity and heterogeneity across species and strains concerning vascular responses involved in pregnancy-related vasodilation. Several methodological aspects deserve consideration. First, many studies (87%), including six in humans [27-32], did not meet the selection criteria shown in Figure 2. As a result, the meta-analysis is based on responses in rodents [rats (WR and SDR), mice (C57BL/6J), and guinea pigs (Hartley)] only. In addition, several of the results showed considerable heterogeneity that resulted mainly from differences in species and strains. Therefore, the findings should not be extrapolated beyond the limitations of these species/strains. Second, we combined responses to an overall G protein-coupled pathway response, as stimuli that share a common receptor-complex induce comparable effects. However, combining results across stimuli, species and strains, may distort reality, especially when responses are qualitatively different. Therefore, the overall effects should be interpreted with caution. Third, publication bias may have affected overall results across species/ strains. Most notably, SDR may have been overrepresented in the meta-analysis. It was the most commonly used animal, possibly due to profound activation of multiple pathways, but not necessarily the best representative of normal pregnancy in general. SDR is a strain known to have vascular dysfunction and SDR are prone to vascular disease later in life. Our meta-analysis shows that SDR employ additional mechanisms to realize the same degree of vascular adaptation to pregnancy, as compared with WR. Therefore, conclusions based on SDR should not be interpreted as representative of healthy pregnancy in general. Fourth, differences in the degree of precontraction between experiments may have affected flow-mediated vasodilation, Gq_{EC}-mediated vasodilation, and myogenic reactivity. This effect is likely to be small, because the degree of precontraction apparently has little effect on EC_{50} [33]. Fifth, most animal experimental studies included in the analysis did not meet the stringent criteria for methodological quality used in systematic reviews of human trials, as details on randomization, blinding, withdrawals, and dropouts were often lacking. This may have affected our results in an unpredictable manner. We recommend that stringent methodological criteria are implemented in animal-experimental studies, through standardized procedures and report guidelines as previously advocated [34]. Sixth, the overall effects should be interpreted with some caution, as the number of studies per subgroup is limited and overall estimates suffer from heterogeneity, this despite efforts to reduce heterogeneity by using a random effects model, performing subgroup analysis and sensitivity analysis.

In conclusion, our meta-analysis shows that in the mesenteric arterial bed, pregnancy increases flow-mediated vasodilation across the rat strains tested, whereas activation of additional pathways is species, strain, and gestational age specific. Therefore, results on vascular adaptation to pregnancy should not be generalized lightly beyond the limits of the study. Second, late pregnancy mediates vasodilation through changes at the receptor

level, for the substances tested. It remains a challenge to decipher the initial steps of vasodilation in early pregnancy.

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CHAPTER 3

FUNCTIONAL VASCULAR CHANGES OF THE KIDNEY DURING PREGNANCY IN ANIMALS: A SYSTEMATIC REVIEW AND META-ANALYSIS

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Submitted

ABSTRACT

Renal vascular responses to pregnancy have frequently been studied, by investigating renal vascular resistance (RVR), renal flow, glomerular filtration rate (GFR), and renal artery responses to stimuli. Nonetheless, several questions remain: 1. Which vasodilator pathways are activated and to what extent do they affect RVR, renal flow and GFR across species, strains and gestational ages, 2. Are these changes dependent on renal artery adaptation, 3. At which cellular level does pregnancy affect the involved pathways? In an attempt to answer the questions raised, we performed a systematic review and meta-analysis.

We included 37 studies (116 responses). At midgestation, RVR and GFR change to a similar degree across species and strains, accompanied by variable change in renal flow. At least in rats, changes depend on NO activation. At late gestation, changes in RVR, renal flow and GFR vary between species and strains. In rats, these changes are effectuated by sympathetic stimulation. Overall, renal artery responsiveness to stimuli is unaffected by pregnancy, except for Sprague Dawley rats in which pregnancy enhances renal artery vascular compliance and reduces renal artery myogenic reactivity.

Our meta-analysis shows that: 1. Pregnancy changes RVR, renal flow and GFR dependent on NO activation and sympathetic deactivation, but adjustments are different among species, strains and gestational ages; 2. These changes do not depend on adaptation of renal artery responsiveness; 3. It remains unknown at which cellular level pregnancy affects the pathways. Our meta-analysis suggests that renal changes during pregnancy in animals are qualitatively similar, even in comparison to humans, but quantitatively different.

INTRODUCTION

Profound vasodilation takes place in early pregnancy, and results in a major reduction in systemic vascular resistance. Vasodilation seems to be maximal at midgestation and slowly decreases towards term pregnancy in both humans [1] and animals [2]. Several local vascular responses, affecting the endothelium cell (EC), smooth muscle cell (SMC) and extracellular matrix, are thought to be responsible for gestational vasodilation by: 1. Up regulation of the endothelium-dependent nitric oxide (NO) pathway [3], 2. Reduction of the responsiveness to vasoconstrictor stimuli [4], 3. Reduction in myogenic reactivity [5], and 4. Increase in arterial compliance [5]. It remains to be determined to what extent each factor contributes to gestational vasodilation at the various stages of pregnancy among different species and strains.

Many studies on the mechanisms of vasodilation in pregnancy have focused on the kidney and its renal arteries. The kidney is a prominent vascular region and major contributor to systemic vascular resistance in the non-pregnant and pregnant condition [6;7]. Vessel tone has been studied by investigating several local vascular responses. These responses can be divided by the stimulus involved: 1. Mechanical stimuli (flow-mediated vasodilation, myogenic reactivity, arterial compliance), and 2. Pharmacological stimuli, which can be subdivided by: a. The agent used (including acetylcholine, norepinephrine, phenylephrine, etc.) or b. The mediating receptor complex involved [8]. Most studies have concentrated on agents that affect the G-protein coupled receptors. These can be divided into three common G-protein coupled receptor pathways: the vasodilator Gq_{EC}-pathway which mediates NO dependent vasodilation, the vasoconstrictor Gq_{SMC}-pathway which induces a calcium rise in the SMC, and the vasodilator Gs_{SMC}-pathway which induces potassium influx in the SMC [8-15].

Various species, strains and gestational age periods have been investigated and often the results have been extrapolated to pregnancy-induced renal adaptation in general and to responsible mechanisms in humans. One may question the validity of such extrapolations. A previous meta-analysis showed that in mesenteric arteries vascular adaptation to pregnancy is strongly dependent on species, strains and gestational ages [8]. This may also be the case for renal arteries.

To investigate which mechanisms are responsible for renal vascular changes in pregnancy and whether they are dependent on species, strains and gestational age periods, we performed a systematic review and meta-analysis on the functional vascular changes of the kidney during pregnancy. Questions to be answered included: 1. Which specific renal vasodilator pathways are activated in pregnancy and to what extent do they affect RVR, renal flow and GFR across species, strains and gestational ages? 2. Are the pregnancy-induced changes in RVR, renal flow and GFR dependent on renal artery adaptation and which specific vasodilator pathways affect the renal artery during pregnancy? and 3. At which cellular level does pregnancy affect the involved pathways?

MATERIALS AND METHODS

Retrieving the literature

In an attempt to identify all original studies on renal vasoactive responses in animals and humans during first pregnancy, we searched the Pubmed (from 1948) and Embase (from 1980) databases until April 2012. We used a three phase search strategy. Tables 1 and 2 depict the search-strategy for Pubmed and Embase, respectively. No language restriction was used in the primary literature search; later in the process non-English studies were excluded. We also searched the reference lists of included studies to identify additional studies. The search strategy was developed in cooperation with an information specialist from the Medical Library of the Radboud University Nijmegen.

Table 1. Literature search-strategy for Pubmed.

Component	Description
Pregnancy	"pregnancy"[MeSH Terms] OR "pregnancy"[tiab] OR "pregnancies"[tiab] OR "gestation"[tiab] OR "pregnant"[tiab] OR "maternal-fetal relations"[tiab]
Renal circulation	"renal artery" [MeSH] OR "renal artery" [tiab] OR "renal arteries" [tiab] OR "kidney artery" [tiab] OR "kidney arteries" [tiab] OR "renal blood vessel" [tiab] OR "renal blood vessels" [tiab] OR "kidney blood vessel" [tiab] OR "kidney blood vessels" [tiab] OR "renal vessel" [tiab] OR "renal vessels" [tiab] OR "kidney vessel" [tiab] OR "kidney vessels" [tiab] OR "arteria renalis" [tiab] OR "arteria renis" [tiab] OR "kidney/blood supply" [MeSH] OR "renal circulation" [MeSH] OR "renal circulation" [tiab] OR "kidney circulation" [tiab] OR "renal blood circulation" [tiab] OR "kidney blood circulation" [tiab] OR "renal blood supply" [tiab] OR "kidney blood supply" [tiab]
Vasoconstrictor and vasodilator responses	"vasoconstriction"[MeSH Terms] OR "vasoconstriction"[tiab] OR "vasoconstrictions"[tiab] OR "vasoconstrictor agents"[MeSH Terms] OR "vasoconstrictor agents"[Pharmacological Action] OR "vascular resistance"[MeSH Terms] OR "vascular resistance"[tiab] OR "vascular capacitance"[MeSH Terms] OR ("vascular"[tiab] AND "capacitance"[tiab]) OR "vasoconstrictor"[tiab] OR "vasoconstrictors"[tiab] OR "vasopressor"[tiab] OR "vasoactive agonist"[tiab] OR "vasoactive agonists"[tiab] OR "vasopressors"[tiab] OR "vasomotor system"[MeSH Terms] OR "vasomotor system"[tiab] OR "peripheral resistance"[tiab] OR "artery constriction"[tiab] OR "vessel constriction"[tiab] OR "vasoconstrictive"[tiab] OR "vasoconstricting"[tiab] OR "vasoconstricted"[tiab] OR "vasodilation"[MeSH Terms] OR "vasodilation"[tiab] OR "vasodilatation"[tiab] OR "vasodilatating"[tiab] OR "vasodilating"[tiab] OR "vasodilative"[tiab] OR "vasodilatative"[tiab] OR "artery dilation"[tiab] OR "vessel dilation"[tiab] OR "artery dilatation"[tiab] OR "vessel dilatation"[tiab] OR "vasodilator agents"[MeSH Terms] OR "vasodilator agents"[Pharmacological Action] OR "vasodilator"[tiab] OR "vasodilators"[tiab] OR "vasorelaxation"[tiab] OR "Vascular Endothelium Dependent Relaxation"[tiab] OR "Endothelium Dependent Relaxation"[tiab] OR "Vascular Endothelium-Dependent Relaxation"[tiab] OR "Endothelium-Dependent-Relaxation"[tiab] OR "hemodynamics"[MeSH Terms] OR "hemodynamics"[tiab]OR "hemodynamic"[tiab] OR "vasodilated"[tiab] OR "vasoactive agent"[tiab] OR "vasoactive drug"[tiab] OR "vasoactive drugs"[tiab] OR "dilation"[tiab] OR "dilatation"[tiab] OR "contraction"[tiab] OR "relaxation"[tiab]

Table 2. Literature search-strategy for Embase.

Component	Description
Pregnancy	exp pregnancy/ OR (pregnancy or pregnant).ti,ab. OR (pregnacies or gestation).ti,ab. OR (pregnancy or pregnant or pregnacies or gestation).ti,ab.
Renal circulation	exp renal artery/ OR renal artery.ti,ab. OR renal arteries.ti,ab. OR arteria renalis.ti,ab. OR exp kidney artery/ OR kidney artery.ti,ab. OR kidney arteries.ti,ab. OR arteria renis.ti,ab. OR kidney blood vessel/ OR renal blood vessel.ti,ab. OR renal blood vessels.ti,ab. OR kidney blood vessel.ti,ab. OR kidney blood vessels.ti,ab. OR intrarenal vessel.ti,ab. OR intrarenal vessels.ti,ab. OR renal vessel.ti,ab. OR renal vessels.ti,ab. OR kidney vessel.ti,ab. OR kidney vessels.ti,ab. OR exp kidney blood flow/ OR kidney blood flow.ti,ab. OR intrarenal blood flow.ti,ab. OR kidney bloodflow.ti,ab. OR renal blood flow.ti,ab. OR kidney blood supply.ti,ab. OR renal blood supply.ti,ab. OR exp kidney circulation/ OR kidney circulation.ti,ab. OR intrarenal circulation.ti,ab. OR kidney blood circulation.ti,ab. OR renal circulation.ti,ab. OR renal blood circulation.ti,ab.
Vasoconstrictor and vasodilator responses	exp vasoconstriction/ OR exp vasoconstrictor agent/ OR vasoconstrict*.ti,ab. OR exp vascular resistance/ OR blood flow resistance.ti,ab. OR blood vessel resistance.ti,ab. OR (peripheral adj1 resistance).ti,ab. OR vascular vessel resistance.ti,ab. OR artery resistance.ti,ab. OR exp hemodynamics/ OR exp blood vessel capacitance/ OR blood vessel capacitance.ti,ab. OR vascular capacitance.ti,ab. OR (hemodynamic or hemodynamics).ti,ab. OR (haemodynamic or haemodynamics).ti,ab. OR vasopressor.ti,ab. OR exp vasoactive agent/ OR (vasoactive agonist OR vasoactive agonists or vasoactive agent or vasoactive agents or vasoactive drug or vasoactive drugs).ti,ab. OR (vessel contriction or vessel contrictions or artery contriction or artery constrictions).ti,ab. OR exp vasodilatation/ OR (vasodilatation or vasodilation or vasodilator or vasodilatator or vasodilating or vasodilative or vasodilatative).ti,ab. OR (vaso dilatation or vaso dilation or vaso dilator or vaso dilatator or vaso dilating or vaso dilative or vaso dilatative).ti,ab. OR vaso relaxation.ti,ab. OR vascular endothelium dependent relaxation.ti,ab. OR vascular endothelium-dependent relaxation.ti,ab. OR endothelium dependent relaxation.ti,ab. OR endothelium-dependent relaxation.ti,ab. OR (vessel dilatation* or vessel dilation* or vascular dilation* or vascular dilatation* or vasorelaxation* or artery dilatation* or artery dilation*).ti,ab. OR (dilatation* or dilation* or relaxation* or contraction*).ti,ab.

Selection of studies and data extraction

The selection and data extraction process was divided into three phases (Figure 2). In the first phase, a. one investigator (first author) selected studies on the basis of title and abstract, and b. two investigators (first and second author) independently screened all remaining abstracts for inclusion criteria. Differences were resolved by mutual agreement. Studies were selected if they met the following criteria: 1. Renal vasoconstrictor/vasodilator responses, 2. First pregnancy versus nulliparous non-pregnant control, 3. Original data, 4.) Healthy animals or humans, 5. Age- or weight-matched subjects, and 6. For *ex vivo* experiments: experiments performed in standard medium (defined as a generally accepted medium for the type of experiments). In the second phase of selection, non-English papers were excluded from further analysis and full articles were analyzed by two investigators (first and second author) independently using the same inclusion criteria as described above. Differences were resolved by mutual agreement. In the third phase, the first author extracted baseline

measurements and responses to specific blockade from *in vivo* and whole organ perfusion experiments that related to renal function changes and the involved pathways. In addition, all vasodilator and vasoconstrictor responses were extracted both in the absence and presence of blockade of NO, prostaglandin (PGI_2), renin-angiotensin-aldosterone (RAAS), endothelin, vasopressin or the sympathetic system, or combinations of blockade, with and without denuded endothelium. Responses had to contain ≥ 5 measurements to be included in further analysis, as analysis of sensitivity to stimuli is not accurate with fewer data points.

From each article, study data were extracted and recorded for species, strain, and gestational age (early, mid- and late pregnancy, defined as first, second and third trimester, respectively), estrous/cycle stage, and experimental setup (*in vivo* /whole organ perfusion, myograph type used). We extracted all reported vascular responses and recorded for each of them the number of subjects, effect size, and standard deviation or standard error of the mean. For *in vivo* and whole organ perfusion experiments we detected baseline renal vascular resistance (RVR), renal flow (combined renal plasma, blood and perfusion flow), glomerular filtration rate (GFR), autoregulatory threshold (lower pressure limit for stable renal flow), and the response to specific blockades. For pharmacological responses, we extracted EC_{50} (concentration inducing 50% of the maximum effect) and E_{max} (maximum response to stimulus) to indicate sensitivity and maximum reactivity, respectively. For the mechanical responses, we determined the direction of the effect (favoring vasodilation, vasoconstriction, or no effect) and used it for descriptive analysis. Parametric comparison of these responses was not possible, as analyses (mostly ANOVA statistics) did not allow calculating overall effect sizes and effect measures were reported in variable ways (for myogenic reactivity and arterial compliance (absolute values, percentages of diameter changes, myogenic tone in relation to passive tone)). In case of missing, incomplete or indeterminate data, we approached the authors by email (response rate 33%).

Quality assessment

The methodological quality of the articles was assessed independently by two reviewers (first and second author). Data were scored on presence or absence of randomization of allocation to groups, blinding of outcome assessment, clearness on nulliparity at entry of study, age or weight of subjects described, and number of animals accounted for at the start and the end of the study. The quality of the responses reported was ranked for the clarity of the number of animals used for analyses, presence of a response graph containing ≥5 measurements, and achievement of E_{max} (defined as the presence of at least two measurements without any further increasing effect). The response quality was expressed as percentage of the number of responses that complied with these items divided by the total number of responses described in the study. It should be noted that the quality assessment determines the quality of the methodology required for comparison of responses to the predefined items and that it should not be interpreted as judgment of the value of the experiment per se.

Quantitative data synthesis and statistical analysis

Extracted data in RVR, renal flow and GFR at baseline, changes induced by specific blockades, and EC_{50} and E_{max} for pharmacological stimuli were analyzed for each

gestational age period, stratified by species and strain, and displayed in forest plots, using Review Manager 5 (The Nordic Cochrane Centre, The Cochrane Collaboration, 2008). Pharmacological stimuli were analyzed by the type of G-protein-coupled pathway (Gq_{EC}, Gq_{SMC} and Gs_{SMC}) involved or by type of stimulus (NO-donors and potassium), when applicable. Combined effect sizes were calculated as weighed mean difference (MD) for RVR, renal flow, GFR and EC_{50}, and as standardized mean difference (SMD) for E_{max}. Both MD and SMD represent the effect sizes in comparison to control values within the same study. In the forest plots data were presented as mean effect sizes with 95% confidence intervals (CI), as provided by Review Manager 5. Combined effect sizes were calculated by using the random effects model (Review Manager 5). P-values <0.05 were considered to represent statistical significance. In the random effects model some heterogeneity beyond sampling errors is allowed to account for anticipated heterogeneity. Heterogeneity was presented as I^2; I^2 <60% represents moderate heterogeneity, I^2 >60% represents considerable to substantial heterogeneity [16]. Publication bias was assessed by linear regression analysis as proposed by Egger et al. (for ≥10 studies) [17] or subjective assessment of funnel plot asymmetry (for ≤10 studies).

For mechanical stimuli, we used the direction of the measured effects for qualitative analysis, and determined the overall weighed direction for each mechanical response, based on the total number of animals used.

We performed sensitivity analysis to assess the robustness of our findings, as is recommended for systematic reviews [16]. This type of analysis shows the influence of the used inclusion and exclusion criteria on the results. In the analysis we repeated all analyses in the presence of the studies that were excluded on the basis of the criterion first pregnancy versus nulliparous non-pregnant control.

References used for analysis included the following: [18-54]

RESULTS

Our systematic literature search identified 1008 studies concerning the effects of pregnancy on vascular changes of the kidney (Figure 1). Based on title and abstract 917 studies were excluded, leaving 91 papers for full article evaluation. Thirty-seven studies did not meet the inclusion criteria. Seventeen studies were excluded based on their non-English language. Finally, 37 studies with 116 vascular responses met the inclusion criteria for meta-analysis.

The study characteristics are presented in Table 3. Most studies (n=28) used rats (Long Evans rats (LER), Sprague Dawley rats (SDR), various strains of Wistar rats (Wistar Kyoto (WKY), Munich Wistar (MWR) and Wistar Hannover (WHR)); other species were used less frequently (sheep=6, rabbits=2 and guinea pigs (GP)=1). Most studies contained *in vivo* experiments (n=25), while whole organ (perfusion), perfusion myograph and wire myograph experiments were less common (n=5, 3 and 4 respectively). Methodological limitations were present in a substantial portion of the included studies (Table 4): 43% (16/37) did not present the number of animals used, 32% (12/37) did not present parity, 97% (36/37) did not report having performed randomization, and 0% reported blinding of the outcome assessment.

Table 3. Characteristics of included studies, arranged by author and year.

Study	Species (strain)	Estrous stage control group	Gestation period	Experiment setup	Number of described baseline measurements	Number of described responses
Annibale et al., 1989	sheep	-	late	WM	n.a.	4
Baylis et al., 1986	rat (MWR)	-	late	IV	4	10
Baylis et al., 1988	rat (MWR)	-	mid	IV	3	0
Baylis et al., 1993	rat (SDR and MWR)	-	mid/late	IV	4	4
Baylis et al., 1995	rat (SDR)#	-	mid/late	IV	2	2
Bobadilla et al., 2001	rat (WR)	-	?	WOP	1	4
Bobadilla et al., 2003	rat (WR)	-	mid/late	WOP	2	16
Bobadilla et al., 2005	rat (WR)	-	late	WOP	1	2
Cha et al., 1993	sheep	-	late	IV	1	0
Cha et al., 1993	sheep	-	late	IV	1	0
Chu et al., 1997	rat (WKY)	-	late	WOP	0	0
Conrad et al., 1989	rat (LE)	-	late	IV	1	1
Conrad et al., 1999	rat (LE)	-	mid	IV	1	2
Danielson et al., 1995	rat (LE)	-	mid	IV	1	3
Danielson et al., 1996	rat (LE)	-	mid	IV	1	2
Fan et al., 1996	sheep	-	late	IV	1	0
Ferris et al., 1983	rabbit	-	late	IV	2	0
Gandley et al., 1997	rat (SDR)	-	late	PM	n.a.	4
Gandley et al., 2001	rat (LE)	-	mid	PM	n.a.	9
Greenberg et al., 1999	sheep	-	late	IV	1	0
Griggs et al., 1993	rat (SDR)	-	late	PM	n.a.	6
Hines et al., 1992	rat (SDR)	-	mid/late	IV	2	4
Hines et al., 2000	rat (SDR)	-	late	IV	1	1
Kim et al., 1994	guinea pig	-	Late	WM	n.a.	12
Knight et al., 2007	rat (SDR)	-	late	IV	0	0
Masilamani et al., 1994	rat (SDR)	-	mid/late	IV	1	0
McElvy et al., 2001	sheep	-	late	IV	1	0
Novak et al., 1997	rat (SDR)	-	mid	IV	1	0
Omer et al., 1999	rat (SDR)	-	late	IV	1	1
Patel et al., 1993	rat (SDR)	-	late	IV	2	0
Reckelhoff et al., 1992	rat (WR)	-	mid	IV	2	2
Sen et al., 1997	rat (?)	-	mid/late	IV	2	0
Sicinska et al., 1971	rat (SDR)	-	late	IV	1	0
Van Drongelen et al., 2011	rat (WR)	-	mid	WOP	1	1
Van Eijndhoven et al., 2003	rat (WR)	-	mid	WM	n.a.	1
Van Eijndhoven et al., 2007	rat (?)	-	mid	WM	n.a.	2
Woods et al., 1987	rabbit	-	late	IV	1	1

WM = wire myograph, PM = pressure myograph, PPM = pressure-perfusion myograph, WOP = whole organ perfusion, IV = *in vivo*. n.a.= not applicable. # data received by email.

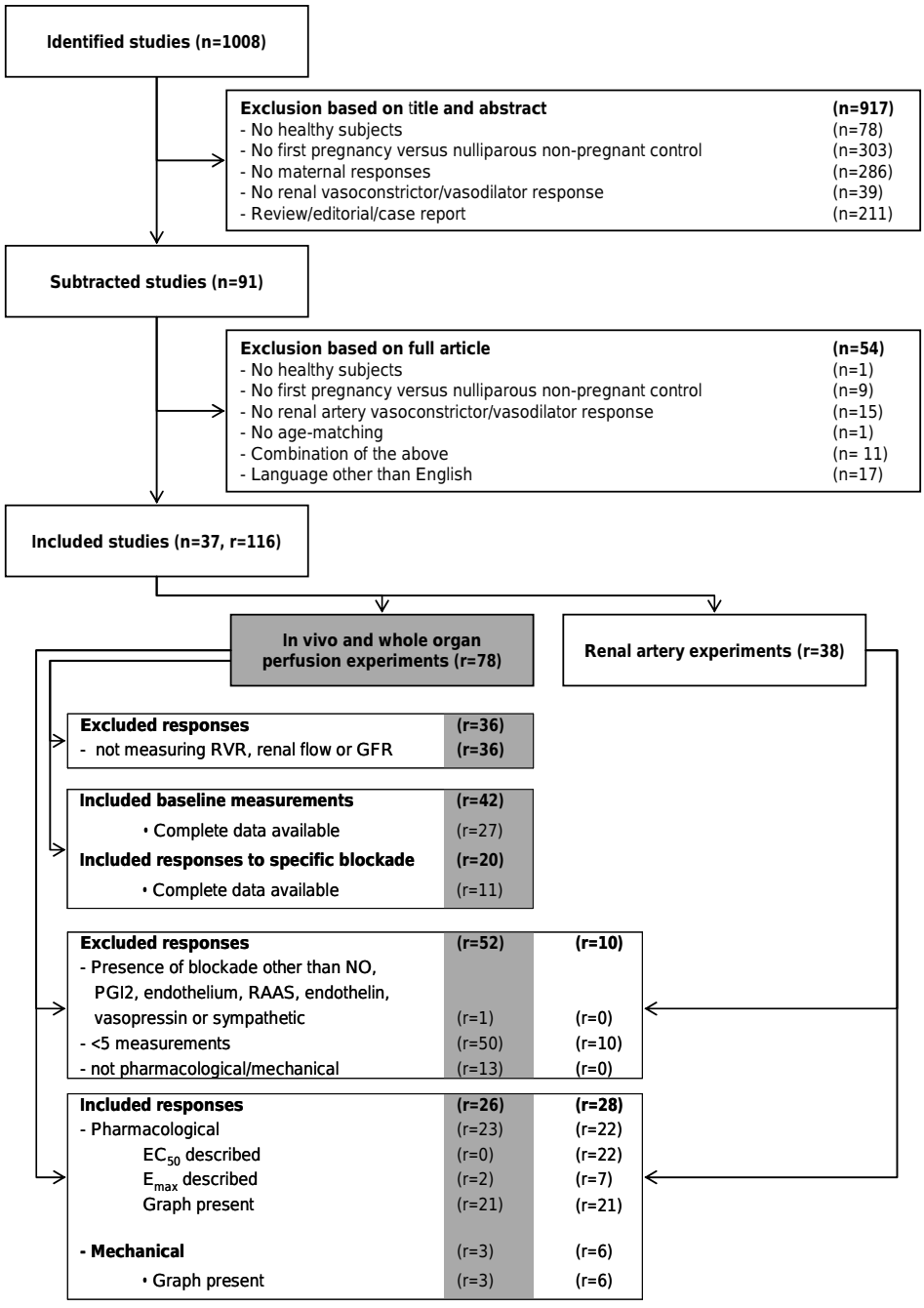

Figure 1. Flow chart for selection, inclusion and exclusion of studies and responses on renal vascular adaptation to pregnancy. n= number of studies; r= number of responses (gray filling for *in vivo* and whole organ perfusion experiments, dotted filling for renal artery responses).

Table 4. Quality assessment of the included studies and subsequent responses.

	Study quality						Response quality			
Study	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**	**Score**	**(6)**	**(7)**	**(8)**	**Score**
Annibale et al., 1989	-	-	?	-	+	1	1.0	1.0	1.0	3.0
Baylis et al., 1986	-	-	+	+	+	3	1.0	0.0	0.0	1.0
Baylis et al., 1988	-	-	+	+	-	2	1.0	0.0	0.0	1.0
Baylis et al., 1993	-	-	+	-	+	2	1.0[#]	0.0	0.0	1.0[#]
Baylis et al., 1995	-	-	+	+	-	2	1.0	0.0	0.0	1.0
Bobadilla et al., 2001	-	-	+	+	-	2	1.0	1.0	0.0	2.0
Bobadilla et al., 2003	-	-	+	+	+	3	1.0	1.0	0.0	2.0
Bobadilla et al., 2005	-	-	+	+	-	2	1.0	1.0	0.5	2.5
Cha et al., 1993[a]	-	-	?	+	+	2	0.0	0.0	0.0	0.0
Cha et al., 1993[b]	-	-	?	-	+	1	0.0	1.0	1.0	2.0
Chu et al., 1997	-	-	?	+	-	1	1.0	0.0	0.0	1.0
Conrad et al., 1989	n.a.	-	+	+	+	3	1.0	0.0	0.0	1.0
Conrad et al., 1999	-	-	+	+	-	2	0.0	0.0	0.0	0.0
Danielson et al., 1995	-	-	+	+	+	3	0.0	0.0	0.0	0.0
Danielson et al., 1996	+	-	+	+	-	3	1.0	0.0	0.0	1.0
Fan et al., 1996	-	-	?	-	+	1	1.0	0.0	0.0	1.0
Ferris et al., 1983	-	-	?	-	+	1	1.0	0.0	0.0	1.0
Gandley et al., 1997	-	-	+	+	-	2	1.0	1.0	0.75	2.75
Gandley et al., 2001	-	-	+	-	-	1	0.9	0.2	0.2	1.3
Greenberg et al., 1999	-	-	?	+	+	2	0.0	0.0	0.0	0.0
Griggs et al., 1993	-	-	+	+	-	2	1.0	0.16	0.33	1.49
Hines et al., 1992	-	-	+	-	-	1	1.0	0.0	0.0	1.0
Hines et al., 2000	-	-	+	-	+	2	0.33	0.66	0.0	1.0
Kim et al., 1994	-	-	?	-	-	0	1.0	1.0	1.0	3.0
Knight et al., 2007	-	-	+	+	+	3	1.0	0.0	0.0	1.0
Masilamani et al., 1994	-	-	+	+	+	3	1.0	0.0	0.0	1.0
Masilamani et al., 1994	-	-	+	+	+	3	1.0	0.0	0.0	1.0
McElvy et al., 2001	-	-	?	+	+	2	1.0	1.0	0.0	2.0
Novak et al., 1997	-	-	?	-	-	0	0.0	0.0	0.0	0.0
Omer et al., 1999	-	-	+	+	-	2	0.0	0.0	0.0	0.0
Patel et al., 1993	-	-	+	-	+	2	1.0	0.0	0.0	1.0
Reckelhoff et al., 1992	-	-	+	+	-	2	1.0	1.0	1.0	3.0
Sen et al., 1997	-	-	?	-	-	0	1.0	0.0	0.0	1.0
Sicinska et al., 1971	-	-	?	+	+	2	0.0	0.0	0.0	0.0
Van Drongelen et al., 2011	-	-	+	+	+	3	1.0	0.0	1.0	2.0
Van Eijndhoven et al., 2003	-	-	+	+	+	3	0.0	0.0	0.0	0.0
Van Eijndhoven et al., 2007	-	-	+	+	+	3	0.5	0.0	0.0	0.5
Woods et al., 1987	-	-	+	-	+	2	1.0	1.0	1.0	3.0

(1) randomization, (2) blinding of outcome assessor, (3) virgin/nulliparous at entry study, (4) age or weight of animals described, (5) number of used animals clear from methods, (6) fraction of responses with clear number of animals used for statistical analyses (as a percentage of the number of described responses), (7) fraction of responses with dose-response curve containing ≥5 measurements, (8) fraction of responses with accomplished E_{max}. n.a.= not applicable. [#] data received by email.

The 116 subtracted vascular responses could be divided into two categories: 1. *In vivo* and whole organ perfusion studies measuring changes in RVR, renal flow and/or GFR, and 2. Renal artery experiments measuring changes in vascular tone. Experiments were performed mainly in mid- (34%, 40/116) and late (63%, 73/116) gestation; for four responses the gestation period was not defined. Early gestation was not investigated by any of the included studies.

We identified 78 *in vivo* and whole organ perfusion experiments. Two types of experiments could be distinguished based on their objective to determine the effects of pregnancy on: 1) RVR, renal flow and GFR, or 2) the response of the kidney to vaso-active substances. For the first type of experiments we extracted 42 baseline measurements and 20 responses to specific blockades. Thirty-six responses were excluded as they did not measure RVR, renal flow or GFR. After exclusion of incomplete data, 27 baseline measurements and 11 responses to specific blockades were suitable for meta-analysis. For the second type of experiments we identified 23 pharmacological and 3 mechanical responses. For pharmacological stimuli, EC_{50} was reported in 0% of the responses, E_{max} was described in 9% and a graph was present in 91%. For mechanical stimuli, all responses were presented graphically without quantified effects.

From the 38 renal artery responses, 28 met the criteria for inclusion. They consisted of 22 pharmacological stimuli (EC_{50}, E_{max} and a graph present in 100%, 32% and 95% of the cases, respectively) and six mechanical stimuli, all with a graph without quantified effects.

In vivo and whole organ perfusion experiments

The influence of mid-pregnancy on kidney function is presented in Figure 2. In mid-pregnancy, *overall* (across species and strains) RVR decreases by 27%, varying from 18% in SDR to 31% in MWR. Renal flow is enhanced to a variable degree, ranging from 13% in WHR to 31% in LER. GFR increases by 17% in SDR to 29% in LER. In LER, blockade of the NO system completely normalizes the pregnancy-induced change in RVR and GFR, while pregnancy-induced increase in renal flow is reduced from 31% to 15%. In WR, NO blockade normalizes renal flow completely. Sympathetic or vasopressin blockade has only been studied in SDR and does not affect the mid-pregnancy-induced changes in RVR, renal flow and GFR. It appears that mid-pregnancy changes in RVR, renal flow and GFR are dependent on up regulation of the NO system and not on sympathetic tone or vasopressin.

The magnitude of pregnancy-induced renal changes in rats decreases from mid-pregnancy towards term. Figures 3 and 4 show renal function in late gestation compared to non-pregnant controls. Late pregnancy affects RVR, renal flow and GFR differently among species and strains. In LER and MWR, pregnancy does not affect any of the renal parameters. Blockade of the renin-angiotensin-aldosteron system (RAAS) did not show any effect in LER, but in MWR reduced RVR by 26% and increased renal flow by 26% without changing in GFR. In SDR, pregnancy decreases RVR by 19% and increases both renal flow and GFR, by 17% and 21% respectively. NO blockade in this strain returns renal flow to non-pregnant values with a persistently increased GFR. Under sympathetic blockade RVR, renal flow and GFR return to non-pregnant values in these rats, while

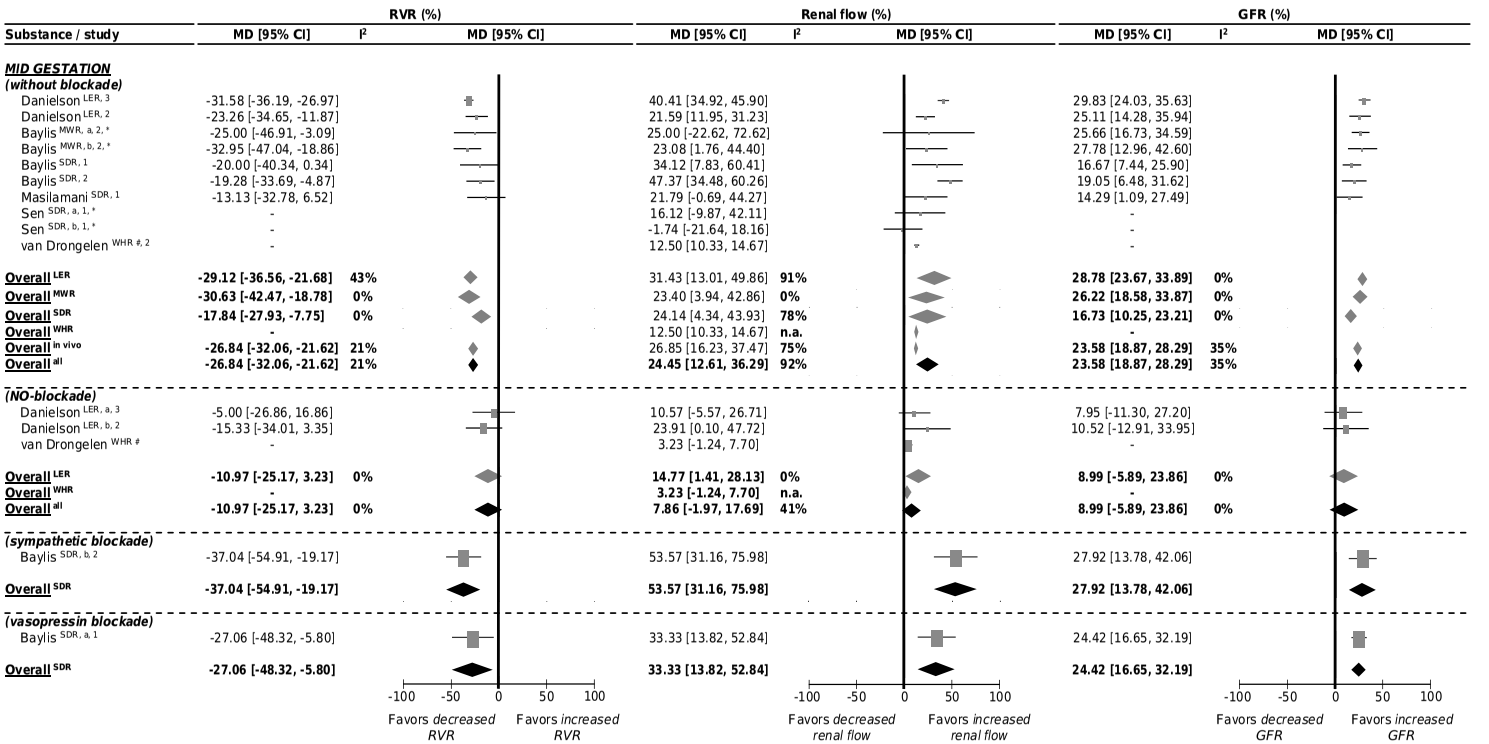

Figure 2. Effect of pregnancy at mid gestation on in vivo and whole organ perfusion# renal vascular resistance (RVR), renal flow and glomerular filtration rate (GFR) in absence and presence of NO and sympathetic blockade.

The effect of pregnancy on RVR, renal flow (effects of renal plasma/blood/perfusion flow (RPF/RBF/RPPF) combined) and GFR is presented as percentage mean difference (MD) and its 95% CI. Studies and totals based on Long Evans rats (LER), Munich Wistar rats (MWR), Sprague Dawley rats (SDR) and Wistar Hannover rats (WHR). 1, 2, 3 represents first, second and third part of mid gestation. # Whole organ perfusion experiments, excluded in the "Overall in vivo" analysis. * Experiments performed under anesthesia. I^2 represents the amount of heterogeneity. n.a. = not applicable.

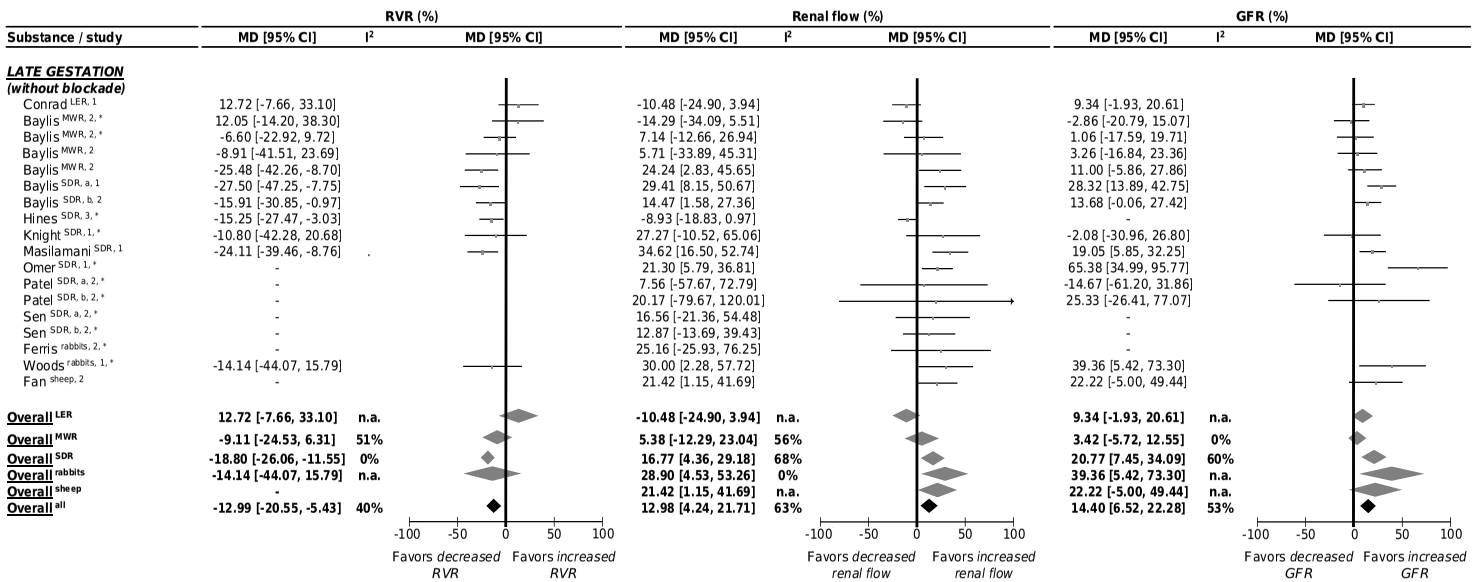

Figure 3. Effect of pregnancy at late gestation on in vivo renal vascular resistance (RVR), renal flow and glomerular filtration rate (GFR) in absence of blockade.

The effect of pregnancy on RVR, renal flow (effects of renal plasma/blood/perfusion flow (RPF/RBF/RPPF) combined) and GFR is presented as percentage mean difference (MD) and its 95% CI. Studies and totals based on Long Evans rats (LER), Munich Wistar rats (MWR), Sprague Dawley rats (SDR), rabbits and sheep. 1, 2, 3 represents first, second and third part of late gestation. * Experiments performed under anesthesia. I^2 represents the amount of heterogeneity. n.a. = not applicable.

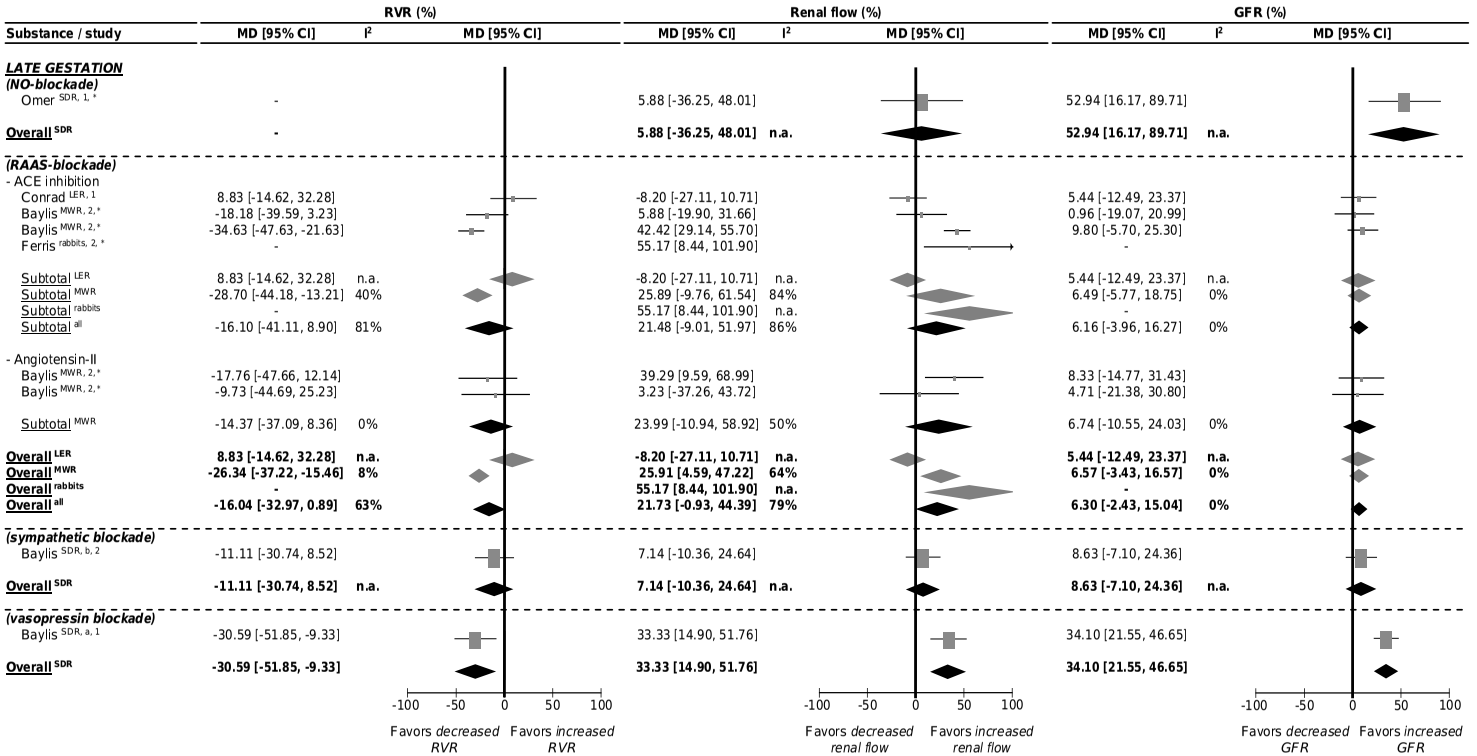

Figure 4. Effect of pregnancy at late gestation on in vivo renal vascular resistance (RVR), renal flow and glomerular filtration rate (GFR) in presence of NO, sympathetic and RAAS blockade.

The effect of pregnancy on RVR, renal flow (effects of renal plasma/blood/perfusion flow (RPF/RBF/RPPF) combined) and GFR is presented as percentage mean difference (MD) and its 95% CI. Studies and totals based on Long Evans rats (LER), Munich Wistar rats (MWR), Sprague Dawley rats (SDR) and rabbits. 1, 2, 3 represents first, second and third part of late gestation. * Experiments performed under anesthesia. I^2 represents the amount of heterogeneity. n.a. = not applicable.

vasopressin blockade has no effect on any of the parameters. In rabbits, late pregnancy does not significantly affect RVR, while renal flow and GFR are increased by 29% and 39% compared to non-pregnant values. These changes are not affected by RAAS-blockade. For late pregnant sheep, no data are available on RVR, but renal flow is 21% higher than in non-pregnant sheep without a significant difference in GFR. In short, there is no consistent pattern of renal changes in late pregnancy across species and strains.

In both mid- and late pregnancy, the overall RVR, renal flow and GFR shows moderate to considerable heterogeneity. This cannot be attributed completely to species or strain specific effects, as stratification by species and/or strain does not reduce heterogeneity to a minimum in all groups. Heterogeneity depends mainly on single responses with very small confidence intervals and responses with an estimated effect opposite to other studies. We could not identify any methodological issue that might explain these effects.

The influence of pregnancy on the autoregulatory threshold was analyzed qualitatively only, as quantitative effect measures of the lower pressure boundary for stable renal flow were not reported in the two relevant studies, which used WR and rabbits [21;54]. From these studies it appears that pregnancy does not affect the renal autoregulatory threshold in either mid- or late pregnancy.

Renal artery experiments

The renal artery changes in response to vasodilator Gq_{EC}-coupled pharmacological stimuli in pregnancy are presented in Figure 5. Our search only detected responses during late gestation, and mainly in guinea pigs. In this species, pregnancy does not affect Gq_{EC}-coupled EC_{50} to acetylcholine and EC_{50} is unaffected by NO and PGI_2 blockade.

The effects of mid- and late pregnancy on the vasoconstrictor Gq_{SMC}-coupled pathway in several species are shown in Figure 6; no data are available on early gestation. In both mid- and late gestation, the Gq_{SMC}-coupled EC_{50} and E_{max} of phenylephrine and U46619 (thromboxane agonist) are unaffected by pregnancy for all species investigated. In guinea pigs under NO and PGI_2 blockade, pregnancy does not affect the response to U46619. In SDR in the absence of endothelium pregnancy increases EC_{50} in response to phenylephrine, which suggests that the pregnancy effect on the SMC is overruled by the endothelium. Apparently, pregnancy does not seem to affect the renal artery responsiveness to Gq_{SMC}-coupled vasoconstrictor stimuli.

The effect of pregnancy on the renal artery responses to NO and potassium are depicted in Figure 7. For NO, only single studies in rats and sheep were available. In mid- and late gestation rats and late pregnant sheep pregnancy does not modify the response to NO or potassium. This suggests that in general pregnancy does not affect the properties of the renal SMC to NO and potassium.

The effect of pregnancy on the renal artery responses to mechanical stimuli was assessed qualitatively only, as shown in Figure 8. We did not find studies that investigated flow-mediated vasodilation of the renal artery during pregnancy, and only two studies that looked at myogenic reactivity and vascular compliance in late gestation. In SDR, late pregnancy reduces myogenic reactivity in an endothelium-dependent manner and enhances vascular compliance. In sheep, late pregnancy does not affect either myogenic

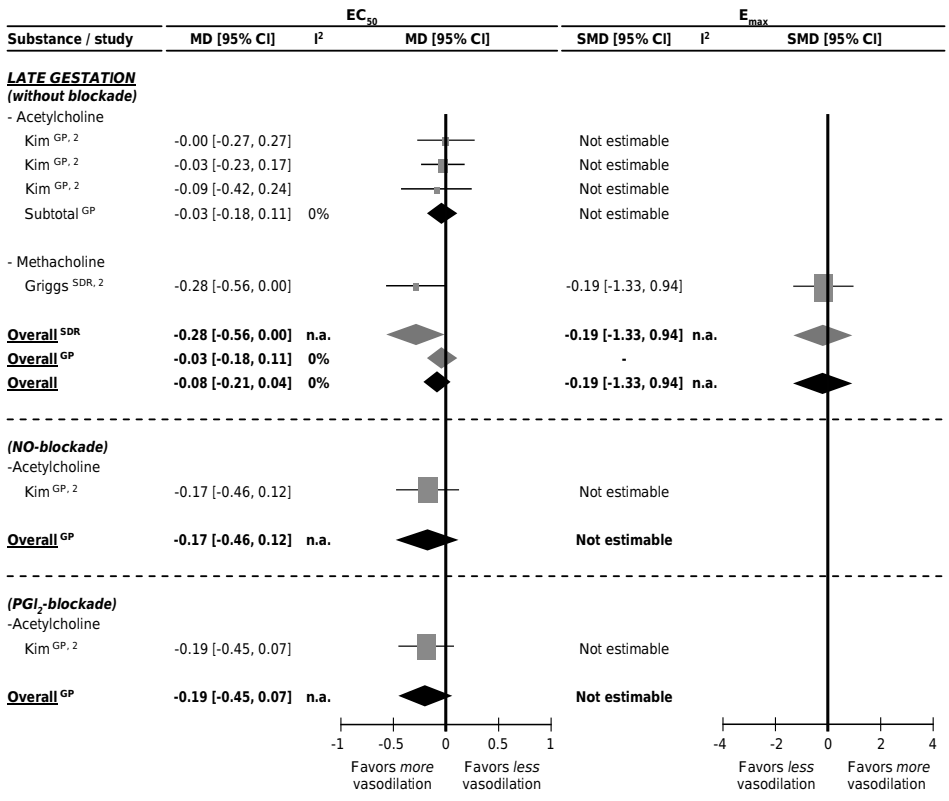

Figure 5. Effect of pregnancy at late gestation on renal artery responses to stimuli involved in vasodilation through the Gq_{EC}-coupled pathway in the presence and absence of blockade.

The effect of pregnancy on EC_{50} (the dose of stimulus inducing 50% response) is depicted as mean difference (MD) and its 95% confidence interval (95% CI). E_{max} (maximum response) is presented as standardized mean difference (SMD) and its 95% CI. Studies and totals based on Sprague Dawley rats (SDR) and guinea pigs (GP). 1, 2, 3 represents first, second and third part of late gestation. I^2 represents the amount of heterogeneity. n.a. = not applicable.

reactivity or vascular compliance. Apparently, pregnancy-induced changes in myogenic reactivity and compliance depend on the species investigated.

Publication bias and sensitivity-analysis

Publication bias was assessed by subjective determination of funnel plot asymmetry, as all analyses consisted of less than 10 studies. We did not detect evidence suggestive for publication bias.

Sensitivity-analysis was performed to assess the influence of inclusion criteria on the results. We extended the inclusion for studies that did not match the criterium "nulliparity" (n=3). The results were not affected by addition of these studies (data not shown). We did not extend the analysis for age-matching, because we had not excluded any study for that reason.

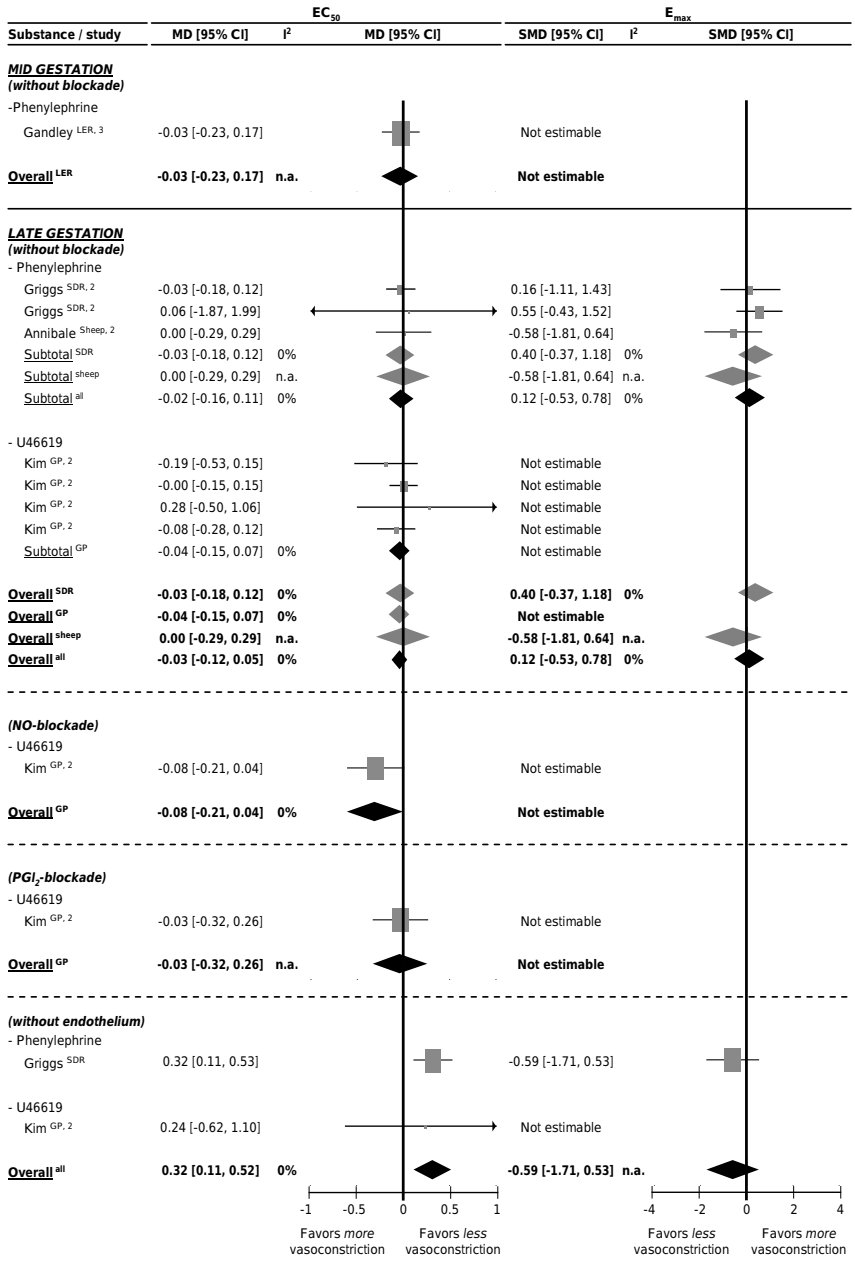

Figure 6. Effect of pregnancy at mid and late gestation on renal artery responses to stimuli involved in vasoconstriction through the Gq_{SMC}- coupled pathway in presence and absence of blockade.

The effect of pregnancy on EC_{50} (the dose of stimulus inducing 50% response) is depicted as mean difference (MD) and its 95% confidence interval (95% CI). E_{max} (maximum response) is presented as standardized mean difference (SMD) and its 95% CI. Studies and totals based on Long Evans rats (LER), Sprague Dawley rats (SDR), sheep and guinea pigs (GP). 1, 2, 3 represents first, second and third part of mid and late gestation. I^2 represents the amount of heterogeneity. n.a. = not applicable.

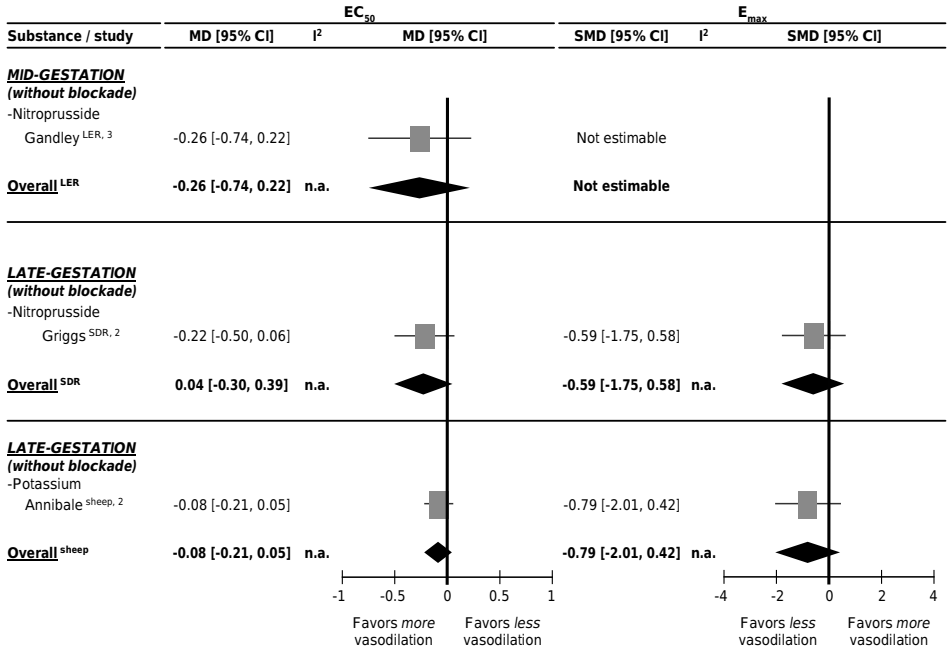

Figure 7. Effect of mid and late pregnancy on renal artery responses to nitric oxide (NO) and potassium.

The effect of pregnancy on EC_{50} (the dose of stimulus inducing 50% response) is depicted as mean difference (MD) and its 95% confidence interval (95% CI). E_{max} (maximum response) is presented as standardized mean difference (SMD) and its 95% CI. Studies and totals based on Long Evans rats (LER), Sprague Dawley rats (SDR) and sheep. 1, 2, 3 represents first, second and third part of mid and late gestation. I^2 represents the amount of heterogeneity. n.a. = not applicable.

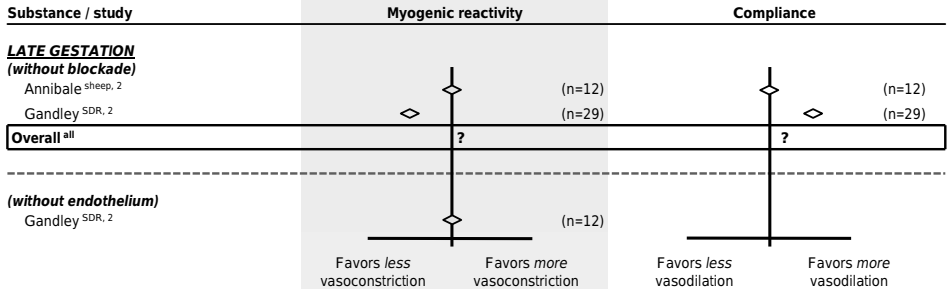

Figure 8. Effect of pregnancy at late gestation on renal artery responses to myogenic response and vascular compliance.

The effect of pregnancy on the response to stimuli, depicted as the difference in direction. ? = unknown or non-conclusive data. Studies and totals based on Sprague Dawley rats (SDR) and sheep. 1, 2, 3 represents first, second and third part of late gestation. n = number of subjects.

DISCUSSION

Our meta-analysis confirms the commonly held view that pregnancy reduces RVR and enhances renal flow and GFR in animals, as it does in humans [1]. Our study has shown that the degree of change depends on the functional parameter, species, strain and gestational age investigated. Our meta-analysis did not detect changes in renal artery responsiveness. More importantly, the underlying mechanisms for the change in RVR, renal flow and GFR is not the same between species, strains and gestational age. Therefore each research question needs careful choice of the animal model and gestational age period of interest.

Across rat strains at mid-gestation, pregnancy decreases RVR and enhances renal flow and GFR, as shown in Figure 2. The effect of pregnancy on RVR and GFR is quite consistent, whereas there is considerable variation in renal flow. This may suggest differences in renal blood pressure between strains. We observed that the common changes in RVR, renal flow and GFR are caused by NO upregulation rather than by change in renal autoregulatory threshold to pressure, or sympathetic or vasopressin-regulation.

At late gestation, the pregnancy effects on RVR, renal flow and GFR are dependent on the species and strain investigated (Figure 3). In some species or strains, renal parameters are not different from non-pregnant values (LER and MWR), while in others late pregnancy affects RVR, renal flow and GFR (SDR, rabbits and sheep). The renal autoregulatory threshold to pressure changes was not affected in any species investigated. The NO, RAAS, sympathetic and vasopressin pathways are differently affected across species and strains. In SDR, NO activation affects renal flow, but not GFR. This implies a difference in response to NO activation between the afferent and efferent glomerular vasculature.

The pregnancy-induced reduction in RVR and the associated increase in renal flow and GFR across the species studied are dependent on gestational age, as shown in Figures 2 and 3. Our analysis includes data on both mid- and late gestation in LER, MWR and SDR (Figure 9). In LER and MWR, all measured renal changes are maximal in mid-gestation and return towards non-pregnant levels in late gestation, whereas in SDR there is no difference between mid- and late pregnancy response. Responses in LER and MWR qualitatively correspond well with human pregnancy, in which renal flow increases in the first and second trimesters and decreases markedly towards term [1]. Quantitatively, in human pregnancy the renal flow increase (around 60%) is more pronounced than in the rat strains included in our meta-analysis (LER 31%, MWR 45% and SDR 14%). One may speculate on the underlying mechanism for this difference. Possibly the more mature human fetus poses a greater demand on the maternal vascular system, than the more immature rat fetus at comparable gestational age period. Our data suggest that renal changes during pregnancy in humans and rats are qualitatively similar but quantitatively different.

It is questionable if SDR is the right model for renal changes in healthy human pregnancy. In contrast to humans in which RVR, renal flow and GFR return towards non-pregnant values near term, the changes in SDR are maximal in mid-pregnancy and remain at maximum level throughout late pregnancy. As shown in Figure 9, this pattern is different from that in the other rat strains investigated (LER, MWR), that matches better with the human pattern. The persistent changes in SDR may reflect their relatively compromised

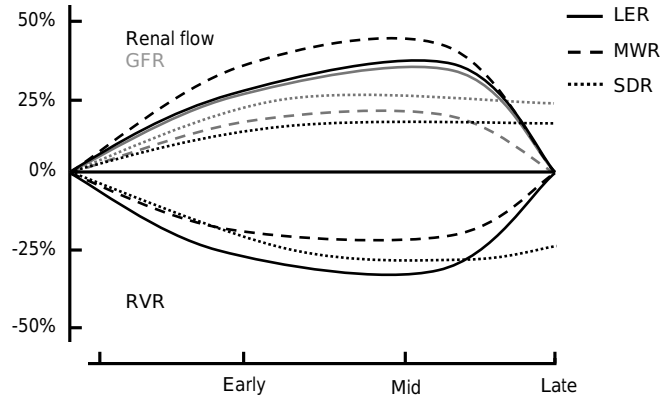

Figure 9. Summary of renal vascular resistance (RVR), renal flow and glomerular filtration rate (GFR) during pregnancy in Long Evens rats (LER), Munich Wistar rats (MWR) and Sprague Dawley rats (SDR).

vascular health. This strain is known to develop severe vascular dysfunction later in life, including chronic progressive nephropathy, peri-/vasculitis, and chronic cardiomyopathy [55]. SDR could be the better model for renal vascular maladaptation, whereas LER and MWR seem the more appropriate models for normal pregnancy-induced renal vasodilation.

Our meta-analysis detected considerable heterogeneity (I^2 >60%) for overall renal flow and moderate heterogeneity (I^2 <60%) for overall GFR and RVR. This can only partially be attributed to differences between species and strains, as stratification reduced heterogeneity substantially only for some subgroups. Additionally, methodological differences in determining renal flow (flow probe or para-aminohippurate clearance) may have contributed to the heterogeneity. The substantial degree of heterogeneity implies that data should not be quantitatively extrapolated to other species or strains.

Renal artery function is not affected by pregnancy, except for SDR. Pregnancy does not change the renal artery responsiveness to pharmacological stimuli (Gq_{EC}- and Gq_{SMC}-mediated stimuli, NO and potassium). One study in SDR showed an endothelium-dependent decrease in renal artery myogenic reactivity and increased vascular compliance in late pregnancy. Apparently SDR activate additional mechanisms to realize the same degree of vascular adaptation to pregnancy, as compared to other strains. This may represent a more generalized pattern, as it has been reported that SDR activate additional vascular adaptive pathways also in mesenteric arteries responses in pregnancy [8].

One may question what mechanisms are responsible for the changes in RVR, renal flow and GFR in pregnancy, given the lack of a role for renal artery adaptation. Our meta-analysis does not provide the answer. Several mechanisms can be considered. Reduced RVR and enhanced renal flow and GFR in pregnancy most likely result from regulation by the small resistance vessels of the kidney, rather than the renal artery [56]. The juxtaglomerular apparatus, which regulates afferent and efferent glomerular artery tone, may also be involved through resetting of the tubuloglomerular feedback system [57]. It seems likely that pregnancy-specific hormones, including relaxin or progesterone, play a role in these processes [58;59].

Several methodological aspects of our meta-analysis deserve discussion. First, the quality of all included studies was scored as poor, in terms of the reported number of animals, parity, randomization, and blinding the outcome assessment. This is a common finding in animal experiments [8], which are primarily concerned with generation and testing of hypotheses rather than with rigorous employment of randomized-controlled-trial methodology. Nonetheless, the quality of animal experimental work, and therefore the reliability of its findings, could benefit from application of strict methodological criteria, standardized procedures and reporting guidelines [60]. Second, our literature search was not designed to identify all studies that reported on pregnancy-induced changes in RVR, renal flow and GFR. Because we were primarily interested in the underlying mechanisms, we restricted our search to studies investigating responses to vaso-active stimuli. Many studies in different animals have investigated RVR, RPF and GFR without using vaso-active stimuli. These studies were not included in our meta-analysis. Our data on pregnancy-induced changes imply species, strain and gestational age differences in RVR, RPF and GFR, without having the intention to be complete on this. Third, despite extensive searches, some of our observations are still based on a limited number of animals and strains of animals. Obviously, these results have to be interpreted with some restraint. Fourth, publication bias may have affected the results. Negative results tend to be underreported, which may therefore lead to overestimated effect sizes. Our funnel-plot analysis did not detect any such effect. Fifth, one may question whether it is legitimate to group together responses to pharmacological stimuli across species and strains. We observed considerable heterogeneity (I^2 >60%), which implies that one should not regard the responses as uniform across species and strains. Sixth, one may question the validity of combining the responses to different stimuli according to their assumed common G-protein coupled pathway. If heterogeneity would have been low, it would have been reasonable to group together the various stimuli to their common pathways, as observed in a former study of our group [61]. The moderate heterogeneity, observed for most responses in the present study, might imply that the assumption is not valid. However, it does not necessarily disprove the validity, because heterogeneity may be affected by many methodological aspects of the experiments. Given the moderate heterogeneity, one should mainly focus on the qualitative similarities.

In conclusion, our meta-analysis shows that pregnancy reduces RVR and increases renal flow and GFR through NO activation and sympathetic deactivation and not through a change in renal artery responsiveness. The cellular level at which pregnancy affects the respective pathways remains unknown. Quantitatively, renal vascular changes in pregnancy vary between species, strains and gestational age. Our meta-analysis suggests that renal changes during pregnancy are qualitatively similar in animals and even in comparison to humans, but quantitatively different.

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CHAPTER 4

CONTRIBUTION OF DIFFERENT LOCAL VASCULAR RESPONSES TO MIDGESTATIONAL VASODILATION

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ABSTRACT

At term pregnancy-induced vasodilation is the resultant of endothelium-dependent vasodilation, decreased myogenic reactivity, increased compliance and reduced sensitivity to vasoconstrictor agents. We hypothesized that these vascular changes are already present at midgestation. In 20 mid-pregnant and 20 nonpregnant Wistar Hannover rats, we measured vascular responses of isolated mesenteric arteries and kidney. In the pregnant rats compared with the nonpregnant rats, mesenteric flow-mediated vasodilation and renal perfusion flow increased 1.52 fold (from 47±5 to 31±4 µl/min) and 1.13 fold (from 12.8±0.1 to 14.4±0.1 ml/min), respectively. Nitric oxide inhibition reduced mesenteric flow-mediated vasodilation to a similar extent in the pregnant and nonpregnant rats; it completely blocked the pregnancy-induced increase in renal perfusion flow. Pregnancy did not change mesenteric artery sensitivity to phenylephrine, myogenic reactivity, nor vascular compliance. At midgestation, alterations in rat mesenteric vascular tone depend primarily on flow-mediated endothelium-dependent changes and not on changes in α-adrenergic vasoconstrictor sensitivity, myogenic reactivity or vascular compliance.

INTRODUCTION

Vasodilation in both human [1] and rat [2] pregnancy is already maximal at midgestation. Under physiological conditions in nonpregnant subjects, the renal and mesenteric vascular beds receive approximately 25% and 30%, respectively, of total cardiac output [3;4]. During pregnancy, renal plasma flow (RPF) increases by 40% and mesenteric perfusion increases by 65% [1;5]. Based on a large body of at term data, vascular adaptation to pregnancy is thought to be the resultant of stimulation of the endothelium dependent NO pathway [6], reduced responsiveness to vasoconstrictive stimuli [7], decreased myogenic reactivity [8], and increased vascular compliance [8], all of which are responses that are likely to interfere with each other. In contrast, data on midgestational isolated vessel function are limited. It has been suggested that some of these responses may not be affected at mid-term [9;10], despite the fully vasodilated state.

Relaxin, a member of the insulin-like growth factor superfamily, is thought to mediate the vasodilation of pregnancy [11;12]. In nonpregnant rats, relaxin simulates the local and systemic vascular adaptations that are present at midgestation [13;14]. Relaxin neutralizing antibodies reverse the renal compensatory vascular changes in mid-pregnancy [15]. These observations suggest a pivotal role of relaxin in gestational vascular adaptation.

We hypothesized that midgestational vasodilation in rats is mediated by the upregulation of endothelium-dependent vasodilation and alteration of vasoconstrictor agent sensitivity, myogenic reactivity, and vascular compliance that is observed at term. To this end, we investigated vascular function in rats in both isolated mesenteric arteries and kidney, because these vascular beds strongly determine peripheral resistance during pregnancy.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Animal Experiments Committee of the Radboud University Nijmegen Medical Centre, the Netherlands, and was performed in accordance to the European guidelines on animal experiments. Forty female virgin Wistar Hannover rats (Harlan Netherlands, Horst, the Netherlands) were studied in 2 groups at an age of 8-10 weeks, followed by 1-week acclimatization. Group 1 (mid-pregnant rats, 10; nonpregnant rats, 10) was used for studies in the Mulvany Halpern myograph and group 2 (mid-pregnant rats, 10, and nonpregnant rats, 10) was used for perfusion-pressure myograph experiments. We used the isolated perfused rat kidney model for experiments on both groups (n=40). We housed all rats by 2 in filter-top cages on a 12/12 hour light/dark cycle and provided them with standard diet (ssniff R/M-H; Ssniff Spezialdieten GmbH, Soest, Germany) and water *ad libitum.* Pregnancy was accomplished by mating with an experienced male of similar age. The presence of a semen plug at the bottom of the cage was considered successful mating and day 1 of pregnancy. Mid-pregnant (midgestation, day 11 of the pregnancy) and nonpregnant animals were anesthetized with an intraperitoneal injection of 6 mg/100 g pentobarbital (Apharmo, Arnhem, the Netherlands). Furosemide (1 mg/100 g, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) was injected intraperitoneally to achieve maximal urethral distention for

optimal catheter placement. We weighed all animals before surgery. During surgery, blood was withdrawn and plasma was stored at -80 °C to measure relaxin concentration with a homologous radioimmunoassay. The assay was performed according to the method of Sherwood and Crnekovic [16]. In our hands, the analytic sensitivity of the assay was 0.51 ng/ml. Dilution experiments showed good parallelism in the range of 25-100 µl.

Pressure-perfusion myograph

Flow-mediated vasodilation, myogenic reactivity to pressure and compliance were analyzed in a pressure-perfusion myograph (Pressure Myograph System-Model P100; J. P. Trading, Aarhus, Denmark). The responses were determined in phosphate-buffered saline solution (119 mM NaCl, 4.69 mM KCl, 25 mM $NaHCO_3$, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 5.5 mM glucose and 10 mM HEPES), to which were added 2 mM ethylene glycol tetraacetic acid and 0.01 mM Na-Nitroprusside (for calcium-free phosphate-buffered saline solution) to measure compliance or 2.5 mM $CaCl_2$, 0.027 mM Na_2 ethylene diamine tetraacetic acid (for calcium phosphate-buffered saline solution) to assess flow-mediated vasodilation and myogenic reactivity. The buffers were oxygenated with 95% O_2 and 5% CO_2 at a temperature of 37 °C. Second-order mesenteric arteries (150-400 µm) were mounted on 2 opposing glass cannulas (125 µm). Vascular inner diameter was measured through video recording (Vessel View; J. P. Trading, Aarhus, Denmark). Arteries were equilibrated at an intraluminal pressure of 60 mmHg for 20 minutes. Subsequently, preconstriction was achieved by the addition of the thromboxane A2 agonist, U46619 (9,11-Dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$; Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands; bath concentration of 10^{-7}-10^{-6} mol/L), thereby reducing the vessel diameter by 30-40% of their basal diameter.

I. Flow-mediated vasodilation

Flow-mediated vasodilation was defined as the vasodilator response to a certain flow increase. After reaching a stable contraction, the flow in the vessel was increased by 16.7 µl/min steps (2-minute interval) from 0 up to 100 µl/min (comparable with a shear-stress range of 0-10 dyne/cm^2), in the absence or presence of 100 µM of the NO antagonist L-NAME (L-Nitro-Arginine Methyl Ester, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands). Meanwhile the intraluminal pressure was maintained at 60 mmHg. Vessels were excluded from the study if they attained <20% preconstriction, <10% vasodilation over the completed flow-range, or instable pressure because of fluid leakage. Flow-mediated vasodilation was expressed as a percentage of the preconstriction status.

II. Mygenic reactivity and vascular compliance

Myogenic reactivity was defined as the vasoconstrictive response to a certain pressure increase in the presence of calcium. Compliance was defined as the vasodilator response to a certain pressure increase in the absence calcium, to avoid smooth muscle cell contraction. To withdraw interference of previous assessments, a new mesenteric vessel was isolated and prepared as described earlier. Intraluminal pressure was increased every two minutes

by 10 mmHg steps, from 20 mmHg up to 110 mmHg, to successively determine myogenic reactivity and compliance (expressed in micrometer μm).

Wire myograph (response tot phenylephrine)

We studied the vasoconstrictor response to phenylephrine (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) with a Mulvany Halpern myograph (Dual Wire Myograph-Model 400A, J. P. Trading, Aarhus, Denmark). The bath was filled with physiologic salt solution (119 mM NaCl, 4.69 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 5.5 mM glucose and 0.027 mM ethylene diamine tetraacetic acid) and oxygenated with 95% O_2 and 5% CO_2 at a temperature of 37 °C. Four second-order mesenteric arteries (150-400 μm) were mounted in 2 wire myographs, which were stabilized as previously described [17], and set at a tension equivalent to that generated at 90% of the inner circumference at 100 mm Hg. The response to 124 mM KCl was measured to allow normalization of the phenylephrine response. The response to phenylephrine was determined in 8 steps over a range from 10^{-7} to 10^{-5} mol/L, in the absence or presence of 100 μM L-NAME. The data for the 2 arteries in each myograph were averaged when available.

Isolated Perfused Rat Kidney model (IPRK)

The intrinsic adaptation of the kidney was assessed in the isolated perfused rat kidney model. Within the time frame of our protocol, this model was known to represent stable renal function [18]. Briefly, the right renal artery was cannulated through the left renal artery and aorta. The right ureter was cannulated for urine collection. Perfusion was started *in situ*, and the kidney was removed and placed in a perfused bath at a temperature of 37.5 °C. The kidney was perfused at a constant pressure of 90 mmHg with oxygenated cell-free Krebs-Ringer-Henseheit (containing 113 mM NaCl, 4.8 mM KCl, 25 mM $NaHCO_3$, 1.4 mM KH_2PO_4, 2.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, and 5 mM Glucose) and 1.7 mM Pluronic F-108 (oxygen carrier and oncotic agent; BASF). Renal perfusion flow (RPFF) was recorded real-time with the use of a computer system (Midac testorganizer, W95 [version 3.0]; Radboud University Nijmegen Medical Centre, the Netherlands).

Stabilization of the RPFF was accomplished during a 40-minute period. From 40-60 minutes the basal RPFF ($RPFF_{baseline}$) was determined. At 60 minutes L-NAME was added to the perfusate (achieving a concentration of 100 μM) to investigate the contribution of NO to the relaxin (or placebo) evoked vasodilator response up to 160 minutes ($RPFF_{160}$). RPFF was normalized for body weight (milliliters per minute per 100 grams of body weight).

The data were expressed as means ± standard error of the mean (SE); n indicates the number of animals. Flow-mediated vasodilation, response to phenylephrine, and RPFF were analyzed by nonlinear regression curve fitting (GraphPad Prism 4.0; Institute for Scientific Information, San Diego, California, USA). Subsequent curve fit estimates were: 1) maximal vascular diameter after flow change ($Diam_{max}$), 2) flow rate inducing 50% dilation; flow-sensitivity ($Flow_{50\%}$), 3) maximum response to phenylephrine (R_{max}), 4) phenylephrine concentration that induced a 50% response ($C_{50\%}$), 5) $RPFF_{baseline}$, and 6) $RPFF_{baseline}$ minus $RPFF_{160}$ ($RPFF_{delta}$). Overall myogenic reactivity ($MR_{overall}$; corrected for

percentage preconstriction) and overall vascular compliance ($VC_{overall}$) were analyzed by using analysis of variance for repeated measures (Greenhouse-Geisser correction; version 16.0.2; SPSS, Chicago, Illinois, USA). Selective straight-line curve fitting was performed over the range of 60 to 110 mmHg, for both myogenic reactivity ($MR_{>60}$) and compliance ($VC_{>60}$), to estimate the hill slope. Baseline characteristics were analyzed with the Student's t-test. A p-value of <0.05 was considered to represent statistical significance.

RESULTS

The study population contained 20 pregnant and 20 nonpregnant female virgin Wistar Hannover rats with a mean age of 94±2 days. Consistent in all experimental settings, pregnant rats were approximately 7% heavier at mid-pregnancy than nonpregnant rats (246±3 g versus 229±4 g, p<0.01). At midgestation, plasma relaxin levels were higher than in nonpregnant animals (2.4±0.3 ng/ml versus 1.1±0.1 ng/ml, p<0.01). Baseline characteristics of isolated mesenteric arteries (basal diameter, percentage preconstriction to U46619, and vasoconstrictor response to 124 mM KCl) were comparable in both groups (Table 1).

Table 1. Baseline vascular characteristics of mesenteric arteries in nonpregnant and pregnant rats.

	Nonpregnant	**(n)**	**Pregnant**	**(n)**
Flow-mediated vasodilation				
L-NAME absence				
- Basal diameter (μm)	274±10	(8)	268±7	(9)
- Preconstriction (%)	41±2	(8)	36±1	(9)
L-NAME presence				
- Basal diameter (μm)	274 ±11	(7)	265±9	(7)
- Preconstriction (%)	35 ±1	(7)	32±2	(7)
Myogenic reactivity				
- Basal diameter (μm)	275 ±8	(10)	270±7	(7)
- Preconstriction (%)	41 ±3	(10)	45±3	(7)
Vascular compliance				
- Basal diameter (μm)	275 ±8	(10)	273±7	(10)
Response to phenylephrine				
L-NAME absence				
- Basal diameter (μm)	262 ±16	(8)	252±10	(10)
- KCl contraction (mN)	13.9 ±0.6	(8)	13.2±1.0	(10)
L-NAME presence				
- Basal diameter (μm)	258 ±10	(9)	263±10	(10)
- KCl contraction (mN)	13.1 ±0.7	(9)	12.4±0.7	(10)

Values presented as means ± SE, n = number of animals.

Flow-mediated vasodilation

Figure 1 represents the flow-mediated vasodilator response and corresponding curve-fit estimates in mesenteric arteries. At midgestation, pregnancy increased sensitivity to flow (decreased $Flow_{50\%}$) 1.52 fold (from 47±5 to 31±4 µl/min, $p<0.05$) without altering maximal reactivity ($Diam_{max}$ 29±2 and 24±2 % in pregnant and nonpregnant rats, respectively). NO blockade reduced maximal reactivity ($Diam_{max}$) in both pregnant and nonpregnant rats by 21±1 and 20±4 %, respectively, although a difference in flow sensitivity persisted ($Flow_{50\%}$ 24±4 and 57±10 µl/min in pregnant and nonpregnant rats, respectively, $p<0.05$). This indicates that the upregulation of flow-mediated vasodilation at midgestation does not depend on stimulation of the NO pathway.

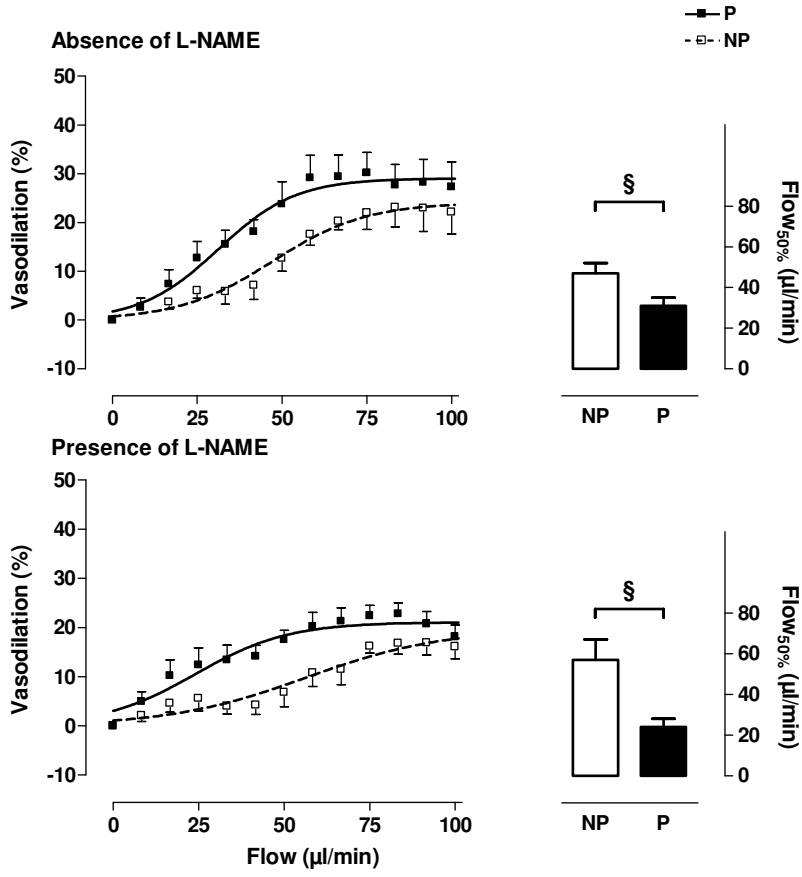

Figure 1. Flow-mediated vasodilator response in mesenteric arteries in mid-pregnant (P) and nonpregnant (NP) rats in absence (n_P=9 and n_{NP}=8) and presence of L-NAME (n_P=7 and n_{NP}=7).

Responses were performed in pressure-perfusion myograph experiments (in absence of L-NAME). Vasodilation is displayed as a percentage of preconstriction to U46619 in non-linear regression curves at the left part of the figure. The derivative $Flow_{50\%}$ (the amount of flow inducing a 50% response) is presented in a bar-graph on the right side. § within weight group difference $p<0.05$.

Myogenic reactivity and vascular compliance

Figure 2 shows myogenic reactivity and vascular compliance. Pregnancy did neither affect myogenic reactivity: $MR_{overall}$ (p=0.19) and $MR_{>60}$ (-2±2 versus -2±2 µm/10 mmHg), nor vascular compliance: $VC_{overall}$ (p=0.56) and $VC_{>60}$ (3±2 versus 3±2 µm/10 mmHg).

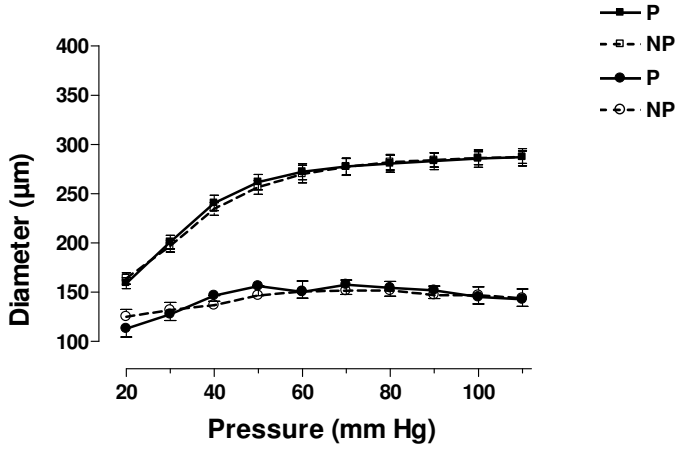

Figure 2. Myogenic reactivity (circles; n_P=7 and n_{NP}=10) and compliance (squares; n_P=10 and n_{NP}=10) in mesenteric arteries in mid-pregnant (P) and nonpregnant (NP) rats.

The response to pressure evaluated in pressure-perfusion myograph experiments. Differences tested by repeated measurement analyses (correction for percentage preconstriction for myogenic reactivity).

Response to phenylephrine

Table 2 depicts the curve-fit estimates of the response to phenylephrine. Both sensitivity ($C_{50\%}$) and maximum response (R_{max}) to phenylephrine were comparable among pregnant and nonpregnant rats. L-NAME increased sensitivity (reduced $C_{50\%}$) to a similar degree in both pregnant and nonpregnant rats.

Table 2. Phenylephrine response curve-fit estimates of the wire myograph experiments in nonpregnant and pregnant rats in absence and presence of 100 µM L-NAME, estimating the concentration of phenylephrine inducing a 50% response ($C_{50\%}$) and the maximum response (R_{max}) as a percentage of the maximum response to 124 mM KCl.

		Nonpregnant	(n)	Pregnant	(n)
* $C_{50\%}$ (µM)	- without L-NAME	1.74±0.12	(9)	1.91±0.13	(10)
	- with L-NAME	1.26±0.11	(9)	1.20±0.08	(10)
* R_{max} (%)	- without L-NAME	114±4	(9)	118±4	(10)
	- with L-NAME	128±6	(9)	120±4	(10)

Values presented as means ± SE, n = number of animals.

Isolated Perfused Rat Kidney model (IPRK)

Figure 3 details the RPFF response curves of pregnant and nonpregnant rats. Pregnancy increased $RPFF_{baseline}$ 1.13 fold (from 12.8±0.1 to 14.4±0.1 ml/min, $p<0.05$) and $RPFF_{delta}$ (NO dependent vasodilation) 1.23 fold (from 6.5±0.2 to 8.0±0.2 ml/min, $p<0.05$).

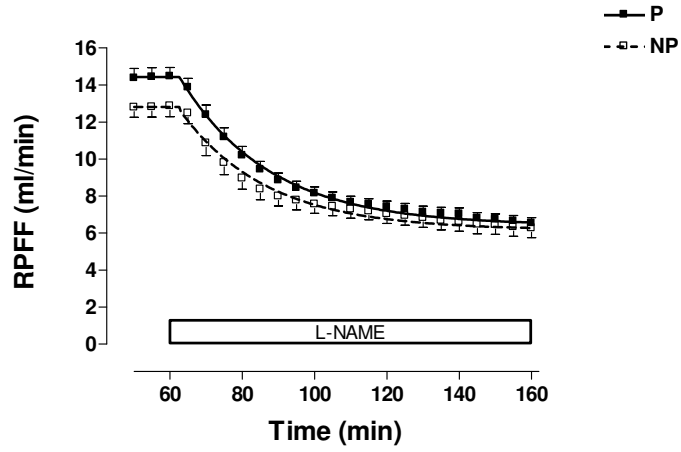

Figure 3. Renal perfusion flow (RPFF) in response to 100 μM L-NAME (at 60 minutes) in mid-pregnant (P; n_P =16) and nonpregnant (NP; n_{NP} =13) rats.

The responses were analyzed in IPRK experiments.

DISCUSSION

The present study investigated the extent to which each of the independent local responses in isolated mesenteric arteries and kidney in rats contributed to mid-pregnancy vasodilation. We showed that midgestation is characterized by enhanced flow-mediated (endothelium-dependent) vasodilation and not by a concomitant increase in vascular compliance, reduced α-adrenergic vasoconstrictor agent sensitivity or myogenic reactivity. As such, our observations at mid-term differ from those observed at term.

In contrast to vascular adaptation in rats at term, mid-term local vascular adaptation seems to rely on endothelium-dependent changes only. Our study does not allow direct comparison of vascular responses at mid-term and term. We did not include a group of rats at term, because this has already been investigated in extent. However, our findings are in line with those of others, who reported that early pregnancy increased (endothelium-dependent) responsiveness to acetylcholine [19] and flow [20]. Apparently, in contrast to observations in rats at term [21], at mid-term the increased responsiveness to flow of mesenteric arteries does not depend on NO upregulation. We speculate that prostaglandins and endothelium-derived hyperpolarizing factor also contribute to midgestational vasodilation. As observed by others at mid-term, we did not detect pregnancy-induced changes in sensitivity to phenylephrine [9]. Possible because of strain differences, and in contrast to others, we did not find changes in

vascular compliance [8]. Although we did not investigate other vasoconstrictor agents, our data imply that midgestational vasodilation directly relates to endothelium-dependent vascular adjustment and not to changes in smooth muscle cell or extra-cellular matrix function.

In vivo data report that RPF increases approximately 1.4 fold in both humans and rodents at mid-term [1;22]. After isolation, RPFF increases only 1.13 fold. The difference may be the consequence of depletion of humoral and autonomic control in *ex vivo*, compared with *in vivo* experiments. However, one may address the effects of impaired oxygen-delivery, which is associated with whole organ perfusion, on renal function. Because we optimized oxygen-delivery by using the oxygen-carrier Pluronic, it seems unlikely that hypoxia-induced changes are responsible for our findings. Our data on RPFF in the isolated rat kidney seem to be in line with *in vivo* observations [23] and in isolated renal arteries [24] at mid-term, in that the gestational rise in RPFF is completely NO dependent. As far as we know, there are no comparable studies that have used the isolated rat kidney model. Our findings show that, in an isolated setting, the intrinsic NO dependent modifications account for only 13% of midgestational renal adaptation. The decrease of mid-term renal vasodilatation, as observed *in vivo*, can be accounted for in the *in vitro* setting itself and subsequent depletion of humoral and autonomic factors.

Our data supply insight in different types of vascular responses that are involved in midgestational local vascular function. Nevertheless, some potential limitations must be addressed. First, we have not randomized rats that did and did not conceive, which may have led to selection bias. Because the Wistar Hannover rat is known to be a uniform outbred line with high reproductive qualities [25], we think that it is unlikely that this may have affected our results. Second, we did not schedule experiments of nonpregnant rats at a particular estrous stage. Although not visible in our results, this could have led to heterogeneity and increased variability of the nonpregnant group.

Changes in endothelium, smooth muscle cell, and extra-cellular matrix function mediate gestational vasodilation. Endothelium-dependent adaptation involves upregulation of 3 parallel mechanisms, that concern the stimulation of NO [6], endothelium-derived hyperpolarizing factor [26] and prostaglandin [27;28] pathways. Possibly depending on the type of vascular bed, one pathway predominates while the others fulfill a supportive role. This may explain the persistence of increased flow-mediated vasodilation under NO blockade, as we observed. Smooth muscle cell adaptation in pregnancy depends mainly on these endothelium-changes [29], although matrix metalloproteases enhance extra-cellular matrix elasticity [30]. There is evidence to suggest that relaxin is an important factor involved in gestational upregulation of endothelium dependent vasodilation and matrix metalloproteases [11;31]. Because we detected only a small increase in relaxin levels at midgestation, we speculate that low levels of relaxin mainly enhance endothelium-dependent vasodilation. As pregnancy proceeds to term, both flow-mediated changes and higher levels of relaxin may promote vessel-remodeling and reduce smooth-muscle cell reactivity.

In summary, these *in vitro* experiments suggest that, in rats, vasodilation in mesenteric arteries at midgestation is mediated mainly by an increase in flow-mediated (endothelium-dependent) changes and not by adjustment of α-adrenergic vasoconstrictor sensitivity, myogenic reactivity or vascular compliance.

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CHAPTER 5

IMPAIRED VASCULAR RESPONSES TO RELAXIN IN DIET-INDUCED OVERWEIGHT FEMALE RATS

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ABSTRACT

Relaxin mediates renal and mesenteric vascular adaptations to pregnancy by increasing endothelium-dependent vasodilation and compliance, and decreasing myogenic reactivity. Diet-induced overweight and obesity are associated with impaired endothelium dysfunction and vascular remodeling leading to a reduction in arterial diameter. In this study, we tested the hypothesis that local vascular responses to relaxin are impaired in diet-induced overweight female rats on a high-fat cafeteria-style diet for 9 weeks. Rats were chronically infused with either relaxin or placebo for 5 days, and vascular responses were measured in isolated mesenteric arteries and the perfused kidney. Diet-induced overweight significantly increased sensitivity to phenylephrine (by 17%) and vessel wall thickness, and reduced renal perfusion flow (RPFF; by 16%), but did not affect flow-mediated vasodilation, myogenic reactivity, and vascular compliance. In the normal weight rats, relaxin treatment significantly enhanced flow-mediated vasodilation (2.67 fold), decreased myogenic reactivity, and reduced sensitivity to phenylephrine (by 28%), but had no effect on compliance or RPFF. NO blockade by L-NAME diminished most relaxin-mediated effects. In diet-induced overweight rats, the vasodilator effects of relaxin were markedly reduced for flow-mediated vasodilation, sensitivity to phenylephrine and myogenic response, compared with the normal diet rats, mostly persistent under L-NAME. Our data demonstrate that some of the vasodilator responses to *in vivo* relaxin administration are impaired in isolated mesenteric arteries and the perfused kidney in diet-induced overweight female rats. This does not result from a decrease in *Rxfp1* (relaxin family peptide receptor) expression but is likely to result from downstream disruption to endothelium-dependent mechanisms in diet-induced overweight animals.

INTRODUCTION

Relaxin, a member of the insulin-like growth factor superfamily, is an important vasodilatory hormone of pregnancy [1-4]. Mostly based on renal vascular studies, four mechanisms are thought to be involved in relaxin-mediated vasodilation: upregulated endothelium dependent NO pathway [5;6], blunted responsiveness to vasoconstrictive stimuli [7], decreased myogenic reactivity [8], and increased compliance [9]. Chronic administration of relaxin to nonpregnant female rats induces vascular adaptations comparable to those observed in pregnancy [5;10], through mechanisms involving matrix metalloproteases, endothelin B receptors and vascular endothelial growth factor [11], whereas relaxin neutralizing antibodies completely abolish the vascular vasodilator effects in rat pregnancy [12]. These observations suggest a pivotal role of relaxin in gestational vascular adaptation.

A high-fat diet, inducing mild overweight in rats, predisposes one to endothelium dysfunction [13] and reflects the human dietary etiology of overweight [14], which clinically translates into an increased risk for gestational hypertensive disease [15]. It has been proposed that attenuated adaptation of the above mentioned mechanisms may precede gestational hypertensive disorders [16;17]. However, it is unknown whether diet-induced overweight affects normal pregnancy-like relaxin-induced vascular changes. The mesenteric and renal vascular beds predominately contribute to peripheral resistance. In nonpregnant healthy conditions, they receive about 25% and 30% of total cardiac output [18;19], whereas during pregnancy renal plasma flow (RPF) and mesenteric vascular perfusion increases by 40% and 65% [20;21]. We hypothesized that diet-induced overweight induces relaxin-resistance, defined as the inability to produce comparable vascular responses to relaxin exposure as observed in healthy controls. To this end, we performed an experimental *ex vivo* study on relaxin-induced vascular responses in isolated mesenteric arteries and kidney in normal weight and diet-induced overweight female rats.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Animal Experiments Committee of the Radboud University Nijmegen Medical Centre, the Netherlands, and was performed in accordance with the European guidelines on animal experiments. Virgin female Wistar Hannover rats (Harlan Netherlands, Horst, the Netherlands) received either a regular diet (ssniff R/M-H, n=20, used in a previous cohort [22]) or a high-fat cafeteria-style diet (ssniff EF R/M acc. D12451 (II), 45%kJ as fat, n=20) *ad libitum* from an age of 21 days onwards. A comparable high-fat diet has previously been shown to result in non significant mild overweight, normal lipid profile, and insulin resistance [23;24]. Rats were housed in filter-top cages on a 12/12 hour light/dark cycle. At an age of 73-85 days, rats were randomly assigned (independent from estrous stage) to chronic infusion for 5 days with either placebo or recombinant human H2 relaxin (both containing 5 mM sodium acetate, pH 5.0; Corthera., San Mateo, California, USA). This readily available human relaxin induces vascular responses in nonpregnant rats comparable to those attributed to endogenous

relaxin in pregnant rats [5;12]. On day 0, an osmotic minipump (Alzet, model 2001, DURECT, Cupertino, California, USA) was implanted subcutaneously under isofluorane anesthesia. The osmotic minipump infused human relaxin at a dose of 4 µg/h for 5 days. This dose of relaxin results in plasma relaxin concentrations equivalent to mid-pregnant rats: 30 ng/ml [5;25]. On day 5, the rats were anesthetized with an intraperitoneal injection of 6 mg/100 g pentobarbital (Apharmo, Arnhem, the Netherlands). Furosemide (1 mg/100g, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) was also injected intraperitoneally to achieve maximal urethral distention for optimal catheter placement. Blood was withdrawn from the vena cava to measure plasma human H2 relaxin concentration levels (Human Relaxin-2 Quantikine ELISA kit DRL200, R&D Systems, Minneapolis, USA). Continuation of chronic relaxin exposure was maintained during all experiments by exposing both mesenteric arteries and isolated kidney to perfusate and tissue bath solutions containing 30 ng/ml of the trial-medication supplied (relaxin or a comparable amount of placebo; incubation time 30 minutes).

Pressure-perfusion myograph

The flow-mediated vasodilation, the myogenic reactivity to pressure and compliance were analyzed in a pressure-perfusion myograph (Pressure Myograph System-Model P100, J. P. Trading, Aarhus, Denmark). The responses were determined in basic phosphate buffered saline (PBS: 119 mM NaCl, 4.69 mM KCl, 25 mM $NaHCO_3$, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 5.5 mM glucose and 10 mM HEPES), with additional 2 mM EGTA and 0.01 mM Na-Nitroprusside (for calcium-free-PBS) to measure compliance and 2.5 mM $CaCl_2$, 0.027 mM Na_2EDTA (for calcium-PBS) to assess flow-mediated vasodilation and myogenic reactivity. The buffers were oxygenated with 95% O_2 and 5% CO_2 at a temperature of 37 °C. Second-order mesenteric arteries (150-400 µm) were mounted on two opposing glass cannulae (125 µm). Vascular inner diameter was measured through video recording (Vessel View, J. P. Trading, Aarhus, Denmark). Arteries were equilibrated at an intraluminal pressure of 60 mmHg during 20 minutes. Subsequently, thromboxane A2 agonist, U46619 (9,11-dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) was added (bath concentration of 10^{-7}-10^{-6} M) to preconstrict vessels to 30-40% of their basal diameter.

I. Flow-mediated vasodilation

Flow-mediated vasodilation is defined as the vasodilator response to a certain flow increase. After reaching a stable contraction, the flow in the vessel was increased every 2 minutes in 16.7 µl/min steps from 0 up to 100 µl/min (comparable to shear-stress range of 0-10 dyne/cm^2), in the absence and presence of 100 µM of the NO antagonist L-NAME (L-Nitro-Arginine Methyl Ester, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands), while maintaining a mean intraluminal pressure of 60 mmHg. Vessels attaining less than 20% preconstriction, less than 10% vasodilation over the completed flow range or with instable pressure due to fluid leakage were excluded from the study. Flow-mediated vasodilation was expressed as a percentage of the preconstriction status.

II. Myogenic reactivity and vascular compliance

Myogenic reactivity is defined as the vasoconstrictive response to a certain pressure increase in the presence of calcium. Compliance is defined as the vasodilator response to a certain pressure increase in the absence of calcium, to avoid smooth muscle cell contraction. A new mesenteric vessel was isolated and prepared as described above. Intraluminal pressure was increased every 2 minutes in 10 mmHg steps from 20 mmHg to 110 mmHg to determine successive myogenic reactivity and compliance. For the latter, outer and inner vessel diameters and wall thickness were measured, and stress-strain relationship was obtained.

Wire myograph (response to phenylephrine)

We studied the vasoconstrictor response to phenylephrine (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) with a Mulvany Halpern myograph (Dual Wire Myograph-Model 400A, J. P. Trading, Aarhus, Denmark). The bath was filled with physiological salt solution (PSS: 119 mM NaCl, 4.69 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 5.5 mM glucose and 0.027 mM EDTA) and oxygenated with 95% O_2 and 5% CO_2 at a temperature of 37 °C. Four second-order mesenteric arteries (150-400 μM) were mounted in two wire myographs, stabilized as previously described [26], and set at a tension equivalent generated at 90% of the inner circumference of 100 mmHg. The response to 124 mM KCl was measured (used for normalization of the phenylephrine response). The response to phenylephrine was determined in eight steps over a range from 10^{-7} M to 10^{-5} M, in the absence or presence of 100 μM L-NAME. The data of the two arteries in each myograph were averaged when available.

Isolated Perfused Rat Kidney Model

Vascular adaptation of the isolated kidney to relaxin was assessed in the isolated perfused rat kidney (IPRK) model. Within the time frame of our protocol, this model shows stable renal perfusion flow (RPFF) and provides an indication of renal function [27], but may not reflect effective renal plasma flow responses *in vivo*. Briefly, the right renal artery was cannulated via the left renal artery and aorta. The right ureter was cannulated for urine collection. Perfusion was started *in situ* and the kidney was removed and placed in a perfused bath at a temperature of 37 °C. The kidney was perfused at a constant pressure of 90 mmHg with oxygenated cell-free Krebs-Ringer-Henselheit (containing 113 mM NaCl, 4.8 mM KCl, 25 mM $NaHCO_3$, 1.4 mM KH_2PO_4, 2.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, and 5 mM Glucose) and 1.7 mM Pluronic F-108 (oxygen carrier and oncotic agent, BASF, Arnhem, the Netherlands). RPFF was recorded in real-time with the use of a computer system (Midac testorganizer, W95 [version 3.0], Radboud University Nijmegen Medical Centre, the Netherlands).

Stabilization of the RPFF was accomplished during a 40 minute period. From 40 to 60 minutes the basal renal plasma flow ($RPFF_{baseline}$) was determined. At 60 minutes L-NAME was added at a final concentration of 100 μM to the perfusate to investigate the contribution of NO to the relaxin (or placebo) evoked vasodilator response up to 160 minutes ($RPFF_{160}$). RPFF was normalized for body weight (ml/min/100g body weight).

Rxfp1 gene expression

Rxfp1 gene expression was analyzed by Quantitative Polymerase Chain Reaction (qPCR), as described previously [28]. Total RNA was extracted from frozen mesenteric arteries with Trizol (GIBCO) according to the manufacturer's instructions. Forward/reverse primers and 6-carboxy fluorescein (FAM)-labeled TaqMan probes were specific for the full-length rat *Rxfp1* from Biosearch Technologies (Novato, California, USA). *Rxfp1* gene expression was compared with expression of the ribosomal 18S reference gene and presented as normalized ΔC_T, transformed by 2^n where $n = (-\Delta C_T)$ [28].

Statistical analysis

The data are expressed as means ± standard error of the mean (SE). Flow-mediated vasodilation, stress-strain relationship, response to phenylephrine and RPFF were analyzed by nonlinear regression curve fitting (GraphPad Prism 4.0, Institute for Scientific Information, San Diego, California, USA). Subsequent curve fit estimates were: $Diam_{max}$ [maximal vascular diameter after flow change], $Flow_{50\%}$ (flow rate inducing 50% dilation, flow-sensitivity), K_1 and K_2 (exponential constants for stress-strain relationship), R_{max} (maximum response to phenylephrine) and $C_{50\%}$ (phenylephrine concentration inducing a 50% response), $RPFF_{baseline}$ (basal RPFF), and $RPFF_{delta}$ ($RPFF_{baseline}$ minus $RPFF_{160}$). Overall myogenic reactivity ($MR_{overall}$; corrected for percentage preconstriction) and overall vascular compliance ($VC_{overall}$) were analyzed by using ANOVA for repeated measures (Greenhouse-Geisser correction; SPSS 16.0.2, SPSS., Chicago, Illinois, USA). Selective straight line curve fitting was performed for both myogenic reactivity ($MR_{>60}$) and compliance ($VC_{>60}$), estimating the slope over the range of 60 to 110 mmHg. Baseline characteristics were analyzed with Student's t-test. In all analyses, we compared relaxin versus placebo in normal weight rats, relaxin versus placebo in diet-induced overweight rats, and normal weight versus diet-induced overweight placebo-treated rats. A p-value of <0.05 was considered to be statistically significant.

RESULTS

The study population contained 20 normal weight female rats on the normal diet and 20 diet-induced female rats on the high-fat diet with average ages of 82±1 and 84±1 days respectively. One rat on the high-fat diet failed to thrive for unknown reasons and was excluded from the study. Overall, the high-fat diet induced mild overweight, as rats on the high-fat diet were 13% heavier than those on the normal diet (234±4 g versus 208±2 g, $p<0.001$). Plasma concentrations of human H2 relaxin in the relaxin-treated female rats were comparable in both weight groups (74±16 ng/ml in normal weight rats versus 61±5 ng/ml in diet-induced overweight rats, $p=0.44$), and were significantly elevated compared with placebo-treated female rats (less than 15.6 pg/ml). Plasma relaxin concentrations were comparable after correction for weight (36±8 ng/ml/100 g in normal weight rats versus 27±3 ng/ml/100 g in diet-induced overweight rats, $p=0.30$) and did not differ significantly between the two groups.

Basal diameter of the mesenteric vessels and percentage precontraction to U46619 did not differ significantly between normal weight and diet-induced overweight female rats or between those chronically infused with relaxin and placebo (Table 1). For the most part, this was also seen in the presence and absence of L-NAME in the different experimental settings. However, under L-NAME, the response to 124 mM KCl was significantly increased in the relaxin-treated diet-induced overweight group, compared with placebo-treated diet-induced overweight female rats (15.1±0.7 versus 12.1±0.6 mN, respectively).

Flow-mediated vasodilation

Flow-mediated vasodilation in the mesenteric arteries of placebo-treated female rats did not differ significantly between normal weight and diet-induced overweight animals either in the absence (p=0.32) or presence (p=0.08) of L-NAME (Table 2). Similarly, although the maximum response to flow ($Diam_{max}$) was slightly higher in the diet-induced

Table 1. Baseline vascular characteristics of mesenteric arteries in normal weight and diet-induced overweight female rats, pre-treated with relaxin (4 µg/h for 5 days) or placebo.

	Normal weight				Diet-induced overweight			
	Placebo	**(n)**	**Relaxin**	**(n)**	**Placebo**	**(n)**	**Relaxin**	**(n)**
Flow-mediated vasodilation								
L-NAME absence								
- Basal diameter (µm)	296±16	(6)	314±15	(7)	295±4	(6)	306±11	(7)
- Precontraction (%)	42±4	(6)	37±2	(7)	43±5	(6)	37±3	(7)
L-NAME presence								
- Basal diameter (µm)	311±16	(5)	330±18	(4)	293±2	(6)	306±8	(8)
- Precontraction (%)	38±2	(5)	36±5	(4)	38±3	(6)	39±5	(8)
Myogenic reactivity								
- Basal diameter (µm)	304±11	(8)	319±11	(10)	294±13	(9)	302±10	(9)
- Precontraction (%)	41±3	(8)	45±3	(10)	38±2	(9)	39±2	(9)
Vascular compliance								
- Basal diameter (µm)	309±8	(8)	318±12	(9)	281±14	(9)	301±10	(9)
Response to phenylephrine								
L-NAME absence								
- Basal diameter (µm)	251±9	(8)	234±10	(10)	269±15	(10)	282±7	(9)
- KCl contraction (mN)	12.8±0.9	(8)	12.7±0.4	(10)	13.0±0.7	(10)	12.3±0.7	(9)
L-NAME presence								
- Basal diameter (µm)	240±9	(9)	243±10	(10)	257±13	(9)	248±10	(9)
- KCl contraction (mN)	13.1±0.5	(9)	12.5±0.5	(10)	12.1±0.6	(9)	15.1±0.7§	(9)

Values presented as mean ± SE, n = number of animals. § p<0.05 between placebo- and relaxin- treated rats.

overweight female rats, it was not significant compared with normal weight female rats in the absence or presence of L-NAME. In normal weight female rats, chronic infusion with relaxin significantly ($p<0.01$) increased flow sensitivity 2.67 fold ($Flow_{50\%}$ from 48±9 to 18±4 µl/min) but had the opposite effect in diet-induced overweight female rats (35±7 to 54±16 µl/min) although this was not significant ($p=0.22$, Figure 1 and Table 2). There was no significant effect of relaxin infusion on mesenteric artery $Diam_{max}$ in either weight group (Table 1). NO blockade blunted the different flow-mediated responses to relaxin in both weight groups but had no effect on $Diam_{max}$ (Table 2).

Table 2. Flow-mediated vasodilation curve-fit estimates in mesenteric arteries of normal weight and diet-induced overweight female rats pre-treated with relaxin (4 µg/h for 5 days) or placebo. Experiments performed in the pressure-perfusion myograph in absence and presence of 100 µM L-NAME.

Flow-mediated vasodilation		**Placebo**	**(n)**	**Relaxin**	**(n)**
* $Flow_{50\%}$ (µl/min)	- normal weight	48±9	(6)	18±4§	(7)
	- diet-induced overweight	35±7	(6)	54±16	(7)
	- normal weight (L-NAME)	28±6	(5)	30±10	(4)
	- diet-induced overweight (L-NAME)	42±5	(6)	38±6	(8)
* $Diam_{max}$ (%)	- normal weight	31±5	(6)	26±2	(7)
	- diet-induced overweight	46±6	(6)	40±12	(7)
	- normal weight (L-NAME)	27±3	(5)	31±5	(4)
	- diet-induced overweight (L-NAME)	35±3	(6)	40±4	(8)

Curve-fit estimates: amount of flow inducing a 50% response ($Flow_{50\%}$), maximum response to flow ($Diam_{max}$) as a percentage of precontraction to U46619. Values presented as mean ± SE, n = number of animals. § $p<0.05$ between placebo- and relaxin- pre-treated rats.

Myogenic reactivity and vascular compliance

Myogenic reactivity was recorded in the presence of calcium (Figure 2). We detected a significant ($p=0.02$) increase in $MR_{overall}$ in the mesenteric arteries of placebo-treated diet-induced overweight female rats compared with those in the normal weight group. This was probably due to smaller basal diameter, as $MR_{>60}$ was not significantly affected (from -1±2 to 0±2 µm/10 mmHg, $p=0.67$). Chronic infusion of relaxin in normal weight female rats reduced both $MR_{overall}$ ($p=0.01$) and $MR_{>60}$ (from -1±2 to 7±3 µm/10 mmHg, $p=0.03$), whereas it did not significantly affect $MR_{overall}$ ($p=0.1$) and $MR_{>60}$ (from 2±2 to 3±2 µm/10 mmHg, $p=0.43$) in diet-induced overweight female rats.

Mesenteric arterial compliance (measured in the absence of calcium) did not differ significantly between the diet-induced overweight and normal weight placebo-treated female rats (Figure 3). However, vessel wall thickness in diet-induced overweight female rats was significantly increased ($p=0.05$) compared with normal weight female rats (Figure 3C). Chronic infusion of relaxin did not significantly alter stress-strain curves, vessel wall thickness, $VC_{overall}$ or $VC_{>60}$ in either weight group.

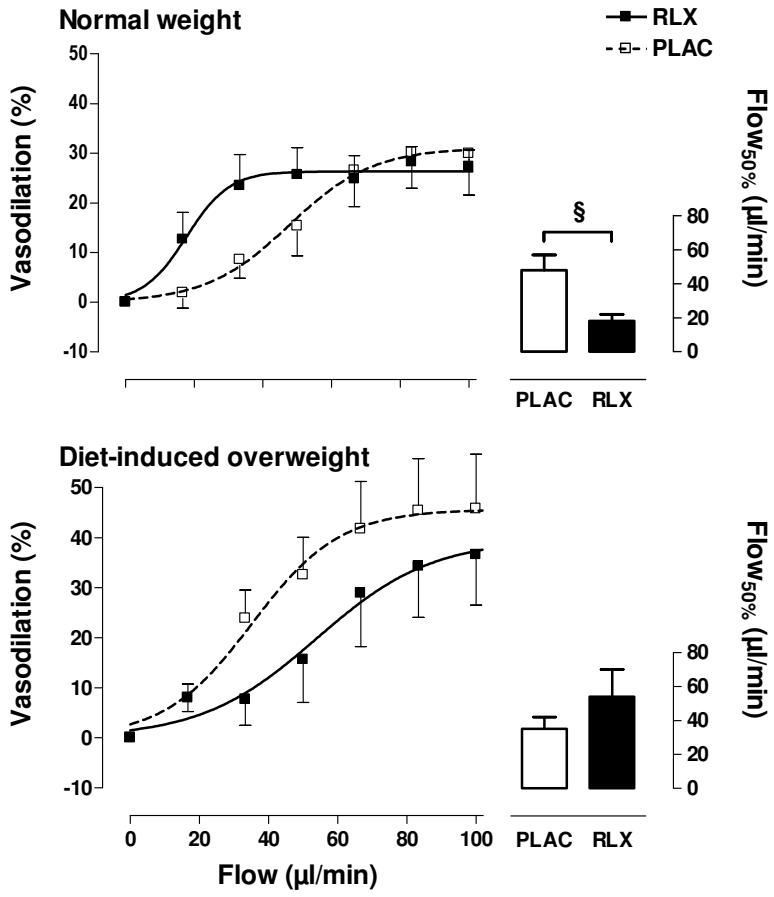

Figure 1. Flow-mediated vasodilator response (left figures) and corresponding $Flow_{50\%}$ (right figures) in normal weight (n_{RLX}=7 and n_{PLAC}=6) and diet-induced overweight (n_{RLX}=7 and n_{PLAC}=6) female rat mesenteric arteries, pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC).

§ p<0.05 between placebo- and relaxin-treated rats.

Response to phenylephrine

Mesenteric arteries of placebo-treated diet-induced overweight female rats had significantly (p=0.04) increased sensitivity to phenylephrine ($C_{50\%}$) by 17 % (from 1.39±0.08 to 1.19±0.02 µM) compared with normal weight female rats (Table 3). Pre-treatment with L-NAME also significantly (p<0.01) diminished R_{max} to phenylephrine in placebo-treated diet-induced overweight female rats. Relaxin treatment significantly (p<0.01) decreased the sensitivity to phenylephrine ($C_{50\%}$) by 28% (from 1.39±0.08 to 1.78±0.10 µM) in normal weight female rats (Table 3). Under NO blockade, this effect was reduced but still significant (from 0.89±0.04 to 1.06±0.07 µM, p=0.04). In diet-induced overweight female rats, relaxin had no effect on $C_{50\%}$. However, in the presence of L-NAME, there was a significant (p<0.01) desensitizing effect of relaxin (from

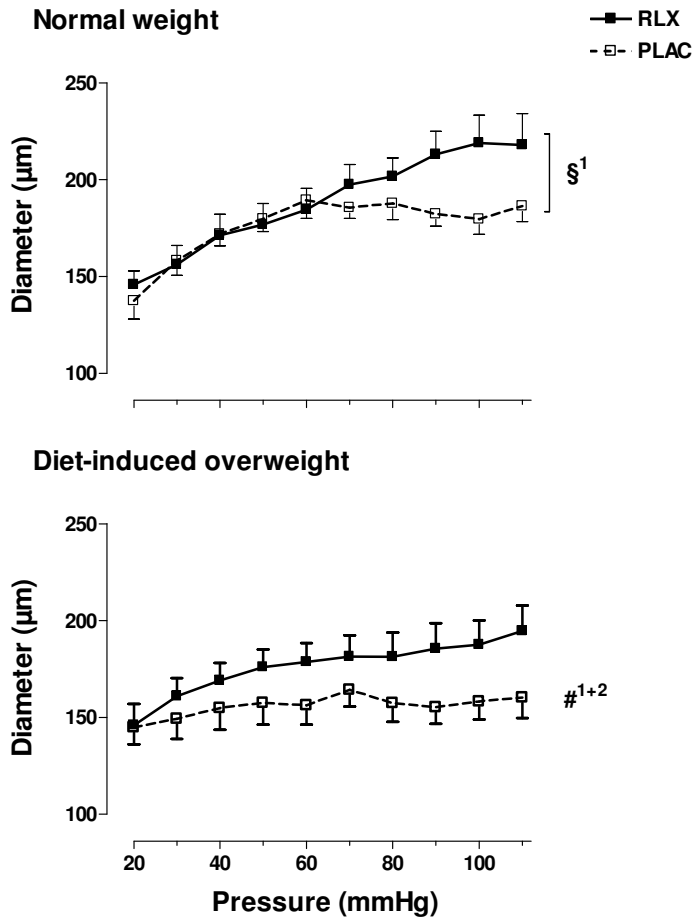

Figure 2. Myogenic reactivity (in presence of calcium) in normal weight (n_{RLX}=10 and n_{PLAC}=8) and diet-induced overweight (n_{RLX}=9 and n_{PLAC}=9) female rat mesenteric arteries, pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC).

§ $p<0.05$ between placebo- and relaxin-treated rats. [1] and [2] represent differences in the within-subjects factor and the between-subjects factor, respectively.

0.84±0.02 to 1.10±0.05 µM). Relaxin did not affect the maximum response (R_{max}) in either weight group, although pretreatment with L-NAME increased R_{max} in diet-induced overweight female rats (p=0.05).

Isolated Perfused Rat Kidney model

The IPRK experiments resulted in RPFF response curves and corresponding curve fit estimates of normal weight and diet-induced overweight female rats (Figure 4). Comparison of the two placebo-treated weight groups demonstrated a significant ($p<0.001$) reduced $RPFF_{baseline}$ by 16% (from 6.9±0.2 to 5.8±0.1 ml/min/100g) in the diet-induced overweight

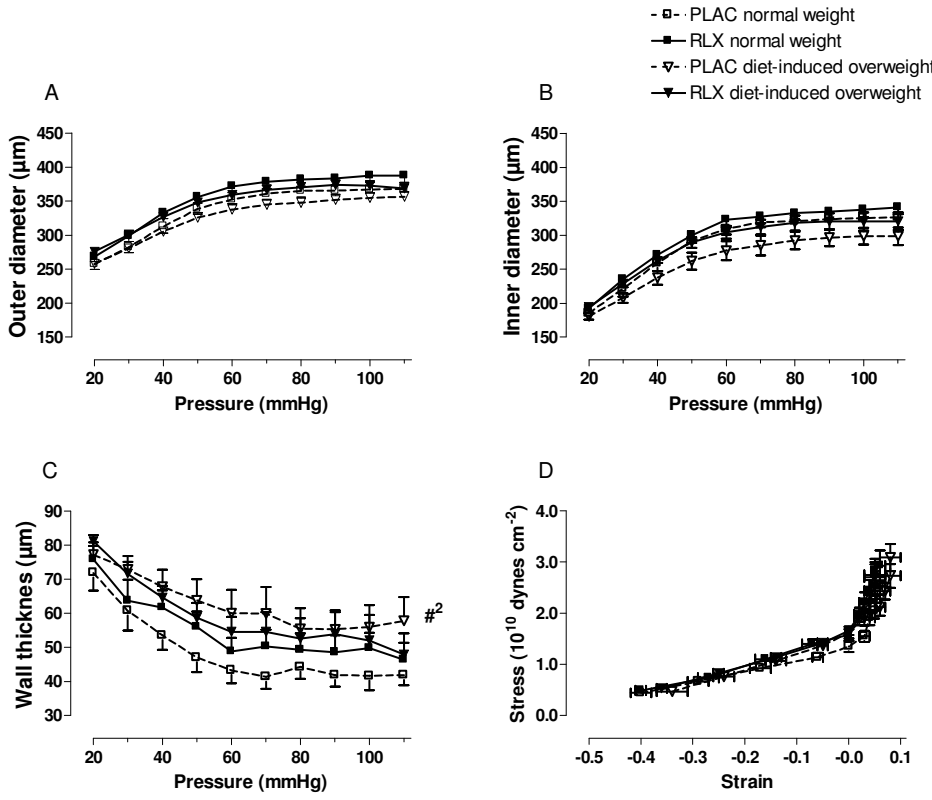

Figure 3. Vascular compliance (in absence of calcium) in normal weight (n_{RLX}=9 and n_{PLAC}=8) and diet-induced overweight (n_{RLX}=9 and n_{PLAC}=9) female rat mesenteric arteries, pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC): outer vessel diameter (A), inner vessel diameter (B), vessel wall thickness (C) and stress-strain curve (D).

# p <0.05 between normal weight and diet-induced overweight rats. [2] represents difference in the between-subjects factor.

female rats, but no effect on NO mediated vasodilation ($RPFF_{delta}$ from 3.9±0.4 to 3.6±0.1 ml/min/100g). Chronic infusion with relaxin had no effect on $RPFF_{baseline}$ (from 6.9±0.2 to 6.7±0.1 ml/min/100g, p=0.38) and $RPFF_{delta}$ (from 3.9±0.4 to 4.1±0.3 ml/min/100g, p=0,78) in normal weight female rats. In the diet-induced overweight female rats, relaxin lowered $RPFF_{baseline}$ by 3% (from 5.8±0.1 to 5.6±0.1 ml/min/100g, p=0.01) but also lowered $RPFF_{delta}$ 14% (from 3.6±0.1 to 3.1±0.1 ml/min/100g, p<0.001).

Rxfp1 gene expression

To confirm that *Rxfp1* was expressed in the mesenteric arteries and test the hypothesis that diet or relaxin treatment could affect expression, we used quantified *Rxfp1* expression. *Rxfp1* was expressed in the mesenteric arteries, with a high degree of variation (Ct value range of 7.79-19.53). We did not find differences in 18S expression among the weight

Table 3. Phenylephrine response curve-fit estimates in mesenteric arteries of normal weight and diet-induced overweight female rats pre-treated with relaxin (4 µg/h for 5 days) or placebo. Experiments performed in the wire myograph in absence and presence of 100 µM L-NAME.

		Placebo	(n)	Relaxin	(n)
* $C_{50\%}$ (µM)	- normal weight	1.39±0.08	(8)	1.78±0.10§	(10)
	- diet-induced overweight	1.19±0.02#	(10)	1.21±0.04	(9)
	- normal weight (L-NAME)	0.89 ±0.04	(9)	1.06 ±0.07§	(10)
	- diet-induced overweight (L-NAME)	0.84 ±0.02	(9)	1.10 ±0.04§	(9)
* R_{max} (%)	- normal weight	115 ±4	(8)	113 ±4	(10)
	- diet-induced overweight	112 ±3	(10)	113 ±3	(9)
	- normal weight (L-NAME)	127 ±3	(9)	122 ±4	(10)
	- diet-induced overweight (L-NAME)	114 ±3#	(9)	124 ±3§	(9)

Curve-fit estimates: concentration of phenylephrine inducing a 50% response ($C_{50\%}$), maximum response (R_{max}) as a percentage of the maximum response to 124 mM KCl. Values presented as mean ± SE, n = number of animals. § $p<0.05$ between placebo- and relaxin- pre-treated rats, # $p<0.05$ between normal weight and diet-induced overweight rats.

and treatment groups. There was no significant difference in expression between the two weight groups, and no effect of relaxin treatment (Figure 5).

DISCUSSION

Chronic relaxin exposure stimulates mesenteric vasodilation by affecting various independent responses involved in local vascular control. These effects are largely NO dependent. The *ex vivo* kidney is insensitive to relaxin. High-fat diet- induced overweight impairs normal mesenteric *in vitro* vasodilation and lowers RPFF in response to high levels of relaxin.

Chronic relaxin-treatment improves the vasodilator state of normal weight female rat mesenteric arteries by affecting several independent pathways. We observed that it raises flow-mediated vasodilation and lowers sensitivity to phenylephrine, both (partially) by enhanced NO mediated vasodilation. This is in line with observations made by others on the relaxin effects on NO dependent vasodilation and angiotensin-II sensitivity [5]. Our data also confirm that relaxin decreases myogenic reactivity [8;9]. Although this effect could be due to reduced vessel compliance rather than decreased myogenic tone [9], we did not observe an effect of relaxin on compliance. The differences in observations between studies may depend on normalization protocol, type, duration and dosage of relaxin, and the pressure range of interest. Overall, in normal weight female rats, we conclude that relaxin enhances vascular dilatation to flow, inhibits sensitivity to vasoconstrictor agents and reduces myogenic reactivity. These effects are predominantly caused by increasing NO mediated vasodilation, without altering the extracellular vascular matrix.

High-fat diet-induced overweight and obesity increase overall myogenic reactivity [29], enhance sensitivity to phenylephrine [30], and reduce endothelium-dependent vasodilation [31],

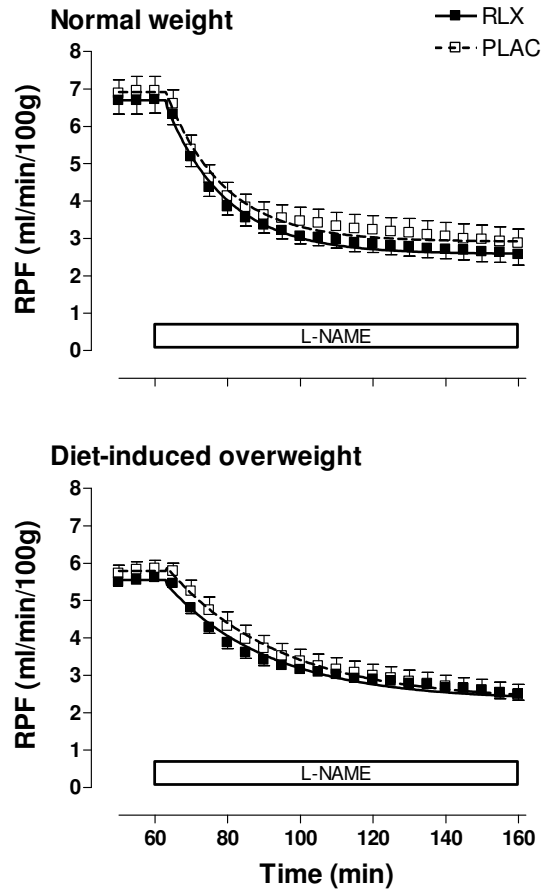

Figure 4. Renal perfusion flow (RPFF) in response to 100 µM L-NAME (at 60 minutes) in *ex vivo* isolated perfused rat kidney experiments in normal weight (n_{RLX}=9 and n_{PLAC}=9) and diet-induced overweight (n_{RLX}=8 and n_{PLAC}=8) female rats, pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC).

whereas arterial compliance is not affected [32]. Additionally, in diet-induced overweight female rats, mesenteric arteries are virtually resistant to relaxin, as we observed that the normal relaxin induced endothelium-dependent NO mediated vasodilation is blunted. The effects of relaxin on the vascular responses in diet-induced overweight female rats have not been studied before. We suggest that diet-induced overweight impairs all the vasodilatory effects of relaxin, in addition to the already present vasoconstriction phenotype in these rats.

In vivo experiments in healthy normal weight female rats have shown that relaxin raises renal plasma flow (RPF) up to 40% by increasing NO availability [5]. In our *ex vivo* experiments, relaxin had no effect on the RPFF in normal weight female rats. Whole organ perfusion is affected by perfusate viscosity and solutions oxygen delivery capacity. As we added oxygen-carrier Pluronic to the perfusate and since we were able to detect small changes in renal function between the weight groups, we think that the absence of a

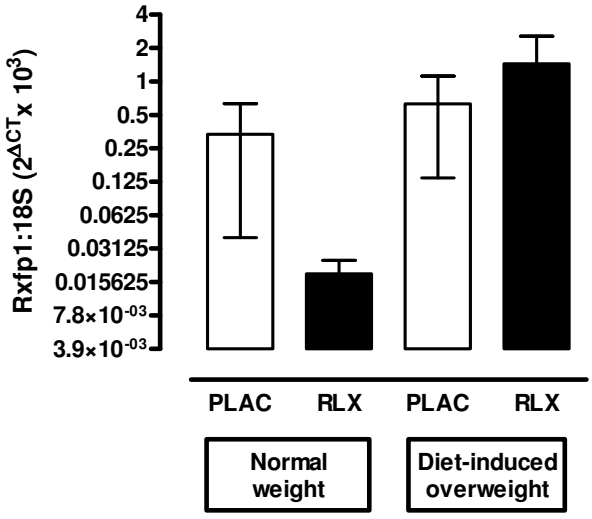

Figure 5. *Rxfp1* gene expression in mesenteric arteries of normal weight (nRLX=8 and nPLAC=7) and diet-induced overweight (nRLX=10 and nPLAC=9) female rats, pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC).

Data are presented as mean ± SE $2^{-\Delta CT}$.

response to relaxin is unlikely due to the IPRK model. Our data therefore suggest that, in healthy female rats, in the absence of humoral and/or autonomic control, relaxin does not intrinsically adjust kidney function.

Being mildly overweight in young subjects induces renal hyperfiltration through hyperinsulinemia [33]. In the isolated setting, we observed that diet-induced overweight reduced RPFF without affecting NO synthase activity [34]. The reduced basal RPFF may be explained by enhanced renal myogenic tone, as observed in overweight-related dyslipidemia [35]. Above the diet-induced overweight related renal impairment, relaxin pre-treatment decreased basal RPFF and lowered NO mediated renal vasodilation.

Our study has several potential limitations. First, our high-fat diet induced only mild overweight. On the analogy of human diet-related overweight, this high-fat diet model has been used in literature to investigate overweight-related vascular changes in rats [14]. As reported by others [23;31], the moderate increase in body weight was sufficient to cause significant differences in vascular responses. Second, the human relaxin plasma levels we detected were a little higher than 30 ng/ml, as reported by others [5]. This may be explained by volume distribution-, relaxin metabolism- or clearance differences among different rat strains. As the acute effects of relaxin predominate [9] and since we exposed both the *ex vivo* kidney and mesenteric artery experiments to the same amount of trial medication as used by others, it is unlikely that this may have confounded our results. Third, rats were enrolled into the experiments independently of their estrous cycle. We think that our randomization-procedure has distributed the estrous stages equally among groups, and therefore the cycle stage does not explain the observed effects of relaxin.

Relaxin is thought to act through the G protein-coupled *Rxfp1* receptor, activating a vasoconstrictive mitogen-activated protein kinase (MAPK) pathway [36] and the normally predominant NO mediated vasodilator phosphatidylinositol 3-kinase (PI_3K) pathway [37], possible by interference of endothelin (ET) and its receptors (ET_A and ET_B) [4]. High-fat diet, similar to that used in the present study, induces overweight related insulin-resistance [38], which leads to inhibition of the PI_3K pathway and overexpression of the MAPK pathway [39]. Although insulin and relaxin induce vasodilation through activation of different receptors (tyrosine kinase- and the *Rxfp1* receptor, respectively), they both use similar down-stream signaling pathways. Our study showed comparable *Rxfp1* receptor expression between normal diet and high-fat diet female rats. In analogy to insulin-resistance, a downregulated PI_3K pathway and an upregulated MAPK pathway could explain our findings on relaxin-resistance in high-fat diet-induced overweight female rats, which results in a relaxin-induced vasoconstrictive state by a relatively impaired NO pathway.

In summary, diet-induced overweight impairs normal mesenteric endothelium-dependent and -independent *in vitro* vasodilation and reduces RPFF in response to relaxin exposure. This suggests the presence of relaxin-resistance in high-fat diet-induced overweight female rats.

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CHAPTER 6

AGING ATTENUATES THE VASODILATOR RESPONSE TO RELAXIN

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ABSTRACT

Relaxin, an insulin-like growth factor peptide, increases endothelium dependent vasodilation and vascular compliance, and decreases myogenic reactivity. These vascular effects significantly contribute to the physiological circulatory adaptations in pregnancy, particularly in the mesentery and kidney. Aging predisposes to vascular maladaptation and gestational hypertensive disease. We hypothesized that mild aging reduces the vascular responses to relaxin. In 20 young (10-12 weeks) and 20 middle aged (40-46 weeks) female Wistar Hannover rats, vascular responses to chronic exposure of relaxin versus placebo (5 days) were quantified in isolated mesenteric arteries and kidney. Vascular responses were evaluated using pressure-perfusion myograph, wire myograph and isolated perfused rat kidney model (IPRK). *Rxfp1* (relaxin family peptide) gene expression was determined by qPCR. In young rats, relaxin stimulated nitric oxide (NO) dependent flow-mediated vasodilation (2.67 fold, from 48±9 to 18±4 µl/min), reduced myogenic reactivity (from -1±2 to 7±3 µm/10 mmHg), decreased mesenteric sensitivity (28%, from 1.39±0.08 to 1.78±0.10 µM), but did not change compliance and renal perfusion flow (RPFF). In aged rats, relaxin did not affect any of the analyzed mesenteric or renal parameters. In aged compared with young placebo-treated rats, all mesenteric characteristics were comparable, while RPFF was lower (17%, from 6.9±0.2 to 5.7±0.1 ml/min/100g) even though NO availability was comparable. *Rxfp1* expression was not different among young and aged rats. Our findings suggest that moderate aging involves normal endothelium function, but blunts the physiological endothelium-dependent and -independent vasodilator response to relaxin.

INTRODUCTION

Relaxin, a member of the insulin-like growth factor superfamily, is an important factor in achieving vasodilation in pregnancy [1]. *In vivo* studies suggest that relaxin-induced renal vascular relaxation is the consequence of an activated endothelium NO release and an attenuated response to angiotensin-II in rats [2]. Additionally, *in vitro* experiments in renal and mesenteric rat arteries have shown that relaxin decreases myogenic reactivity [3], and increases compliance [4]. Relaxin infusion induces vasodilation comparable to that observed in pregnancy [5-7], whereas relaxin neutralizing antibodies completely diminish pregnancy-induced vascular vasodilation [8]. These findings suggest that relaxin is involved in gestational vascular adaptation.

Moderate aging is an independent risk factor for gestational hypertensive disease, doubling the risk above a maternal age of 40 years [9]. It relates to reduced flow-mediated vasodilation [10] and increased sensitivity to phenylephrine in female rat mesenteric arteries [11]. It also reduces myogenic reactivity without affecting vascular compliance in mice [12]. Other studies report beneficial vascular effects of relaxin treatment in aged subjects [13;14]. Attenuation of the pregnancy-induced increase in flow-mediated vasodilation, and reduction in phenylephrine sensitivity and myogenic reactivity, possibly induced by impaired response to relaxin, may be coupled to the development of gestational hypertensive disease [15;16].

We hypothesized that moderate aging (at the end of the reproductive lifespan) blunts the vascular responses to relaxin and subsequently induces relaxin-resistance, defined as the inability to produce comparable vascular responses to relaxin exposure as observed in young subjects. For this reason, we studied the effect of chronic exposure to relaxin in young versus moderately aged rats. The mesenteric and renal vascular beds are both important determinants of pregnancy-induced changes in peripheral resistance, increasing their perfusion by 65% and 40% respectively [17;18]. Therefore, we addressed our hypothesis by investigating the vascular responses to relaxin in isolated mesenteric arteries and the IPRK.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Animal Experiments Committee of the Radboud University Nijmegen Medical Centre, the Netherlands, and was performed in accordance to the European guidelines on animal experiments. Forty female virgin Wistar Hannover rats (Harlan Netherlands, Horst, the Netherlands) were used in the experiments. Young rats (n=20, 10-12 weeks old) and aged rats (n=20, 40-46 weeks old) received standard rodent chow (ssniff R/M-H) and water *ad libitum*. We housed all rats in pairs in filter-top cages on a 12/12 h light/dark cycle. Rats were randomly assigned (independent from estrous stage) to treatment for 5 days with either placebo or recombinant human relaxin (both containing 5mM sodium acetate, pH 5.0; Corthera, San Mateo, California, USA). Human recombinant relaxin induces vascular responses comparable to those exerted by rat relaxin [2;8]. On day 0, an osmotic minipump (Alzet, model 2001, DURECT, Cupertino, California, USA) was placed subcutaneously under isoflurane anesthesia. The osmotic minipump delivered the study medication at a dose of 4 µg/h during 5 days to achieve

plasma relaxin concentrations normally present on day 11 of gestation in rats [2]. On day 5, the rats were anaesthetized with an intraperitoneal injection of pentobarbital (6 mg/100 g body weight (BW), Apharmo, Arnhem, the Netherlands). Furosemide (1 mg/100g BW, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) was also injected intraperitoneally to achieve maximal urethral distention for optimal catheter placement. Blood was withdrawn from the vena cava to measure human recombinant relaxin plasma levels (Human Relaxin-2 Quantikine ELISA kit DRL200, R&D Systems Inc, Minneapolis, USA). In all experiments, we used perfusate and bath solutions containing 30 ng/ml of the trial-medication supplied.

Pressure-perfusion myograph

The flow-mediated vasodilation, the myogenic reactivity to pressure and the compliance were analyzed in a pressure-perfusion myograph (Pressure Myograph System-Model P100, J. P. Trading, Aarhus, Denmark). The responses were determined in basic phosphate buffered saline (PBS: 119 mM NaCl, 4.69 mM KCl, 25 mM $NaHCO_3$, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 5.5 mM glucose and 10 mM HEPES), with additional 2 mM EGTA and 0.01 mM Na-Nitroprusside (for calcium-free-PBS) to measure compliance and 2.5 mM $CaCl_2$, 0.027 mM Na_2EDTA (for calcium-PBS) to assess flow-mediated vasodilation and myogenic reactivity. The buffers were oxygenated with 95% O_2 and 5% CO_2 at a temperature of 37 °C. Second-order mesenteric arteries (150-400 µm) were mounted on two opposing glass canules (125 µm). Vascular inner diameter was measured through video recording (Vessel View, J. P. Trading, Aarhus, Denmark). Arteries were equilibrated at an intraluminal pressure of 60 mmHg during 20 minutes. Subsequently, thromboxane A2 agonist, U46619 (9,11-Dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) was added (bath concentration of 10^{-7}to10^{-6} M) to preconstrict vessels to 30-40% of their basal diameter.

I. Flow-mediated vasodilation

Flow-mediated vasodilation is defined as the vasodilator response to a certain flow increase. After reaching a stable contraction, the flow in the vessel was increased every to 2 minutes in 16.7 µl/min steps from 0 up to 100 µl/min (comparable to shear-stress range of 0-10 dyne/cm^2), in absence and presence of 100 µM of the NO antagonist L-NAME (L-Nitro-Arginine Methyl Ester, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands), while maintaining a mean intraluminal pressure of 60 mmHg. Vessels attaining less than 20% preconstriction, less than 10% vasodilation over the completed flow-range or with instable pressure due to fluid leakage were excluded from the study. Flow-mediated vasodilation was expressed as a percentage of the preconstriction status.

II. Myogenic reactivity and vascular compliance

Myogenic reactivity is defined as the vasoconstrictor response to a certain pressure increase in the presence of calcium. Compliance is defined as the vasodilator response to a certain pressure increase in the absence of calcium, to avoid smooth muscle cell contraction. A new

mesenteric vessel was isolated and prepared as described above. Intraluminal pressure was increased every 2 minutes in 10 mmHg steps from 20 mmHg up to 110 mmHg to determine successive myogenic reactivity (measuring inner vessel diameter) and compliance (measuring outer/inner vessel diameter and vessel wall thickness and the stress-strain relationship [19]).

Wire myograph (response to phenylephrine)

We studied the vasoconstrictor response to phenylephrine (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) with a Mulvany Halpern myograph (Dual Wire Myograph-Model 400A, J. P. Trading, Aarhus, Denmark). The bath was filled with physiological salt solution (PSS: 119 mM NaCl, 4.69 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 5.5 mM glucose and 0.027 mM EDTA) and oxygenated with 95% O_2 and 5% CO_2 at a temperature of 37 °C. Four second-order mesenteric arteries (150-400 µm) were mounted in two wire myographs, stabilized as previously described [20], and set at a tension equivalent generated at 90% of the inner circumference of 100 mmHg. The response to 124 mM KCl was measured (used for normalization of the phenylephrine response). The response to phenylephrine was determined in eight steps over a range from 10^{-7} M to 10^{-5} M, in the absence or presence of 100 µM L-NAME. The data of the two arteries in each myograph were averaged when available.

Isolated Perfused Rat Kidney model (IPRK)

The intrinsic adaptation to relaxin of the kidney was assessed in the IPRK. Within the time frame of our protocol, this model is known to present stable renal function [21], although one has to be restrictive to comparison of absolute values observed *in vivo*. Briefly, the right renal artery was cannulated via the left renal artery and aorta. The right ureter was cannulated for urine collection. Perfusion was started *in situ* and the kidney was removed and placed in a perfused bath at a temperature of 37 °C. The kidney was perfused at constant pressure of 90 mmHg with oxygenated cell-free Krebs-Ringer-Henselheit (containing 113 mM NaCl, 4.8 mM KCL, 25 mM $NaHCO_3$, 1.4 mM KH_2PO_4, 2.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, and 5 mM Glucose) and 1.7 mM Pluronic F-108 (oxygen carrier and oncotic agent, BASF, Arnhem, the Netherlands). Renal perfusion flow (RPFF) was recorded in real time with the use of a computer system (Midac testorganizer, W95 [version 3.0], Radboud University Nijmegen Medical Centre, the Netherlands).

Stabilization of the RPFF was accomplished during a 40-minute period. From 40 to 60 minutes the basal renal plasma flow ($RPFF_{baseline}$) was determined. At 60 minutes L-NAME was added at a final concentration of 100 µM to the perfusate to investigate the contribution of NO to the relaxin (or placebo) evoked vasodilator response up to 160 minutes ($RPFF_{160}$). RPFF was normalized for body weight (ml/min/100g body weight).

Rxfp1 gene expression

The *Rxfp1* gene expression was analyzed by Quantitative Polymerase Chain Reaction (qPCR), according to the procedure described by Vodstrcil et al [22]. Total RNA was extracted from frozen

mesenteric arteries with Trizol (GIBCO-BRL) according to the manufacturer's instructions. Forward/reverse primers and 6-carboxy fluorescein (FAM)-labeled TaqMan probes specific for the full-length rat *Rxfp1* were from Biosearch Technologies (Novato, California, USA).

Statistical analysis

The data were expressed as means ± standard error of the mean (SE); n indicates the number of animals. Flow-mediated vasodilation and the responses to phenylephrine and RPFF were analyzed by nonlinear regression curve fitting (GraphPad Prism 4.0, Institute for Scientific Information, San Diego, California, USA). Subsequent curve fit estimates were: $Diam_{max}$ [maximal vascular diameter after flow change], $Flow_{50\%}$ (flow rate inducing 50% dilation, flow-sensitivity), R_{max} (maximum response to phenylephrine) and $C_{50\%}$ (phenylephrine concentration inducing a 50% response), $RPFF_{baseline}$ (basal RPFF), and $RPFF_{delta}$ ($RPFF_{baseline}$ minus $RPFF_{160}$). Overall myogenic reactivity ($MR_{overall}$; using the inner vessel diameter corrected for percentage preconstriction) and overall vascular compliance ($VC_{overall}$; using the inner vessel diameter) were analyzed by using ANOVA for repeated measures (Greenhouse-Geisser correction; SPSS 16.0.2, SPSS, Chicago, Illinois, USA). Selective straight line curve fitting was performed for both myogenic reactivity ($MR_{>60}$) and compliance ($VC_{>60}$) of the inner vessel diameter, estimating the hill slope over the range of 60-110 mmHg. Baseline characteristics were analyzed by using Student's t-test. In all analyses, we compared relaxin versus placebo in young rats, relaxin versus placebo in aged rats, and young versus aged in placebo-treated rats. *Rxpf1* gene expression was presented as normalized ΔC_T, transformed by 2^n where $n = (-\Delta C_T)$ [22]. A p-value <0.05 was considered to be statistically significant.

RESULTS

The study population contained 20 young and 20 aged female virgin Wistar Hannover rats (mean age respectively 83±1 and 309±3 days). Aged rats weighed 29% more than young rats (208±2 g versus 268±5 g, p<0.01). Plasma concentrations of human relaxin after infusion were comparable (74±16 ng/ml versus 94±8 ng/ml, p=0.30, in young and aged rats, respectively). At baseline, aged rats showed larger vessel diameters than observed in young rats (reaching statistical difference in two experimental settings: myogenic reactivity and response to phenylephrine) with a comparable percentage preconstriction (Table 1). Basal diameter, percentage preconstriction and response to 124 mM KCl of mesenteric vessels were comparable for relaxin versus placebo, and for the presence versus absence of L-NAME for each experimental setting (Table 1).

Flow-mediated vasodilation

In young rats, relaxin enhanced flow-sensitivity (decreased $Flow_{50\%}$) 2.67 fold (from 48±9 to 18±4 µl/min, p=0.001) without changing maximal reactivity ($Diam_{max}$ 26±2 and 31±5 % in relaxin- and placebo-treated rats, respectively; Figure 1 and 2). L-NAME blunted the relaxin effect on flow-sensitivity ($Flow_{50\%}$ 30±10 and 28±6 µl/min in relaxin- and placebo-treated rats, respectively). In aged rats, relaxin did not change flow-sensitivity nor maximal

Table 1. Baseline vascular characteristics of mesenteric arteries in young and aged rats pre-treated with relaxin (4 µg/h for 5 days) or placebo.

	Young				Aged			
	Placebo	**(n)**	**Relaxin**	**(n)**	**Placebo**	**(n)**	**Relaxin**	**(n)**
Flow-mediated vasodilation								
L-NAME absence								
- Basal diameter (µm)	296±16	(6)	314±15	(7)	341±15	(6)	316±9	(9)
- Preconstriction (µm)	173±17	(6)	197±7	(7)	207±6	(6)	201±10	(9)
- Preconstriction (%)	42±4		37±2		39±3		36±2	
L-NAME presence								
- Basal diameter (µm)	311±16	(5)	330±18	(4)	340±16	(5)	325±8	(7)
- Preconstriction (µm)	191±7	(5)	219±15	(4)	227±4	(5)	194±17	(7)
- Preconstriction (%)	38±2		36±5		35±3		41±5	
Myogenic reactivity								
- Basal diameter (µm)	304±11	(8)	320±11	(10)	341±7#	(7)	329±14	(8)
- Preconstriction (µm)	182±12	(8)	176±11	(10)	216±11	(7)	209±9	(8)
- Preconstriction (%)	41±3		45±3		37±3		36±2	
Vascular compliance								
- Basal diameter (µm)	309±8	(8)	320±11	(9)	335±11	(7)	322±12	(8)
Response to phenylephrine								
L-NAME absence								
- Basal diameter (µm)	254±9	(8)	234±10	(10)	282±9#	(10)	261±7	(10)
- Contraction to KCl (mN/mm^2)	9.1±0.6	(8)	9.8±0.4	(10)	9.4±0.5	(10)	8.6±0.5	(10)
L-NAME presence								
- Basal diameter (µm)	237±9	(9)	245±9	(10)	285±9#	(10)	281±8	(10)
- Contraction to KCl (mN/mm^2)	9.8±0.4	(9)	9.3±0.3	(10)	8.9±0.4	(10)	8.4±0.5	(10)

Values presented as mean ± SE, n = number of animals. # $p<0.05$ between young and aged rats.

reactivity ($Flow_{50\%}$ 50±10 and 40±9 µl/min and $Diam_{max}$ 34±6 and 23±4 % in relaxin- and placebo-treated rats, respectively), independent of NO blockade ($Flow_{50\%}$ 47±8 and 56±3 µl/min in relaxin- and placebo-treated rats, respectively). Aging itself only decreased flow-sensitivity 2.00 fold (from 28±6 to 56±3 µl/min, $p=0.001$) when NO production is blocked.

Myogenic reactivity and vascular compliance

In young rats, chronic relaxin infusion reduced $MR_{overall}$ and $MR_{>60}$ (from -1±2 to 7±3 µm/10 mmHg), but did not change $MR_{overall}$ and $MR_{>60}$ (from -1±4 to -1±2 µm/10 mmHg) in aged rats (Figure 3). Aging itself did not alter $MR_{overall}$ and $MR_{>60}$ (from -1±2 to -1±4 µm/10 mmHg), compared with young placebo-treated rats.

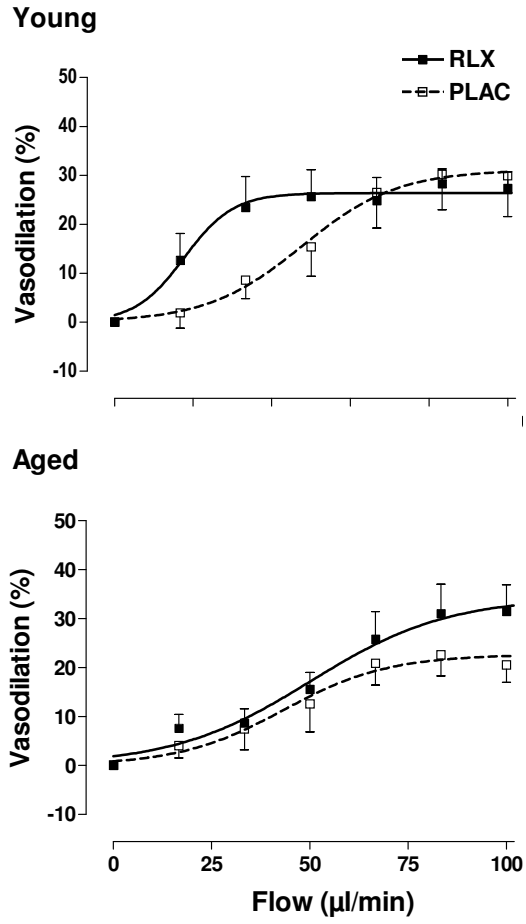

Figure 1. Flow-mediated vasodilator response of young (n_{RLX}=7 and n_{PLAC}=6) and aged (n_{RLX}=9 and n_{PLAC}=6) rat mesenteric arteries, pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC).

In young and aged rats (Figure 4), relaxin exposure did not affect stress-strain curves, $VC_{overall}$ and $VC_{>60}$ (young: from 3±1 to 3±2 µm/10 mmHg; aged: from 4±2 to 3±3 µm/10 mmHg). Aging itself increased $VC_{overall}$ ($p<0.01$) without affecting $VC_{>60}$ (from 3±1 to 4±2 µm/10 mmHg), compared with young placebo-treated rats. However, this is rather dependent upon enlarged basal diameter than changes in vessel elasticity, since both curves run parallel and the stress-strain relationship was comparable.

Response to phenylephrine

In young rats, relaxin reduced the sensitivity to phenylephrine ($C_{50\%}$) by 28% (p=0.01; Table 2), an effect partially persistent after NO blockade (19%, p=0.03). Relaxin did not affect R_{max} to phenylephrine. In aged rats, relaxin neither affected $C_{50\%}$ nor R_{max}, whereas

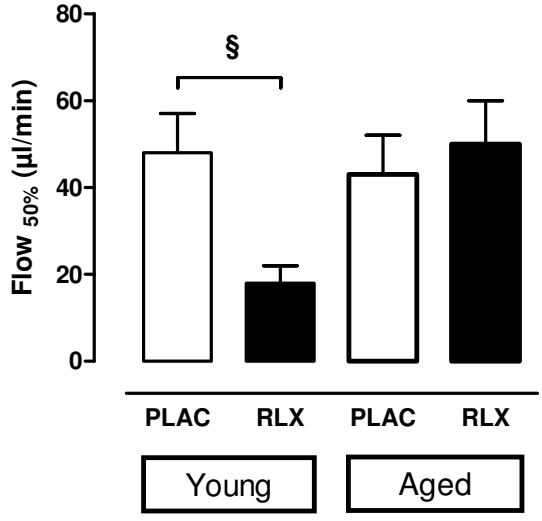

Figure 2. $Flow_{50\%}$ of young (n_{RLX}=7 and n_{PLAC}=6) and aged (n_{RLX}=9 and n_{PLAC}=6) rat mesenteric arteries, pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC). Data are presented as mean ± SE. § within age group difference p<0.05.

under NO blockade relaxin decreased $C_{50\%}$ by 67% (p=0.05). Aging itself did not alter either sensitivity or the maximum response to phenylephrine, although aging reduced the maximum response under L-NAME (p=0.05).

Isolated Perfused Rat Kidney model (IPRK)

In the kidney (Figure 5), in both young and aged rats, relaxin did not affect $RPFF_{baseline}$ (young: 6.7±0.1 and 6.9±0.2 ml/min/100g; aged: 6.0±0.1 and 5.7±0.1 ml/min/100g in relaxin- and placebo-treated rats, respectively), nor did it change the contribution of NO to the vascular tone, measured in $RPFF_{delta}$ (young: 4.1±0.3 and 3.9±0.4 ml/min/100g; aged: 4.3±0.2 and 4.1±0.2 ml/min/100g in relaxin- and placebo-treated rats, respectively). Aging reduced $RPFF_{baseline}$ by 17% (from 6.9±0.2 to 5.7±0.1 ml/min/100g, p=0.001) without affecting NO mediated vasodilation ($RPFF_{delta}$: 6.7±0.1 and 6.0±0.1 ml/min/100g), when comparing young versus aged placebo-treated rats.

Rxfp1 gene expression

Rxfp1 was expressed in the mesenteric arteries. However, there was no significant difference in expression between the young and aged rats treated with relaxin or placebo (Figure 6).

DISCUSSION

The present study reveals three important observations. Relaxin promotes mesenteric flow-mediated vasodilatation by stimulating the NO pathway, and reduces myogenic tone

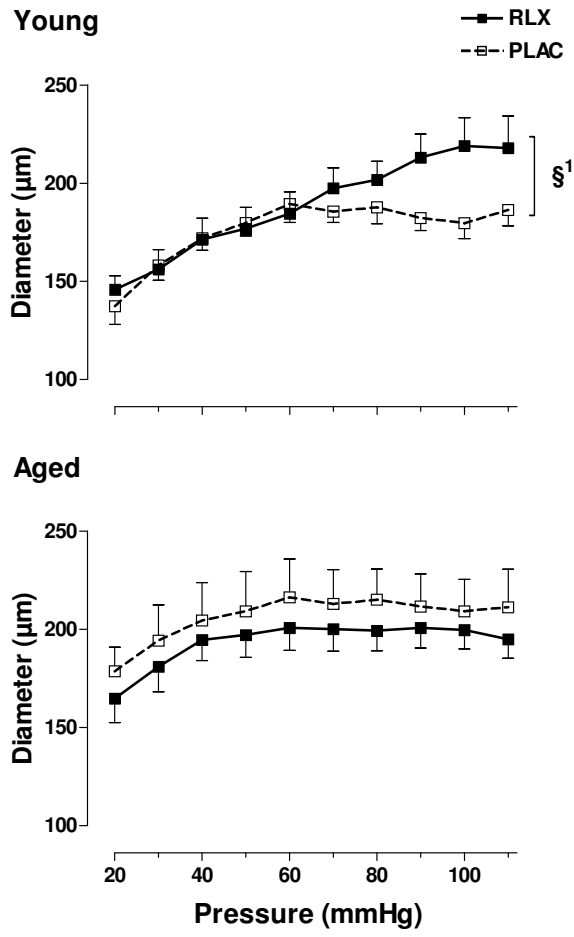

Figure 3. Myogenic reactivity of young (n_{RLX}=10 and n_{PLAC}=8) and aged (n_{RLX}=8 and n_{PLAC}=7) rat mesenteric arteries, pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC). § $p<0.05$ between placebo- and relaxin-treated rats. [1] represents difference in the within-subjects factor.

and responsiveness to phenylephrine. Interestingly, moderate aging appears to attenuate all of these relaxin-mediated effects. Finally, relaxin does not affect *ex vivo* RPFF in either young or aged rats.

Our data on functional responses in mesenteric arteries of young rats are mostly in line with observations made by others. Relaxin enhances flow-mediated vasodilation by increasing NO production at lower flow rates and decreases myogenic reactivity [3;4]. The increased NO production seems to be a direct effect of relaxin, since relaxin exposure to isolated cells directly stimulates NO synthesis [23]. Relaxin NO dependently reduces sensitivity to angiotensin-II [2], which is in line with our phenylephrine data. We did not detect any influence of relaxin on vascular compliance, although one study suggests that relaxin decreases compliance [4]. This discordance may be the result of a variety of differences in

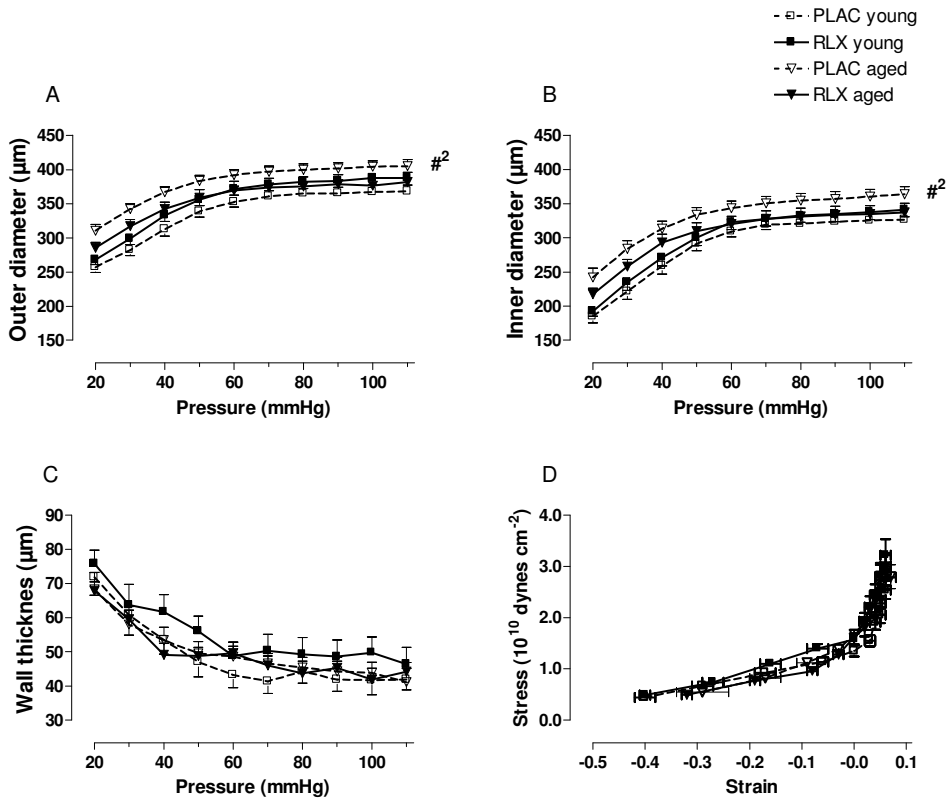

Figure 4. Vascular compliance of young (n_{RLX}=9 and n_{PLAC}=8) and aged (n_{RLX}=9 and n_{PLAC}=8) rat mesenteric arteries, pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC): outer vessel diameter (A), inner vessel diameter (B), vessel wall thickness (C) and stress-strain curve (D). # $p<0.05$ between young and aged rats. [2] represents difference in the between-subjects factor.

protocol, as Li et al. used Long Evans rats, supplies porcine relaxin at a rate of 4 µg/h for three days, and sets the pressure range of interest at 20-60 mmHg. Considering the extent of all changes, relaxin enhances the vasodilator state by affecting different local vascular responses, mainly by upregulation of NO mediated vasodilation.

Vascular effects of aging in female rodents is still a topic of debate, since some observed reduced NO dependent flow-mediated vasodilation [24] and myogenic reactivity [25], and increased phenylephrine reactivity [26], while others did not detect any age-dependent effects on these parameters [27]. We were also unable to observe an age-induced effect on NO dependent vasodilation, myogenic reactivity or sensitivity to phenylephrine. Additionally, aging enhances vascular compliance [25]. In line with morphological data [28], this is mainly due to increased basal vessel diameter rather than changes in vessel elasticity, considering the shape of the curve (Figure 4). As far as we know, this is the first study addressing effects of relaxin on mesenteric vascular function in relation to aging. We observed that, at the onset of reproductive senescence, basal responses of mesenteric arteries are comparable to those

Table 2. Phenylephrine response curve-fit estimates of young and aged rat mesenteric arteries pre-treated with relaxin (4 µg/h for 5 days) or placebo. Experiments performed in the wire myograph in absence and presence of 100 µM L-NAME.

		Placebo	(n)	Relaxin	(n)
* $C_{50\%}$ (µM)	- young	1.39±0.08	(8)	1.78±0.10§	(10)
	- aged	1.07±0.10	(10)	1.31±0.24	(10)
	- young (L-NAME)	0.89±0.04	(9)	1.06±0.07§	(10)
	- aged (L-NAME)	0.72±0.07	(10)	1.20±0.29§	(10)
* R_{max} (%)	- young	115±4	(8)	113±4	(10)
	- aged	111±6	(10)	118±11	(10)
	- young (L-NAME)	127±3	(9)	125±4	(10)
	- aged (L-NAME)	115±5#	(10)	122±14	(10)

Curve-fit estimates: concentration of phenylephrine inducing a 50% response ($C_{50\%}$), maximum response (R_{max}) as a percentage of the maximum response to 124 mM KCl. Values presented as mean ± SE, n = number of animals. § $p<0.05$ between placebo- and relaxin- pre-treated rats, # $p<0.05$ between young and aged rats.

observed in young rats, implying normal endothelium function in the moderately aged rat. However, all relaxin-induced mesenteric vasodilator responses, mostly NO dependent, are blunted. These findings suggest that, despite normal endothelium function, the endothelium in aged subjects suffers from endothelium-dependent resistance to relaxin.

Relaxin is known to increase *in vivo* renal plasma flow up to 40% by stimulating the NO pathway [2] and to reduce myogenic reactivity in isolated renal arteries [3]. In aged rats, the increase in renal plasma flow is even more pronounced [13]. We observed that in isolated kidney, relaxin did not affect RPFF in either young or aged rats. One may allege that renal plasma flow and RPFF are not the same parameters, although likely to be strongly related, and that perfusate viscosity and solutions oxygen delivery capacity are important factors jeopardizing whole organ perfusion function. In our model the oxygen-carrier Pluronic was added to the perfusate to optimize both factors. In the presence of this supplement we were able to detect a subtle aging-induced reduction in RPFF without concomitant changes in NO availability, as observed *in vivo* studies [13], possibly as a consequence of age-related loss in the number of functional glomeruli [29]. We therefore think that merits of the model are unlikely to explain the absence of response to relaxin. Our findings indicate that, in the presence of both myogenic and junxtaglomerular control, the isolated kidney is insensitive to relaxin in both young and aged subjects and that extra-renal factors mediate relaxin-dependent renal vasodilation.

There are some limitations to our study design. First, our human relaxin plasma levels were a little higher than 30 ng/ml, as reported by others [2]. This may be explained by differences in volume distribution or relaxin metabolism and clearance among different rat strains. Second, literature reports different physiological levels of relaxin among humans and rats (1 ng/ml and 30 ng/ml, respectively) [30]. It is difficult to interpret these differences and make comparison to our study, since there is no international standardization in the various relaxin assays used.

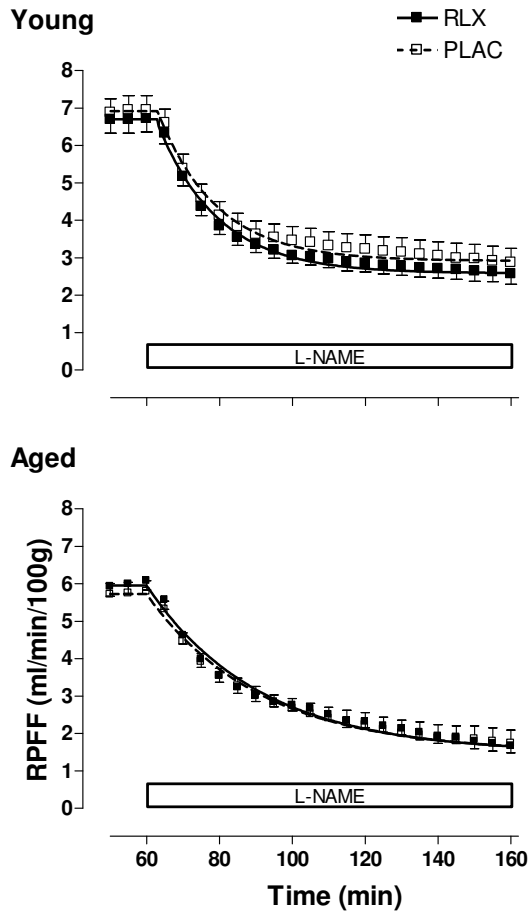

Figure 5. Renal perfusion flow (RPFF) in response to 100 μM L-NAME (at 60 minutes) in young (n_{RLX}=9 and n_{PLAC}=9) and aged (n_{RLX}=8 and n_{PLAC}=8) rats, pre-treated with relaxin (RLX, 4 μg/h for 5 days) or placebo (PLAC).

Third, the female rats were not selected for a particular stage of their estrous cycle. This may have affected our results, since high levels of estrogen could affect vascular tone. However, our randomization procedure was designed to distribute the estrous stages equally among groups, and this is unlikely to account for the observed effects of relaxin.

It is thought that the vascular effects of relaxin are mediated through activation of the G protein-coupled relaxin family peptide receptor (*Rxfp1*), initiating both an inferior vasoconstrictor and a predominant vasodilator pathway. Activation of the mitogen-activated protein kinase (MAPK) pathway induces vasoconstriction [31], where stimulation of the NO synthase-mediated phosphatidylinositol 3-kinase (PI_3K) pathway enhances vasodilation [32]. Moreover, matrix metalloprotease-2 is also involved [33]. The influence of relaxin on the vascular tone is likely a combined result of these pathways. It is unlikely that aging attenuates vascular *Rxfp1* expression because we did not detect any differences between

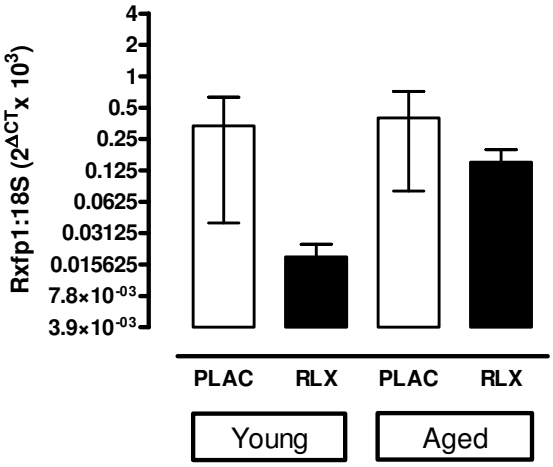

Figure 6. *Rxfp1* gene expression in mesenteric arteries in young (n_{RLX}=8 and n_{PLAC}=7) and aged (n_{RLX}=5 and n_{PLAC}=5) rats, pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC). Data are presented as mean ± SE $2^{-\Delta CT}$.

young and aged rats. Because aging is associated with down regulation of the vasodilatory PI_3K pathway at the Akt/protein kinase B level [34;35], this implies that aging affects the down-stream pathway. This may explain the aging-induced relaxin-resistance, as relaxin equally activates both the vasodilator and vasoconstrictor pathway. Whether higher concentrations of relaxin may be capable of overcoming the attenuated response, seen at a lower dose, remains to be elucidated. Nevertheless, one may speculate that resistance to relaxin relates aging-associated circulatory maladaptation with gestational hypertensive disease.

In summary, aging abolishes the physiological vasodilator response to relaxin, without affecting baseline vascular function. The disability to develop a pronounced relaxin-induced vasodilator state supports our concept of relaxin-resistance in aged subjects.

ACKNOWLEDGEMENTS

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CHAPTER 7

IMPAIRED EFFECT OF RELAXIN ON VASOCONSTRICTOR REACTIVITY IN SPONTANEOUS HYPERTENSIVE RATS

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ABSTRACT

Relaxin is thought to be involved in vasodilation to pregnancy by increasing endothelium-dependent vasodilation and compliance, and decreasing myogenic reactivity. Primary (essential) hypertension predisposes to circulatory maladaptation and subsequent gestational hypertensive disease. This study aimed to determine that vascular responses to chronic exposure to relaxin are impaired in young female rats with primary hypertension. In 10-12 weeks old Wistar-Hannover rats (WHR) and spontaneous hypertensive rats (SHR), we determined vascular responses in isolated kidney and mesenteric arteries after 5 days of chronic exposure to relaxin (4 µg/h) or placebo. SHR show decreased sensitivity to phenylephrine (by 67%, $p<0.01$) and renal perfusion flow (RPFF, by 19%, $p<0.01$), but no changes in flow-mediated vasodilation, myogenic reactivity or vascular compliance. In WHR, relaxin stimulated flow-mediated vasodilation (2.67 fold, from 48±9 to 18±4 µl/min, $p=0.001$), inhibited myogenic reactivity (from -1±2 to 7±3 µm/10 mmHg, $p=0.01$), and decreased sensitivity to phenylephrine (28%, from 1.39±0.08 to 1.78±0.10 µM, $p<0.01$), but left compliance and RPFF unchanged. NO blockade by L-NAME diminished most relaxin-mediated responses. In SHR, the vasodilator effects of relaxin were blunted for myogenic reactivity and sensitivity to phenylephrine, with similar effects on flow-mediated vasodilation, compliance, RPFF and equal *Rxfp1* (relaxin family peptide receptor) gene expression, as compared with WHR. Primary hypertension blunts both the relaxin-induced inhibition of myogenic reactivity and α-adrenergic vasoconstrictor response, independent from *Rxfp1* gene expression, while the relaxin-dependent enhanced flow-mediated vasodilation remains intact. This implies selective resistance to relaxin in young subjects suffering from primary hypertension.

INTRODUCTION

Relaxin, a member of the insulin-like growth factor superfamily, is thought to play an important role in pregnancy-associated vasodilation [1]. *In vivo*, relaxin induces renal vasodilation by activation of endothelium nitric oxide (NO) release and attenuation of the response to angiotensin-II in rats [2]. Additionally, *in vitro*, relaxin decreases myogenic reactivity [3], and increases compliance [4] in renal and mesenteric rat arteries. Overall, relaxin infusion in nonpregnant female rats accomplishes vasodilation comparable to that observed in pregnancy [5-7], whereas relaxin neutralizing antibodies completely blunt pregnancy-induced vasodilation [8].

In humans, preeclampsia predisposes to the development of chronic hypertension later in life [9], which poses individuals at risk for cardiovascular disease [10]. Primary (essential) hypertension, in turn, increases the risk of subsequent preeclampsia by 25% [11;12]. In male hypertensive rats, relaxin exposure reduces the systemic arterial load, but also shows a relative insensitivity on the cellular level [13-16]. As preeclampsia is generally preceded by circulatory maladaptation in early pregnancy, these findings raise the question whether primary hypertension affects the normal pregnancy-associated relaxin-mediated vascular adaptation.

In this study, we tested the hypothesis that primary hypertension in spontaneous hypertensive rats (SHR) induces resistance to chronic exposure to relaxin, defined as the inability to produce comparable vascular responses to chronic relaxin exposure as observed in non-susceptible Wistar Hannover rats (WHR). Wistar Kyote rats (WKY), often seen as the usual SHR control, do not respond to relaxin as seen in other strains [17], show genetic dissimilarity to SHR of 50% [18] and were therefore not used as control. Both the mesenteric and renal vascular beds largely determine peripheral resistance in nonpregnant and pregnant condition [19-22]. To this end, we performed an experimental study on vascular responses in isolated mesenteric arteries and isolated kidney in nonpregnant female WHR and SHR, chronically exposed to relaxin or placebo.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Animal Experiments Committee of the Radboud University Nijmegen Medical Centre, the Netherlands, and was performed in accordance with the European guidelines on animal experiments. Twenty female virgin WHR and 20 virgin SHR (Harlan Netherlands, Horst, the Netherlands) were used for approximately parallel experiments. The WHR that were used also served as controls for other studies, their results have been previously reported [23;24]. SHR are known to have primary hypertension from 6 weeks of age [25]. Both groups received regular diet (ssniff R/M-H) and water *ad libitum*. We housed all rats in filter-top cages on a 12/12 hour light/dark cycle. At an age of 73-85 days, rats were randomly assigned (independent from estrous stage) to chronic infusion for 5 days with either placebo or recombinant human H2 relaxin (both containing 5mM sodium acetate, pH 5.0; Corthera, San Mateo, California, USA). Nonpregnant rats, exposed to 4 µg/h human relaxin for 5 days, present serum relaxin levels

of ±30 ng/ml and vascular responses comparable to those induced by chronic exposure to endogenous relaxin in mid-term pregnant rats [2;8]. The experimental protocol was similar to the one described before [24]. In short, on day 0, an osmotic minipump (Alzet, model 2001, DURECT Corporation, Cupertino, California, USA) was implanted subcutaneously, and infused human relaxin at a dose of 4 µg/h for 5 days. On day 5, the rats were anesthetized with an intraperitoneal injection pentobarbital (Apharmo, Arnhem, the Netherlands). Blood was sampled from the caval vein for plasma human H2 relaxin measurement (Human Relaxin-2 Quantikine ELISA kit DRL200, R&D Systems, Minneapolis, USA). We continued chronic exposure of the vessels to human relaxin or placebo during all experiments, by adding 30 ng/ml of the trial-medication supplied to the perfusate and bath solutions.

PRESSURE-PERFUSION MYOGRAPH

Flow-mediated vasodilation, myogenic reactivity and compliance were analyzed in a pressure-perfusion myograph (Pressure Myograph System-Model P100, Danish Myo Technology, Aarhus, Denmark). The responses were determined in basic phosphate buffered saline (PBS: 119 mM NaCl, 4.69 mM KCl, 25 mM $NaHCO_3$, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 5.5 mM glucose and 10 mM HEPES), with additional 2 mM EGTA and 0.01 mM Na-Nitroprusside (for calcium-free-PBS) to measure compliance and 2.5 mM $CaCl_2$, 0.027 mM Na_2EDTA (for calcium-PBS) to assess flow-mediated vasodilation and myogenic reactivity. The buffers were oxygenated with 95% O_2 and 5% CO_2 at a temperature of 37 °C. Second-order mesenteric arteries (150-400 µm) were mounted on two opposing glass cannulae (125 µm). Vascular inner and outer diameter was measured through video recording (Vessel View, Danish Myo Technology, Aarhus, Denmark). Arteries were equilibrated at an intraluminal pressure of 60 mmHg and basal diameter was measured. Thereafter, vessels were preconstricted to 30-40% of their basal diameter by use of the thromboxane A2 agonist, U46619 (9,11-Dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands).

I. Flow-mediated vasodilation

After reaching a stable contraction, the flow in the vessel was increased every 2 minutes in 16.7 µl/min steps from 0 up to 100 µl/min (comparable to shear-stress range of 0-10 dyne/cm^2), in the absence and presence of 100 µM of the NO antagonist L-NAME (L-Nitro-Arginine Methyl Ester, Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands). Intraluminal pressure was maintained at 60 mmHg. Vessels attaining less than 20% preconstriction, less than 10% vasodilation over the completed flow-range or with instable pressure due to fluid leakage were excluded from the study. Flow-mediated vasodilation was expressed as a percentage of the preconstriction status.

II. Myogenic reactivity and vascular compliance

A new mesenteric vessel was isolated and prepared as described above. Intraluminal pressure was increased every 2 minutes in 10 mmHg steps from 20 mmHg up to 110 mmHg

to determine successive myogenic reactivity and compliance. After the 2-minute time interval vessels have reached a stable vascular tone. For compliance, outer and inner vessel diameters, and wall thickness were measured, and stress-strain relationship was obtained.

Wire myograph (response to phenylephrine)

The vasoconstrictor response to phenylephrine (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) was analyzed in a Mulvany Halpern myograph (Dual Wire Myograph-Model 400A, Danish Myo Technology, Aarhus, Denmark). The bath contained a physiological salt solution (PSS: 119 mM NaCl, 4.69 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 5.5 mM glucose and 0.027 mM EDTA) and was oxygenated with 95% O_2 and 5% CO_2 at a temperature of 37 °C. Four second-order mesenteric arteries (150-400 µm) were mounted in two wire myographs. The response to 124 mM KCl was measured and used for normalization of the phenylephrine response. The response to phenylephrine was determined in eight steps over a range from 10^{-7} M to 10^{-5} M, in the absence or presence of 100 µM L-NAME. The data of the two arteries in each myograph were averaged when available.

Isolated Perfused Rat Kidney model (IPRK)

We assessed relaxin-induced vascular adaptation of the isolated kidney in the IPRK. Within the time frame of our protocol, this model shows stable RPFF and provides an indication of renal function [26], but may not reflect effective renal plasma flow responses *in vivo*. Briefly, the right renal artery and the right ureter were cannulated. Perfusion was started *in situ* and the kidney was removed and placed in a perfused bath. The kidney was perfused at constant pressure of 90 mmHg and a temperature of 37 °C with oxygenated cell-free Krebs-Ringer-Henselheit (containing 113 mM NaCl, 4.8 mM KCl, 25 mM $NaHCO_3$, 1.4 mM KH_2PO_4, 2.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, and 5 mM Glucose) with 1.7 mM Pluronic F-108 (oxygen carrier and oncotic agent, BASF, Arnhem, the Netherlands). RPFF was recorded real-time (Midac testorganizer, W95 [V3.0], Radboud University Nijmegen Medical Centre, the Netherlands).

Stabilization of the RPFF was accomplished during a 40-minute period. From 40 to 60 minutes basal renal plasma flow ($RPFF_{baseline}$) was determined. At 60 minutes L-NAME was added at a final concentration of 100 µM to the perfusate. At 160 minutes a stable RPFF was observed ($RPFF_{160}$). RPFF was normalised for body weight (ml/min/100g body weight).

Rxfp1 gene expression

Rxfp1 gene expression was analyzed by Quantitative Polymerase Chain Reaction (qPCR), as described previously [27]. Total RNA was extracted from frozen mesenteric arteries with Trizol (GIBCO-BRL) according to the manufacturer's instructions. Forward/reverse primers and 6-carboxy fluorescein (FAM)-labeled TaqMan probes were specific for the full-length rat *Rxfp1* from Biosearch Technologies (Novato, California, USA). *Rxfp1* gene expression was compared with expression of the ribosomal 18S reference gene and presented as normalized ΔC_T, transformed by 2^n where $n = (-\Delta C_T)$ [27].

Statistical analysis

The data were expressed as mean ± standard error of the mean (SE). Flow-mediated vasodilation, stress-strain relationship, response to phenylephrine and RPFF were analyzed by nonlinear regression curve fitting (GraphPad Prism 4.0, Institute for Scientific Information, San Diego, USA). Subsequent curve fit estimates were: $Diam_{max}$ [maximal vascular diameter after flow change], $Flow_{50\%}$ (flow rate inducing 50% dilation, flow-sensitivity), K_1 and K_2 (exponential constants for stress-strain relationship), R_{max} (maximum response to phenylephrine) and $C_{50\%}$ (phenylephrine concentration inducing a 50% response), $RPFF_{baseline}$ (basal RPFF), and $RPFF_{delta}$ ($RPFF_{baseline}$ minus $RPFF_{160}$). Overall myogenic reactivity ($MR_{overall}$; corrected for percentage preconstriction) and overall vascular compliance ($VC_{overall}$) were analyzed by using analysis of variance (ANOVA) for repeated measures (Greenhouse-Geisser correction; SPSS 16.0.2, SPSS, Chicago, Illinois, USA). Straight line curve fitting was performed for both myogenic reactivity ($MR_{>60}$) and compliance ($VC_{>60}$), estimating the slope over the range of 60 to 110 mmHg. Baseline characteristics were analyzed with Student's t-test. In all analyses, we compared relaxin versus placebo in WHR, relaxin versus placebo in SHR, and placebo-treated WHR versus placebo-treated SHR. A p-value <0.05 was considered to be statistically significant.

RESULTS

The study population contained 20 WHR and 20 SHR female virgin rats (4-10 per group for each measurement) with average ages of 82±1 days in both groups when sacrificed. Overall, body weight was significantly lower (13%) in SHR, as compared with WHR (180±2 g versus 208±2 g, $p<0.001$). Plasma concentration of human H2 relaxin was comparable in both strains exposed to relaxin (74±16 ng/ml in WHR versus 77±9 ng/ml in SHR, $p=0.88$).

Vascular baseline characteristics were generally comparable between WHR and SHR (Table 1). For flow-mediated vasodilation experiments in the absence of L-NAME, the amount of preconstriction was slightly less in placebo-treated SHR compared with placebo-treated WHR (32±1 versus 42±4 %, $p=0.01$). For myogenic reactivity experiments, the diameter after preconstriction was higher in relaxin- versus placebo-treated SHR, although the percentage preconstriction was comparable. For the vascular compliance experiments, placebo-treated SHR showed a smaller basal diameter as compared with placebo-treated WHR. The concentration U46619 used for preconstriction was comparable for all groups and experiments. For the response to phenylephrine, the contraction to 124 mM KCl was increased in placebo-treated SHR, compared with placebo-treated WHR.

Flow-mediated vasodilation

Flow-mediated vasodilation in mesenteric arteries of placebo-treated rats was comparable between SHR and WHR in the absence ($p=0.28$) or presence ($p=0.92$) of L-NAME (Table 2). In both WHR and SHR (Figure 1 and Table 2), relaxin stimulated flow-sensitivity (decreased $Flow_{50\%}$) 2.67 fold ($p=0.001$) and 3.78 fold ($p=0.001$), respectively, without affecting maximum dilated diameter ($Diam_{max}$). This effect was completely NO dependent, as L-NAME blunted the increase in sensitivity to relaxin in both WHR and SHR.

Table 1. Baseline vascular characteristics of mesenteric arteries in female WHR and SHR pre-treated with relaxin (4 µg/h for 5 days) or placebo.

	WHR				SHR			
	Placebo	**(n)**	**Relaxin**	**(n)**	**Placebo**	**(n)**	**Relaxin**	**(n)**
Flow-mediated vasodilation								
L-NAME absence								
- Basal diameter (µm)	296±16	(6)	314±15	(7)	274±9	(7)	279±12	(9)
- U46619 for preconstriction (µM)	0.20±0.08		0.14±0.03		1.58±0.93		0.66±0.18	
- Preconstriction (µm)| (%)	173±17 42±4		197±7 37±2		187±7 32±1#		182±8 35±1	
L-NAME presence								
- Basal diameter (µm)	311±16	(5)	330±18	(4)	276±6	(10)	281±10	(10)
- U46619 for preconstriction (µM)	0.38±0.12		0.10±0.01		2.38±0.98		0.72±0.17	
- Preconstriction (µm) (%)	191±7 38±2		219±15 36±5		161±13 42±4		175±12 38±3	
Myogenic reactivity								
- Basal diameter (µm)	304±11	(8)	320±11	(10)	266±15	(6)	294±10	(10)
- U46619 for preconstriction (µM)	0.18±0.05		0.18±0.07		0.66±0.28	(6)	0.26±0.04	
- Preconstriction (µm) (%)	182±12 41±3		176±11 45±3		168±5 36±2	(6)	199±7§ 32±1	
Vascular compliance								
- Basal diameter (µm)	309±8	(8)	320±11	(9)	278±12#	(9)	294±10	(10)
Response to phenylephrine								
L-NAME absence								
- Basal diameter (µm)	254±9	(8)	234±10	(10)	244±7	(10)	237±7	(10)
- Contraction to KCl (mN/mm^2)	9.1±0.6		9.8±0.4		12.3±0.8#		10.0±0.6§	
L-NAME presence								
- Basal diameter (µm)	237±9	(9)	245±9	(10)	232±6	(10)	228±9	(10)
- Contraction to KCl (mN/mm^2)	9.8±0.4		9.3 ±0.3		10.3±0.5		11.6 ±0.5	

Values presented as mean ± SE, n = number of animals. § $p<0.05$ between placebo- and relaxin- pre-treated rats, # $p<0.05$ between WHR and SHR.

Myogenic reactivity and vascular compliance

In the presence of calcium, myogenic reactivity was analyzed (Figure 2). In placebo-treated SHR and WHR myogenic reactivity was comparable ($MR_{overall}$ ($p=0.11$) and $MR_{>60}$ (respectively 5±3 and -1±2 µm/10 mmHg, $p=0.07$)), although SHR do not show a clear vasoconstrictor

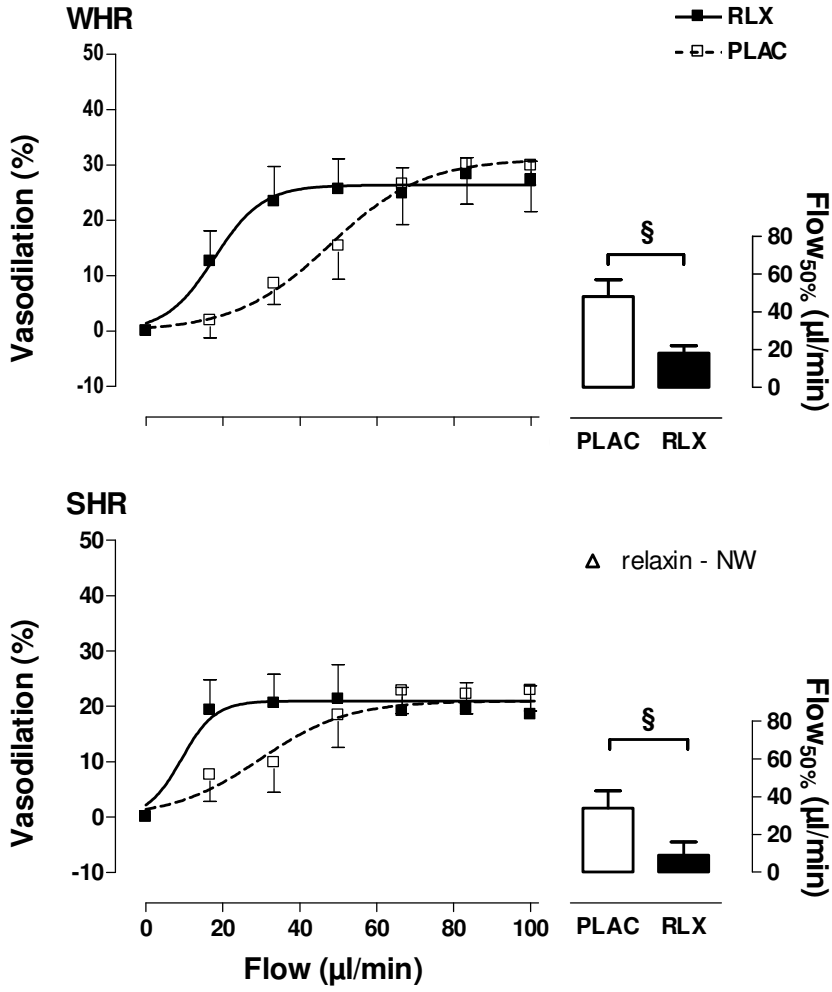

Figure 1. Flow-mediated vasodilator response (left figures) and corresponding $Flow_{50\%}$ (right figures) in female WHR (n_{RLX}=7 and n_{PLAC}=6) and SHR (n_{RLX}=9 and n_{PLAC}=7) mesenteric arteries, pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC).

§ $p<0.05$ between placebo- and relaxin-treated rats.

response at high pressure levels. Chronic infusion of relaxin in WHR reduced myogenic reactivity ($MR_{overall}$ (p=0.01) and $MR_{>60}$ (from -1±2 to 7±3 µm/10 mmHg, p=0.03)), while in SHR it did not ($MR_{overall}$ (p=0.83) and $MR_{>60}$ (from 5±3 to 4±2 µm/10 mmHg, p=0.75)).

In the absence of calcium, mesenteric arterial compliance was comparable between placebo treated SHR and WHR: $VC_{>60}$ (respectively 7±3 and 3±1 µm/10 mmHg, p=0.23), vessel wall thickness (p=0.07) and stress-strain relationship (K_1: p=0.38; K_2: p=0.38) (Figure 3C and 3D). Outer and inner diameters in SHR were significantly reduced over the whole pressure range of interest ($VC_{overall}$ outer diameter: p=0.01, $VC_{overall}$ inner diameter:

Table 2. Flow-mediated vasodilation curve-fit estimates of the pressure-perfusion myograph experiments in female WHR and SHR in the absence and presence of 100 µM L-NAME.

Flow-mediated vasodilation		**Placebo**	**(n)**	**Relaxin**	**(n)**
* $Flow_{50\%}$ (µl/min)	- WHR	48±9	(6)	18±4§	(7)
	- SHR	34±9	(7)	9±7§	(9)
	- WHR (L-NAME)	28±6*	(5)	30±10	(4)
	- SHR (L-NAME)	26±9	(10)	24±7	(10)
* $Diam_{max}$ (%)	- WHR	31±5	(6)	26±2	(7)
	- SHR	23±3	(7)	20±2	(9)
	- WHR (L-NAME)	27±3	(5)	31±5	(4)
	- SHR (L-NAME)	50±7	(10)	39±4	(10)

Experiments performed in the pressure-perfusion myograph in absence and presence of 100 µM L-NAME. Curve-fit estimates: amount of flow inducing a 50% response ($Flow_{50\%}$), maximum response to flow ($Diam_{max}$) as a percentage of preconstriction to U46619. Values presented as mean ± SE, n = number of animals. § $p<0.05$ between placebo- and relaxin- pre-treated rats, * $p<0.05$ between absence and presence of L-NAME.

p=0.02, Figure 3A and 3B). Chronic infusion of relaxin did not significantly alter stress-strain curves, vessel wall thickness, $VC_{overall}$ or $VC_{>60}$ in both WHR and SHR.

Response to phenylephrine

Mesenteric arteries of placebo-treated SHR had a significantly reduced sensitivity to phenylephrine ($C_{50\%}$, $p<0.01$) by 67% and maximum response (R_{max}, p=0.02) by 10%, compared with WHR (Table 3). NO blockade blunted the R_{max} differences, while lower sensitivity to phenylephrine persisted in SHR versus WHR placebo-treated rats. Relaxin exposure reduced sensitivity to phenylephrine by 28% ($p<0.01$) in WHR (Table 3). Under NO blockade, this effect decreased but was still significant (p=0.04). This suggests that NO is responsible for approximately half of the relaxin-induced reduction in sensitivity to phenylephrine. In SHR, relaxin did not alter $C_{50\%}$ (p=0.21), independent from NO synthase inhibition. Relaxin did not affect the maximum response (R_{max}) in both strains, independent from NO blockade.

Isolated Perfused Rat Kidney model (IPRK)

In placebo-treated rats, SHR showed 19% (5.6±0.1 versus 6.9±0.2 ml/min/100g, $p<0.01$) lower $RPFF_{baseline}$ and 18% (3.2±0.1 versus 3.9±0.4 ml/min/100g, p=0.03) less reduction in flow in response to L-NAME ($RPFF_{delta}$), when compared with WHR (Figure 4). The L-NAME induced reduction in flow as a percentage of $RPFF_{baseline}$ was comparable among SHR and WHR: both 57%. In both SHR and WHR, chronic infusion of relaxin did not change $RPFF_{baseline}$ (SHR: from 5.6±0.1 to 5.6±0.1 ml/min/100g; WHR: from 6.9±0.2 to 6.7±0.1 ml/min/100g) or $RPFF_{delta}$ (SHR: from 3.2±0.1 to 3.2±0.1 ml/min/100g; WHR: from 3.9±0.4 to 4.1±0.3 ml/min/100g).

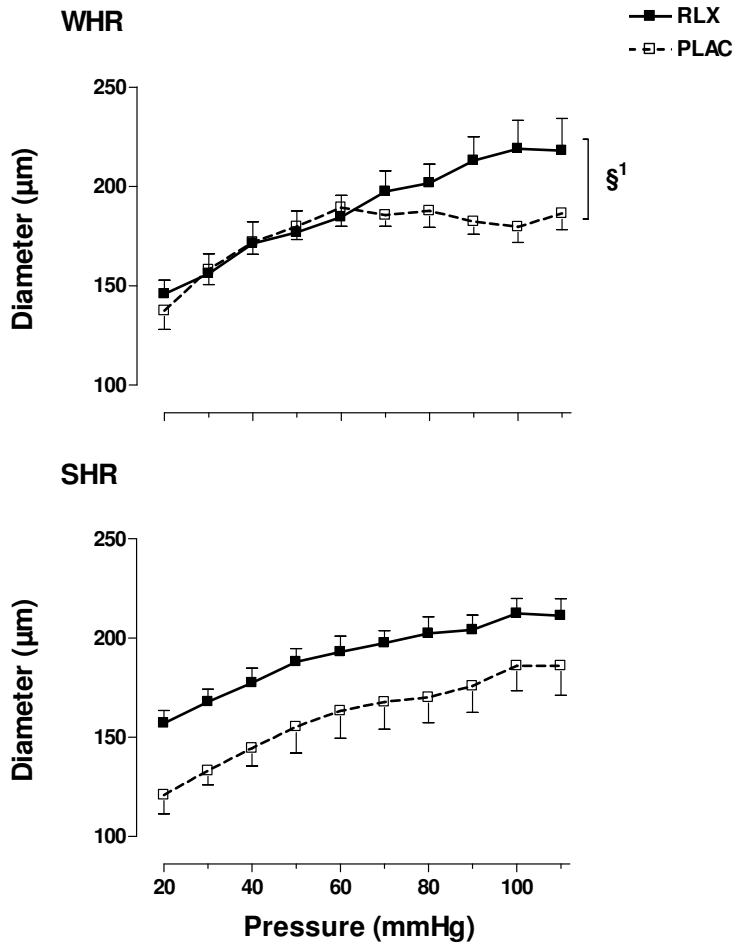

Figure 2. Myogenic reactivity (in presence of calcium) in female WHR (n_{RLX}=10 and n_{PLAC}=8) and SHR (n_{RLX}=10 and n_{PLAC}=6) mesenteric arteries, pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC).

§ p<0.05 between placebo- and relaxin-treated rats. [1] represent differences in the within-subjects factor.

Rxfp1 gene expression

Mesenteric arteries expressed *Rxfp1* with a high degree of variation (Ct value range 8.79-18.49). 18S expression was comparable among WHR and SHR, independent from relaxin exposure. We did not detect any difference in *Rxfp1* expression between WHR and SHR, and no effect of relaxin exposure (Figure 5).

DISCUSSION

Relaxin has been reported to enhance vasodilation. Our study implies that young SHR, which have primary hypertension, do not show the relaxin-induced inhibition of the

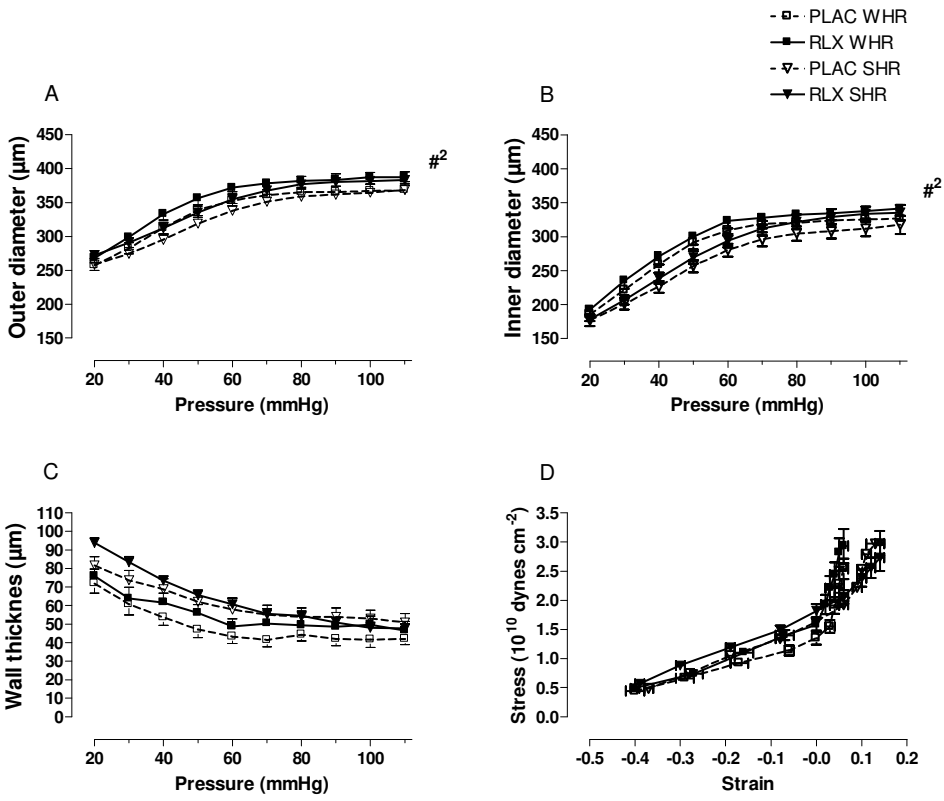

Figure 3. Vascular compliance (in absence of calcium) in female WHR (n_{RLX}=9 and n_{PLAC}=8) and SHR (n_{RLX}=10 and n_{PLAC}=9) mesenteric arteries, pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC): outer vessel diameter (A), inner vessel diameter (B), vessel wall thickness (C) and stress-strain curve (D).

# $p<0.05$ between WHR and SHR rats. [2] represents difference in the between-subjects factor.

myogenic reactivity and α-adrenergic vasoconstrictor response, as observed in healthy controls, while the enhanced flow-mediated vasodilation to relaxin remains intact.

Evaluation of mesenteric arteries in healthy female rats shows that relaxin affects several independent vascular responses. In agreement with the literature, our data show that relaxin reduces sensitivity to α-adrenergic activation by stimulation of the NO pathway [2] and decreases myogenic reactivity [3;4]. Additionally, we observed that relaxin enhances flow-mediated vasodilation without affecting vascular compliance. Flow-mediated vasodilation seems to be NO dependent, although this is based on a small amount of animals. Others have reported a relaxin-induced increase in vascular compliance [4]. Dissimilarities with our observation might be explained by differences in exposure protocol (using porcine relaxin at 4 µg/h for three days instead of human recombinant relaxin for 5 days) and methods to quantify vascular compliance (using Long Evans rats instead of WHR and setting the pressure range of interest at 20-60 mmHg compared with 60-110 mmHg). As the physiological mean arterial pressure in mesenteric arteries is around 90 mmHg, our data suggest that

Table 3. Phenylephrine response curve-fit estimates of the wire myograph experiments in female WHR and SHR in the absence and presence of 100 µM L-NAME.

		Placebo	(n)	Relaxin	(n)
* $C_{50\%}$ (log µM)	- WHR	1.39±0.08	(8)	1.78±0.10§	(10)
	- SHR	2.32±0.13#	(9)	2.26±0.17	(10)
	- WHR (L-NAME)	0.89±0.04*	(9)	1.06±0.07§	(10)
	- SHR (L-NAME)	1.80±0.14#*	(9)	1.66±0.14	(10)
* R_{max} (%)	- WHR	115±4	(8)	113±4	(10)
	- SHR	103±4#	(9)	113±5	(10)
	- WHR (L-NAME)	127±3*	(9)	125±4	(10)
	- SHR (L-NAME)	121±6*	(9)	117±6	(10)

Experiments performed in the wire myograph in absence and presence of 100 µM L-NAME. Curve-fit estimates: concentration of phenylephrine inducing a 50% response ($C_{50\%}$), maximum response (R_{max}) as a percentage of the maximum response to 124 mM KCl. Values presented as mean ± SE, n = number of animals. § $p<0.05$ between placebo- and relaxin- pre-treated rats, # $p<0.05$ between WHR and SHR rats, * $p<0.05$ between absence and presence of L-NAME.

relaxin does not change compliance, but induces vasodilation by affecting flow-mediated vasodilation, myogenic reactivity and sensitivity to α-adrenergic vasopressors.

Relaxin is known to have several positive influences on the cardiovascular status in senescent SHR: it reduces cardiac fibrosis, cardiac hypertrophy, cardiac after load and blood pressure, and increases cardiac output and arterial compliance [13;15;16;28;29]. To our knowledge, the effects of relaxin on the local vascular responses have not been investigated before. We found that, in young female rats, primary hypertension abolishes the relaxin-mediated inhibition of the myogenic reactivity and response to phenylephrine, with intact vasodilator effects of relaxin on flow-mediated vasodilation. This explains why others showed a relaxin-induced desensitizing effect to vasopressors in presence of continuous flow [14;17;30]. On systemic level this translates to persistence of relaxin-mediated systemic vasodilation in conscious hypertensive rodents [13]. Apparently, primary hypertension blunts the relaxin-desensitizing effect on myogenic reactivity and vasoconstrictor agents, but does not affect flow-mediated normal vascular adjustments.

The strain of rats used in our study may have affected the results. SHR is a widely accepted animal model for insulin-resistance and primary hypertension at young age. Based on strain similarities, Wistar Kyoto Rats (WKY) generally function as a control for SHR. However, WKY do not respond to relaxin as seen in other strains [17]. Above that, the WKY has genetic dissimilarity of 50%, as compared with SHR, questioning its role as a optimal control for SHR [18]. Therefore, we unconventionally used WHR as a control for SHR. In correspondence to literature comparing WKY and SHR [31-33], we observed that SHR do not differ from WHR in response to flow-mediated vasodilation, myogenic reactivity, vascular compliance, but show reduced α-adrenergic sensitivity.

The *in vivo* effects of relaxin on the renal plasma flow (RPF) are different from those observed *in vitro*. *In vivo*, relaxin NO dependently enhances RPF by 40% in normotensive

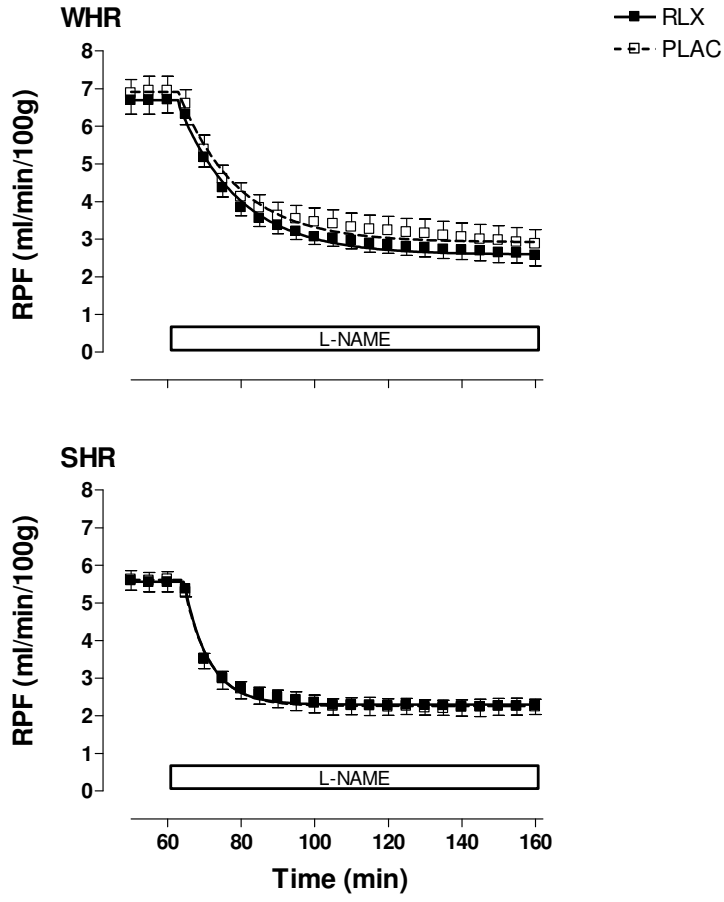

Figure 4. Renal perfusion flow (RPFF) in response to 100 µM L-NAME (at 60 minutes) in female WHR (n_{RLX}=9 and n_{PLAC}=9) and SHR (n_{RLX}=9 and n_{PLAC}=10) rats, pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC).

female rats [2], where female SHR show increased NO availability, as compared with WKY [34]. In isolated setting, RPFF is unaltered after 5 days exposure to relaxin in both female WHR and SHR, while NO activity is reduced in placebo-treated SHR, as compared with placebo-treated WHR. One might argue that these differences could relate to reduced oxygen delivery *ex vivo* as a consequence of whole organ perfusion. We used the oxygen-carrier Pluronic in our experiments and, under these conditions, we were able to detect known differences in RPFF between WHR and SHR [35]. Since the *in vitro* kidney is depleted from extra-renal factors (like humoral and autonomic influences), our observations imply that relaxin does not intrinsically affect the kidney.

We would like to address several limitations to our study. First, the relaxin plasma levels in our study were higher than 30 ng/ml, as reported by others [2]. It has been described that relaxin levels comparable to ours are ineffective [36]. Differences in volume distribution, relaxin metabolism and clearance among different rat strains may be held responsible for

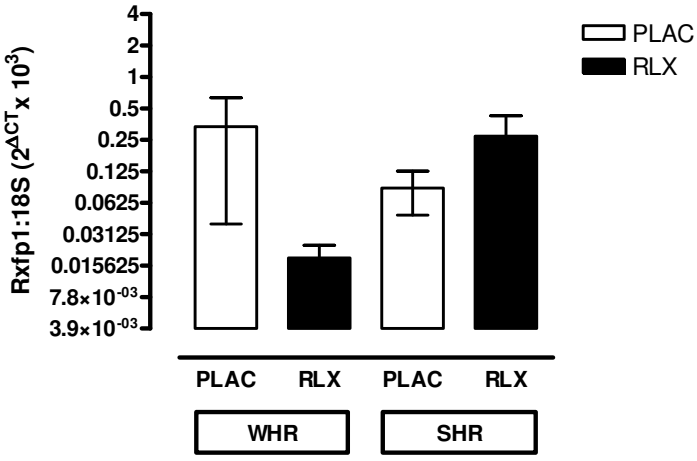

Figure 5. *Rxfp1* gene expression in mesenteric arteries in female WHR (n_{RLX}=8 and n_{PLAC}=7) and SHR (n_{RLX}=9 and n_{PLAC}=9), pre-treated with relaxin (RLX, 4 µg/h for 5 days) or placebo (PLAC).
Data are presented as mean ± SE $2^{-\Delta CT}$.

these dissimilarities. The different non-standardized techniques to measure the level of relaxin may have led to differences in relaxin levels between studies and make appropriate comparison not possible. As we observed significant effects in the mesenteric arteries at comparable "higher" relaxin levels in WHR and SHR, we do not think this explains the unresponsiveness of the kidney. Second, we started experiments independent of the estrous cycle, which may have affected vascular function. Since we performed randomization, the stages should have been equally distributed among groups and therefore we expect this does not explain the observed effects of relaxin. Third, the surgical procedure for placement of the osmotic minipumps may have influenced our observations. This is unavoidable and has affected both relaxin- and placebo-treated WHR and SHR equally.

Our findings on relaxin in WHR and SHR can be explained by the cellular pathway involved and may have clinical implications. Relaxin is thought to accomplish vasodilation by activation of the endothelium cell (EC) Gq_{EC}-protein-coupled relaxin family peptide receptor (*Rxfp1*). This pathway upregulates NO production, by activation of phosphatidylinositol 3-kinase (PI_3K) [37], which enhances vasodilation in the smooth muscle cell (SMC). This pathway is intact in both WHR and SHR. The direct effects of relaxin on the SMC remain unclear, but may be induced by affecting the Gq_{SMC}-coupled pathway for vasoconstrictor stimuli, activation of a vasodilator pathway or desensitizing calcium-channels. Possibly, one or more of its actions are less functional in SHR, which leads to resistance for relaxin-mediated inhibition of the myogenic reactivity and response to α-adrenergic stimuli. It is unlikely that the changes depend on *Rxfp1* receptor expression, as we did not detect differences between female WHR and SHR. The presence of insulin-resistance in SHR also does not explain our findings, as one might expect that it would affect both EC- and SMC-dependent responses and not SMC-dependent responses only. Although relaxin-induced flow-mediated vasodilation remains unchanged in

SHR, the impaired effects on myogenic reactivity and response to α-adrenergic stimuli may have implications for therapeutic use of relaxin, and may possibly be a contributing factor jeopardizing vascular function in pregnancy in hypertensive subjects.

In summary, primary hypertension relates to inhibition of the relaxin-mediated decrease in myogenic reactivity and desensitization to phenylephrine as present in young female healthy rats, while flow-mediated relaxin-induced vasodilation remains intact. This suggests selective relaxin resistance in female SHR.

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CHAPTER 8

GENERAL DISCUSSION

This thesis posed three research questions: 1. To what extent are the pregnancy-induced changes in the renal and mesenteric vasculature similar among species, strains and gestational periods (Chapter 2 and 3)? 2. To what extent is midgestational vasodilation mediated by upregulation of endothelium-dependent mechanisms and vascular compliance, and downregulation of vasoconstrictor agent sensitivity and myogenic reactivity (Chapter 4)? and 3. To what extent are local vascular responses to relaxin impaired in overweight, aged and hypertensive female rats (Chapter 5, 6 and 7).

The studies have shown that renal and mesenteric vascular changes to pregnancy are markedly different among species, strains and gestational periods. Midgestational vasodilation is mediated by upregulation of flow-dependent mechanisms, no change in vascular compliance, and downregulation of sensitivity to phenylephrine and myogenic reactivity. Local vascular responses to relaxin are impaired in overweight, aged and hypertensive female rats, which suggests resistance to relaxin.

Studies on vasodilation important to pregnancy have been performed on pregnant and nonpregnant animals, *ex vivo* and *in vivo*, in healthy subjects and subjects with vascular risk factors. We used the resulting four categories in the further discussion. At the end of the chapter we describe possible pathways of vasodilation in healthy and complicated pregnancies, with special emphasis on the role of relaxin.

I. Ex vivo local vascular responses to pregnancy

We used the technique of systematic review and meta-analysis to investigate pathways involved in pregnancy-induced vasodilation *ex vivo*, thus in the absence of the normal humoral and autonomic environment. This methodology of systematic review is commonly used in human medicine, but is relatively new in the field of experimental animal research. We used a novel application in which we quantified alterations in vessel sensitivity and reactivity through stratification by species, strain, gestational age and pathways of receptor complex families.

The meta-analyses (Chapter 2 and 3) have shown that renal and mesenteric vascular changes to pregnancy are markedly different among species, strains and gestational periods. This raises the question which animal model best simulates human pregnancy and various health states, if any.

For our meta-analyses we aimed to include all species. Yet, by lack of data for most species, only rats, rabbits, guinea pigs, mice and pigs were included in the analyses. Between these species responses differed markedly. We could not determine which animal model best simulates human pregnancy, because no human studies on *ex vivo* vessel responses in aged-matched pregnant versus nonpregnant subjects are available.

Rats were best represented in the studies and involved various strains. We observed remarkable differences in responses between species. In Wistar rats (WR), a strain known for its cardiovascular health, pregnancy induces upregulation of endothelium-dependent vasodilation only, without affecting the other pathways. In contrast, Sprague Dawley rats (SDR), which are prone to develop cardiovascular disease, activate multiple local vascular responses in mid- and late pregnancy. These include not only endothelium-dependent vasodilation, but also enhanced compliance and deactivation of vasoconstrictor sensitivity

and myogenic reactivity. Impaired gestational circulatory adaptations precede preeclampsia, which in turn is associated with cardiovascular disease later in life. Based on these human observations, we speculate that the WR is a better model for healthy pregnancy and SDR for vascular compromised pregnancy.

Five studies in rats have systematically investigated the gestational age effects on vascular adaptation [1-5]. These studies, all in SDR and in line with other studies on this strain, showed that pregnancy affects the pathways involved in vascular tone differently across gestational ages. Limited data suggest that NO activation is more important in mid-pregnancy than in late pregnancy. Studies on WR, including our own (Chapter 4), compared pregnancy responses at a certain gestational age to nonpregnant responses. Those studies show that responses in mid-pregnancy do not differ much from those in late pregnancy. Apparently, depending on the strain, vascular adaptation is virtually complete at midgestation but the underlying mechanisms may differ.

Ex vivo responses of mesenteric and renal arteries have not been compared directly within studies. Renal artery responses have mainly been studied in SDR. Our meta-analyses (Chapters 2 and 3) show that in mesenteric arteries pregnancy enhances the endothelium cell- (EC) dependent vasodilator Gq_{EC}-pathway and reduces the smooth muscle cell- (SMC) dependent vasoconstrictor Gq_{SMC}-coupled pathway, while in renal arteries both pathways are unaffected by pregnancy. One may speculate that this is due to the fact that small downstream resistance vessels have been used to study mesenteric arteries, while the renal artery represents a major blood supplying vessel [6]. Therefore, studies on pregnancy-induced vasodilation should take into account not only the vascular region, but also the type of vessel.

Experiments on isolated vessels in the absence of hormonal and autonomic influences have their intrinsic limitations. First and foremost, responses on isolated vessels cannot safely be extrapolated to whole organ responses because of the absence of humoral and autonomic influences. Second, methodological differences between studies (e.g. type of myograph, perfusate) may affect results. Third, most studies are limited to a small number of animals. These methodological limitations can affect the overall outcome and conclusions from experiments. Therefore, results from single studies should be interpreted with caution, especially when results are extrapolated to more generalized conclusions. Meta-analysis can overcome these limitations to some extent, as their conclusions are based on multiple studies and they identify outlier results.

II. In vivo local vascular responses to pregnancy

In vivo studies have the advantage of investigating an integrated system in the presence of humoral and autonomic control. Data on *in vivo* adaptations to pregnancy were too limited to allow meta-analysis of mesenteric vascular responses. Our meta-analysis on renal adaptations included data on rats, sheep, rabbits and guinea pigs (Chapter 3). It showed marked differences for strains of rats and for gestational age. At midgestation, renal vascular resistance (RVR) is decreased by 27%, varying from 18% in SDR to 31% in Munich Wistar rats (MWR). Renal flow is increased to a variable degree, ranging from 13% in Wistar Hannover rats (WHR) to 31% in Long Evans rats (LER). GFR is enhanced

by 17% in SDR to 29% in LER (Chapter 3). At late gestation, RVR, renal flow and GFR remain at mid-pregnancy levels in SDR, whereas in LER these renal variables return to nonpregnant values. The reasons for the differences across strains and gestational ages have not been systematically investigated. One may speculate that the degree of vascular change depends on strain-specific differences in vascular control mechanisms, as observed *ex vivo*. This suggests that the vascular impaired SDR has a reduced hemodynamic reserve capacity compared with the healthier LER.

Our meta-analysis on MWR and LER data are in line with human data, showing reduced RVR, and increased renal flow and GFR in pregnancy (Chapter 3) [7]. The underlying pathways have not been studied in detail in humans because this requires blockade of pathways and would be unethical. In LER, changes in RVR, flow and GFR depend on upregulation of the NO system. In SDR, these changes are induced by both NO activation and deactivation of the autonomic nervous system (Chapter 3). These adaptations are likely secondary to humoral changes, as blockade of specific humoral antibodies can completely overcome the pregnancy-induced systemic vascular changes [8].

III. Humoral effects on vascular function in healthy subjects

Several hormones are involved in normal pregnancy-induced vasodilation, most notably estradiol, progesterone, human chorionic gonadotrophin and relaxin. These hormones have been investigated mainly in rats.

Estradiol induces systemic vasodilator responses as observed during pregnancy [9]. Its effects are region specific as it enhances uterine, renal, coronary and iliac perfusion, while the mesenteric region is relatively insensitive [9;10]. Estradiol reduces vascular tone through the estrogen receptors that are located on both the EC and SMC [11;12]. Stimulation of the EC receptor induces NO release which mediates the increase in uterine blood flow [13]. Estradiol also affects the SMC through activation of potassium channels [14]. Estrogens apparently are only partly responsible for the decrease in systemic vascular resistance in pregnancy as the mesenteric region shows profound pregnancy-induced vasodilation despite a lack of response to estradiol.

Progesterone has some vasodilator capacity, but its contribution to vasodilation in pregnancy is controversial [15]. On the one hand, progesterone induces vasodilation by increasing EC NO production [16;17], on the other hand it induces vasoconstriction through the SMC [18]. Some studies suggest that progesterone is the primary dilator in pregnancy, but other studies suggest that it is involved in the control of spatial distribution of the uterine blood flow rather than in generalized vasodilation [19]. Apparently progesterone is not the major vasodilator of pregnancy that it was once considered to be.

Human chorionic gonadotrophin (hCG) is a vasodilator [2]. It exerts its effect not by affecting the EC, but through deactivation of the SMC [2;20]. As hCG levels peak during the first trimester and are much lower thereafter, it is unlikely to be responsible for maintaining the vasodilator state later in pregnancy.

Relaxin induces vascular changes that mimic those of pregnancy [4;21;22]. Relaxin affects local vascular tone through various pathways. In healthy WR it stimulates flow-mediated

vasodilation, and downregulates myogenic reactivity and sensitivity to phenylephrine without affecting vascular compliance (Chapter 5, 6, 7, and Table 1). In other strains, including Long Evans rats (LER), relaxin also increases vascular compliance [23]. Isolated vessels from pregnant rats, in the absence of relaxin in the perfusate, only show increased flow-mediated vasodilation, whereas vessels continuously exposed to relaxin show activation of several additional vasodilator pathways (Chapters 4, 5, 6, and 7). This suggests that maximal dilatation requires not only intrinsic adaptation of the EC to pregnancy, but also the continuous presence of relaxin. *In vivo*, relaxin-specific antibodies block all the pregnancy-induced systemic vascular changes. These findings imply that relaxin is one of the most important hormones involved in vasodilation in pregnancy [8].

IV. Humoral effects on vascular function in subjects with vascular risk factors

Overweight or obesity, moderately advanced age (>40 years) and primary hypertension, are associated with increased risk for hypertensive disorders in pregnancy [24], which may lead to fetal and maternal morbidity and mortality. In addition, they are associated with increased risk for cardiovascular complications later in life [25-27]. One may speculate that impaired vascular adaptations in pregnancy are in part mediated by resistance to pregnancy-related hormones. The vascular effects of the pregnancy hormones estradiol, progesterone, hCG and relaxin have only been studied to a limited extent in subjects with these cardiovascular risk conditions. In our studies on the vascular effects of relaxin in overweight, aged and hypertensive rats, we showed that overweight rats, or those with advanced age or hypertension have impaired mesenteric *in vitro* responses to relaxin as compared with healthy controls (Table 1). In fact, overweight and aged rats do not show any vessel adaption to relaxin, while hypertensive rats lack the normal adaptation in myogenic reactivity or α-adrenergic response and respond to relaxin only by activation of flow-mediated vasodilation. This suggests the presence of relaxin- resistance in overweight, aged and hypertensive rats. One may question if the relaxin levels reached in our experiments reflect physiologic concentrations. We intended to simulate human recombinant relaxin levels comparable to those observed during human pregnancy (around 30 ng/ml). Despite an identical methode of relaxin exposure as used in other studies in rats, we measured higher levels of relaxin in our rats (around 70 ng/ml). Considering no shortcomings in continues infusion systems, several variables may have affected this finding, such as differences in volume distribution among stains of rats (compared to reported studied rats) or differences in test methodology, affinity or test tube degradation of human recombinant relaxin. This may imply that we detected relaxin-resistance at little higher supra-physiologic levels, but could also be the result of the different non-standardized techniques available to measure the level of relaxin. Because we performed our experiments similarly across groups, at least results can be compared throughout our study. Additionally, one may question the importance of relaxin-resistance in vascular impaired rats, as they seem to reproduce without having clearly compromised pregnancies as seen in humans. There are at least two factors that may contribute to the different response by limiting the metabolic and vascular demands for adaptation. First, severe experimental vascular impairment in rats

Table 1. Overview of the effects of relaxin- (RLX) and placebo- (PCB) treatment on flow-mediated vasodilation, vascular compliance, renal perfusion flow, response to phenylephrine and myogenic reactivity. Experiments performed in healthy, diet-induced overweight, advanced aged and hypertensive rats in the presence and/or absence of L-NAME.

	Healthy		Diet induced overweight		Advanced aged		Hypertensive	
	PCB	**RLX**	**PCB**	**RLX**	**PCB**	**RLX**	**PCB**	**RLX**
Vasodilation								
- Flow-mediated vasodilation:								
- in the absence of L-NAME	c	↑↑	↔	↔	↔	↔	↔	↑↑
- in the presence of L-NAME	c	↔	↔	↔	↓	↔	↔	↔
- Vascular compliance	c	↔	↔	↔	↔	↔	↔	↔
- Renal perfusion flow	c	↔	↓	↓	↓	↔	↓	↔
Vasoconstriction								
- Phenylephrine								
- in the absence of L-NAME	c	↓↓	↑	↔	↔	↔	↓	↔
- in the presence of L-NAME	c	↓	↔	↓	↔	↓	↓	↔
- Myogenic reactivity	c	↓	↔	↔	↔	↔	↔	↔

Effects of relaxin depicted as increased (↑),decreased (↓) or no change (↔) compared with placebo of the specific subgroup. Effects of the specific subgroup presented as increased (↑),decreased (↓) or no change (↔) compared with healthy placebo (c). Gray filling shows the effects favoring vasodilation. Striped filling shows the effects favoring vasoconstriction.

usually results in reduced litter size. Second, rats deliver their pups relatively premature. The limited litter size and short duration of pregnancy may well protect them from developing hypertension in pregnancy. Although the rat may not be the ideal model for human hypertensive pregnancy, the presence of relaxin-resistance in overweight, aged and hypertensive rats can be seen as a precursor for vascular impairment, which in humans may lead to poor placentation and hypertensive disease in pregnancy.

VASODILATOR PATHWAYS IN PREGNANCY

Gestational vasodilation is most likely regulated by several hormones, through activation and deactivation of a limited set of down-stream pathways. In this paragraph we discuss the known pathways involved in vascular tone in healthy nonpregnant, pregnant, relaxin-treated, and placebo-treated rats with vascular risk factors (overweight, advanced age and hypertension) (Figure 1). Vascular tone in nonpregnant healthy subjects is regulated by the EC (by flow-mediated vasodilation), SMC (through myogenic reactivity and the response to vasodilator and vasoconstrictor agents) and the extra-cellular matrix (ECM, by compliance) (Figure 1A). At midgestation, pregnancy upregulates only flow-mediated vasodilation (Figure 1B).

Relaxin stimulates the flow-mediated vasodilation through activation of the Gq_{EC}-pathway, and by deactivation of α-adrenergic constriction and myogenic reactivity

through downregulation of the Gq_{SMC}-pathway and calcium-influx in the SMC (Figure 1C). The vasodilator Gq_{EC}-pathway can be upregulated through four possible mechanisms: 1) enhanced sensitivity of the EC flow-receptors and Gq_{EC}-receptors which leads to increased NO production, 2) stimulation of specific down-stream Gq_{EC}-receptor reactions (stimulation of Akt/PKB and NO availability) which optimizes the response to flow and Gq_{EC}-specific substances, 3) direct stimulation of the relaxin specific Gq_{EC}-receptors and additional formation of NO, and 4) a combination of 1, 2 and 3. All these mechanisms result in upregulation of NO production, which can be regarded as an increase in sensitivity to flow and Gq_{EC}-receptor specific stimuli. Relaxin also changes the vasoconstrictor Gq_{SMC}-receptor pathway and myogenic reactivity, which are both partially dependent on NO production by the EC. As both pathways depend on an increase in intracellular calcium, it is possible that adaptation of these pathways to relaxin occurs through a common mechanism that has not yet been elucidated. It would be important to differentiate between the various mechanisms in order to better understand what happens in the overweight, aged and hypertensive rats. This would require relaxin specific Gq_{EC}- and Gq_{SMC}-receptor blocking agents, which have not yet been developed.

Subjects with vascular risk conditions show impaired relaxin-induced vascular responses. The various pathways involved are shown in Table 1. Overweight and aged rats show impaired upregulation of the flow-mediated vasodilator/Gq_{EC}-coupled response and reduced downregulation of the α-adrenergic and myogenic reactivity constrictor responses (Figure 1D and E). Hypertensive subjects have intact activation of the flow-mediated vasodilator/Gq_{EC}-coupled response, but reduced deactivation of the α-adrenergic and myogenic reactivity constrictor responses (Figure 1F). This does not seem to depend on relaxin receptor expression, as we did not detect any differences in *Rxfp1* (relaxin family peptide) gene expression. The mechanisms, as outlined above, indicate how cardiovascular risk conditions may negatively affect relaxin-mediated vasodilation through resistance to relaxin. As overweight, aging and hypertension carry an increased risk of hypertensive disease in pregnancy, one may speculate that relaxin-resistance may also be causally involved in the development of hypertensive disorders in human pregnancy.

CONCLUSIONS

This thesis provides two main insights. First, the extent of vascular changes in pregnancy depends on the species, strain, gestational age period and vascular region investigated. This implies that most of our current knowledge is based on animal models that hardly resemble other species and strains. Therefore, from a scientific point of view, extrapolation towards human pregnancy has severe limitations. Second, vascular risk conditions, such as overweight, advanced age and hypertension, impair the vascular adjustments to relaxin in pregnancy. This suggests that relaxin-resistance may well play role in the development of hypertensive disease in human pregnancy.

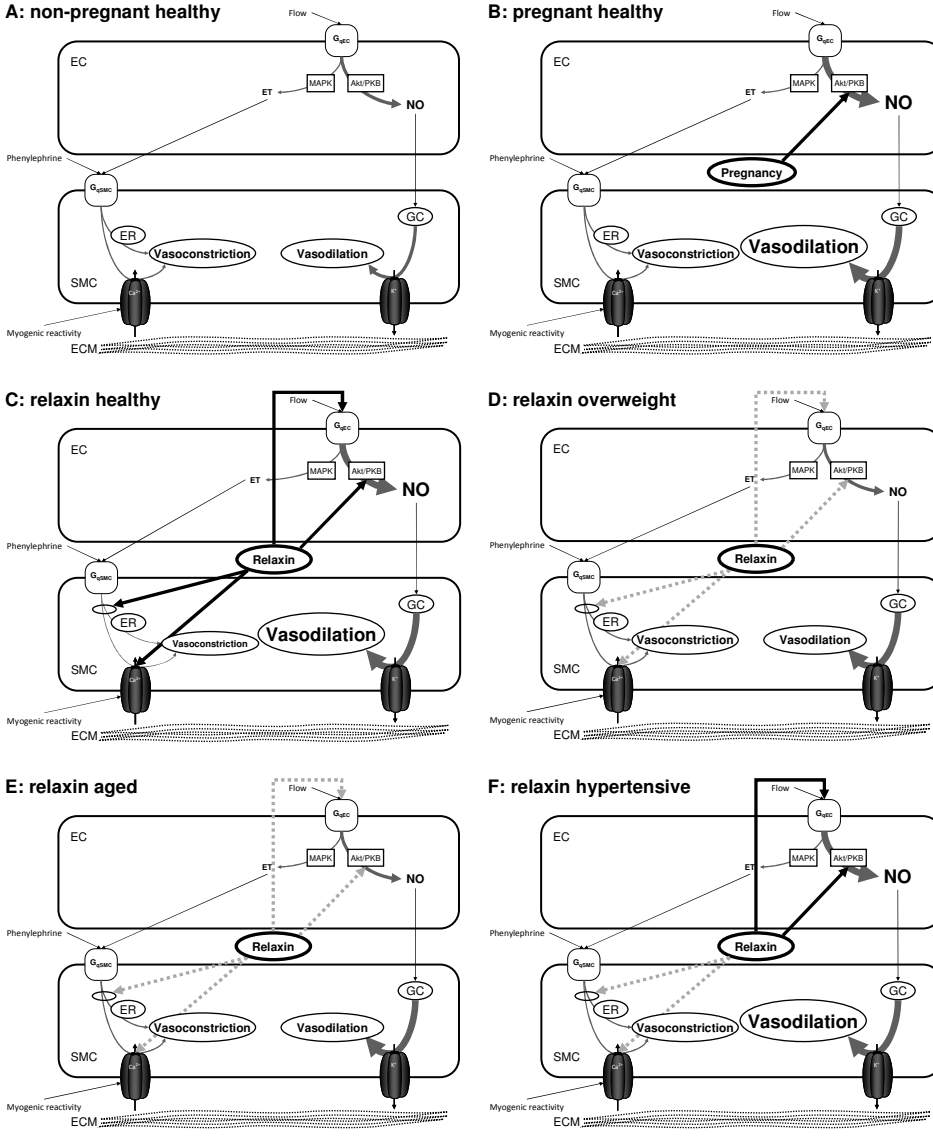

Figure 1. Simplified schematic overviews of the effects of pregnancy and relaxin exposure on the endothelium cell (EC) and smooth muscle cell (SMC) pathways involved in local vascular tone under different conditions.

In the absence of additional humoral and autonomic environment, the different conditions are: nonpregnant/ placebo-treated healthy subjects (A), mid-pregnant healthy subjects (B), relaxin-exposed state in healthy subjects (C), relaxin-exposed state in overweight subjects (D), relaxin-exposed state in aged subjects (E), relaxin-exposed state in hypertensive subjects (F).

FUTURE RESEARCH

Scientific research is a continuous process. The conclusions from one study induce new ideas for future research. Poignant questions that remain after completion of this thesis include the following:

1. Is it possible to identify women at risk for hypertensive disease in their first pregnancy, through a simple first-line vascular test before conception?
2. To what extent is relaxin-resistance really important in the development of hypertensive disease in human pregnancy?
3. Can we indentify an ideal animal model to simulate vascular adaptation in human pregnancy?

Identifying women at risk for hypertensive disease in their first pregnancy is most important but also most challenging. This type of research needs large numbers of seemingly healthy women before their first pregnancy. In addition one may raise the ethical question how legitimate it is to expose a large group of healthy women to testing for a disorder that will occur in only 1:20 tested individuals. If coupled to an effective treatment, such a test could potentially reduce the incidence of some of the biggest problems in obstetrics today, including preeclampsia, fetal growth restriction and iatrogenic prematurity.

A first step towards an effective treatment would be to resolve the underlying mechanism(s) of disease, which should include the role of relaxin-resistance in the development of hypertensive disease in human pregnancy. There are tools available to study this question, such as the forearm model, which may be used to test women with vascular risk factors (e.g. formerly preeclamptic, obese, over 40 years of age, hypertensive, diabetic, renal impaired) for possible impaired responses to relaxin-exposure as compared with healthy controls. Such a study should start by testing for relaxin-resistance prior to pregnancy and should continue until after the outcome of the next pregnancy is known. This would require a large cohort study and sufficient financial support. Yet, it seems a necessary first step before embarking on further studies on the importance of such a test in nulliparous women or on the efficacy of any interventions in relaxin-resistant women in reducing the chance of hypertensive disease in their next pregnancy.

It would be most useful if we could study the mechanisms of hypertensive disease in pregnancy in an animal model instead of in humans, as it would reduce the duration and costs of experimentation. Unfortunately, however, all animal models have limitations in mimicking human pregnancy, either in the healthy or the vascular impaired state. Preeclampsia is almost unique to primates, which are not readily available for experimental research. Rats and mice deliver their pups much more immaturely than humans. Nonetheless, vascular adaptations to pregnancy in selected species and strains are quite similar to those in humans. However, if we want to use an animal model to investigate mechanisms similar to vascular (mal)adaptation in human pregnancy, one has to be really careful in the choice of species, strain, gestation age and vascular bed. Meta-analysis on animal data is most helpful in differentiating between models that are appropriate to study mechanisms of vascular adaptation comparable to human pregnancy in health and disease. The uterine

artery, aorta and carotid artery are examples of vessels supplying vascular beds that may be relevant in this respect. Careful meta-analysis of such responses will result in overall reduction, replacement and/or refinement of animal use in pregnancy-specific research.

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CHAPTER 9

SUMMARY
SAMENVATTING
LEGENDS TO ABBREVIATIONS
DANKWOORD
CURRICULUM VITAE

SUMMARY

Pregnancy is known to induce a significant systemic vasodilator response in both humans and rodents, most frequently investigated in the mesenteric and renal vascular beds. The change reaches a maximum at midgestation and is likely affected by vascular risk factor (such as overweight, advanced age and hypertension). The substance responsible for this change is still unidentified, although it has been suggested that the hormone relaxin is involved. Both pregnancy and exposure to relaxin induce adaptation of several endothelium cell (EC) and smooth muscle cell (SMC) dependent local vascular responses in mesenteric and renal arteries: 1. Upregulation of the endothelium-dependent nitric oxide (NO) pathway, 2. Reduction of the responsiveness to vasoconstrictor stimuli, 3. Decrease in myogenic reactivity, and 4. Increase in arterial compliance. From published work, three observations can be made. First, it is unclear if one may generalize effects across species, strains and the gestational age periods, as results from various studies show substantial differences and inconsistencies. Second, data mainly focus on term pregnancy, and for some strains data on midgestational effects are not available. Third, relaxin may be an important factor in pregnancy-induced vasodilation. As certain risk conditions predispose to vascular maladaptation and subsequent hypertensive disease, one may propose the idea that vascular risk factors relate to poor vessel reactivity to relaxin and subsequent vascular maladaptation during pregnancy.

Based on the three observations, this thesis aimed to provide additional information on pregnancy-induced vasodilation by investigating three hypotheses:

1. The extent of renal and mesenteric vascular adaptation to pregnancy is similar across species, strains and the course of pregnancy.
2. Midgestational vasodilation in rats is mediated by upregulation of endothelium-dependent vasodilation, and alteration of vasoconstrictor agent sensitivity, myogenic reactivity and vascular compliance as observed at term.
3. Local vascular responses to relaxin are impaired in common risk associates with gestational maladaptation (overweight, increased age and hypertension).

The first hypothesis was tested in two separate meta-analyses. The first meta-analysis focused on the pregnancy-induced changes of local vascular control in mesenteric arteries (Chapter 2). Through a systematic literature search and selection process, we identified 55 (out of 398) studies with 188 vascular mesenteric responses to stimuli, mostly investigated in rodents at term. We observed that pregnancy facilitates flow-mediated vasodilation at term, among all investigated species. The contribution of additional local vascular responses is species- and strain specific. Late pregnancy mediates vasodilation through changes at the receptor level, for the substances tested. As the initial steps of vasodilation in early pregnancy have been investigated sparseley, this period deserves attention in the future.

The second meta-analysis investigated the pregnancy-induced vascular changes of the kidney (Chapter 3). We identified 37 (out of 1008) studies with 116 responses by performing a systematic literature search and selection process. We showed that pregnancy changes renal vascular resistance (RVR), renal flow and glomerular filtration rate (GFR) dependent

on NO activation and sympathetic deactivation, but adjustments are different among species, strains and gestational ages. These changes do not depend on adaptation of renal artery responsiveness. It remains unknown at which cellular level pregnancy affects the pathways. Our meta-analysis suggests that renal changes during pregnancy in animals are qualitatively similar, even in comparison to humans, but quantitatively different.

The second hypothesis was investigated in one experimental study on the pregnancy-induced vascular changes in mesenteric arteries and the isolated perfused rat kidney at midgestation (Chapter 4). For this, we used 20 mid-pregnant and 20 nonpregnant Wistar Hannover rats (WHR). We demonstrated that, at midgestation, alterations in rat mesenteric vascular tone primarily depend on flow-mediated endothelium-dependent changes and not on changes in α-adrenergic vasoconstrictor sensitivity, myogenic reactivity or vascular compliance.

The third hypothesis was analyzed in three separate experimental studies investigating the relaxin-induced vascular changes in mesenteric arteries and the isolated perfused rat kidney in healthy, overweight, aged and hypertensive rats. The first study focused on the effects in normal weight versus overweight WHR (Chapter 5). Rats were fed a regular diet or a high-fat cafeteria-style diet. We found that, in overweight rats, the vasodilator effects of relaxin were markedly reduced for flow-mediated vasodilation, sensitivity to phenylephrine and myogenic response, as compared with the normal diet rats, mostly persistent under L-NAME. Our data demonstrate that some of the vasodilator responses to *in vivo* relaxin administration are impaired in isolated mesenteric arteries and the perfused kidney in overweight female rats, suggestive for relaxin-resistance. This does not result from a decrease in *Rxfp1* (relaxin family peptide receptor) expression but is likely to result from downstream disruption to endothelium-dependent mechanisms in overweight animals.

The second study looked at the effects of relaxin in young versus moderately aged WHR (Chapter 6). The young (n=20) and moderately aged (n=20) rats were 10-12 and 40-46 weeks old, respectively. We observed that moderate aging involves normal endothelium function, but blunts the physiological endothelium-dependent and -independent vasodilator response to relaxin.

The third study investigated the effects of relaxin in normotensive WHR (n=20) and spontaneous hypertensive rats (SHR, n=20) (Chapter 7). We illustrated that primary hypertension blunts both the relaxin-induced inhibition of myogenic reactivity and α-adrenergic vasoconstrictor response, independent from *Rxfp1* gene expression, while the relaxin-dependent enhanced flow-mediated vasodilation remains intact.

In Chapter 8 we discussed our findings and the implications, propose possible pathways through which vascular adjustments appear in vascular uncomplicated and complicated pregnancies and suggest opportunities for future research. We conclude that the involved pathways in vascular adaptation to pregnancy depend on the species, strain, gestational age period and vascular bed investigated. This raises the question which animal model, if any, best simulates vascular uncomplicated and complicated human pregnancy. Although it remains a topic of debate which substance or substances induce pregnancy-induced vasodilation, the humoral environment seems to play an essential role. Possibly it is not one hormone but the mixture of hormones that share comparable vasodilator mechanisms, but have diverged sensitivity to different vascular beds. Vascular risk factors

(like overweight, advanced age, and hypertension) relate to impaired vascular adaptation to the hormone relaxin. This insinuates relaxin-resistance among these risk factors and a possible mechanism through which vascular maladaptation leads to the development of hypertensive disorders in pregnancy. The above generates new insights and creates several hypotheses that need to be explored in future research.

SAMENVATTING

Zwangerschap gaat gepaard met belangrijke systemische aanpassingen van de bloedvaten bij zowel de mens als het knaagdier. Deze aanpassingen zijn het meest onderzocht in het vaatsysteem van de darm en de nieren. De veranderingen bereiken halverwege de zwangerschap een maximum en de mate van verandering is waarschijnlijk afhankelijk van vasculaire risicofactoren, zoals overgewicht, veroudering en hypertensie. De oorzaak van deze vaataanpassingen is nog niet geheel bekend, maar het lijkt dat het hormoon relaxine hierbij betrokken is. Zowel zwangerschap als relaxine induceren aanpassingen van meerdere endotheelcel- (EC) en gladde spiercel- (SMC) afhankelijke lokale responsen van de arteriën van de darm en de nieren: 1. Activatie van de endotheelafhankelijke productie van stikstofmonoxide (NO), 2. Onderdrukking van de respons op vaatvernauwende stimuli, 3. Verlaging van de myogene reactiviteit, en 4. Toename van de elasticiteit van de bloedvaten. Vanuit de literatuur vallen drie observaties op. Ten eerste is het onduidelijk of men mag generaliseren tussen diersoorten, dierstammen en verschillende zwangerschapstermijnen. Ten tweede is het meest bekend over de late zwangerschapsperiode, terwijl voor meerdere dierstammen gegevens ontbreken over het midden van de zwangerschap. Ten derde lijkt relaxine een belangrijke factor voor zwangerschapsgeïnduceerde vaatverwijding. Omdat bepaalde risicofactoren predisponeren voor vasculaire maladaptatie en hypertensieve aandoeningen, zou men zich kunnen voorstellen dat deze risicofactoren leiden tot verminderde relaxine-geïnduceerde vaataanpassingen. Dit zou dan weer kunnen leiden tot vasculaire maladaptatie in de zwangerschap.

Gebaseerd op deze drie observaties tracht dit proefschrift aanvullende informatie te verstrekken omtrent zwangerschapsgeïnduceerde vaatverwijding. Hiervoor zijn drie hypotheses onderzocht:

1. De mate van vaataanpassingen in de darm en de nieren ten gevolge van zwangerschap zijn vergelijkbaar tussen diersoorten, dierstammen en zwangerschapstermijnen.
2. Vaatverwijding bij ratten in het midden van de zwangerschap wordt veroorzaakt door toename van endotheelafhankelijke vaatverwijding en verandering van de respons op vaatvernauwende stoffen, myogene reactiviteit en elasticiteit van de arteriën, zoals geobserveerd in de a terme periode.
3. Lokale vasculaire responsen ten gevolge van relaxineblootstelling zijn verminderd bij risicofactoren voor maladaptatie in de zwangerschap (overgewicht, oudere leeftijd en hypertensie).

De eerste hypothese werd getest door middel van twee meta-analyses. De eerste meta-analyse richtte zich op de zwangerschapsgeïnduceerde veranderingen van de lokale vasculaire controle in arteriën van de darm (Hoofdstuk 2). Een systematische zoekstrategie van de literatuur en een gestructureerd selectieproces identificeerden 55 (van de 398) studies met 188 arteriële responsen van de darm op stimuli, voornamelijk uitgevoerd bij de a terme zwangerschap van de rat. Wij zagen dat zwangerschap, in alle onderzochte diersoorten, de flow-gemediëerde vaatverwijding versterkt. Beïnvloeding van andere locale vasculaire responsen is afhankelijk van de onderzochte diersoort en dierstam. De aanpassingen in

de late zwangerschap zijn voor de onderzochte stimuli afhankelijk van veranderingen op receptorniveau. Omdat de initiële stappen van vaatverwijding in de vroege zwangerschap slechts zeer beperkt onderzocht zijn, verdient deze periode nader onderzoek.

De tweede meta-analyse onderzocht de zwangerschapsgeïnduceerde aanpassingen van arteriën van de nier (Hoofdstuk 3). Wij identificeerden 37 (van de 1008) studies met 116 responsen, eveneens door middel van een systematische zoekstrategie en een gestructureerd selectieproces van de literatuur. Deze studie liet zien dat zwangerschap de renale vaatweerstand, renale doorbloeding en glomerulaire filtratiesnelheid van de nier beïnvloedt door toename in NO productie en deactivatie van het sympathisch systeem, maar dat de aanpassingen verschillen tussen diersoorten, dierstammen en zwangerschapstermijnen. De veranderingen zijn niet afhankelijk van aanpassing in de reactiviteit van de nierarterie. Het blijft nog onduidelijk op welk celniveau zwangerschap de adaptatie bewerkstelligt. Onze meta-analyse suggereert dat de renale aanpassingen tijdens zwangerschap in dieren kwalitatief vergelijkbaar zijn, zelfs in vergelijking met de mens, maar kwantitatief verschillen.

De tweede hypothese werd onderzocht in een experimentele studie, waarin de arteriële vaataanpassingen van de darm en het geïsoleerde niermodel werden bestudeerd in het midden van de zwangerschap (Hoofdstuk 4). Hiervoor werden 20 zwangere en 20 niet-zwangere Wistar Hannover ratten (WHR) gebruikt. Wij zagen dat de verandering in tonus van de arteriën van de darm in het midden van de zwangerschap voornamelijk afhankelijk is van aanpassing van de flow-gemedieerde vaatverwijding, en niet van aanpassingen in de gevoeligheid voor α-adrenerge stimuli, myogene reactiviteit of vasculaire elasticiteit.

De derde hypothese werd onderzocht door middel van drie experimentele studies. Hierin analyseerden we de relaxine-geïnduceerde arteriële vaataanpassingen van de darm en het geïsoleerde niermodel in ratten met overgewicht, verouderde ratten en ratten met hypertensie. De eerste studie richtte zich op de effecten van relaxine in WHR met een normaal gewicht versus overgewicht (Hoofdstuk 5). Hiervoor kregen de ratten een normaal of een hoog vethoudend dieet. Wij zagen een afname van de vaatverwijdende effecten van relaxine op flow-gemedieerde vaatverwijding, gevoeligheid voor phenylephrine en myogene reactiviteit van ratten met overgewicht ten opzichte van ratten met een normaal gewicht. Deze observatie was grotendeels onafhankelijk van NO blokkade. Deze studie laat zien dat meerdere vaatverwijdende responsen ten gevolge van *in vivo* blootstelling aan relaxine zijn aangetast in de geïsoleerde darmarteriën en de nier van ratten met overgewicht. Dit suggereert dat overgewicht gepaard gaat met relaxine-resistentie. Dit lijkt niet te berusten op een afname van de relaxine-receptor genexpressie, maar mogelijk op een afname van 'down-stream' endotheelafhankelijke processen in ratten met overgewicht.

De tweede studie bekeek de effecten van relaxine in jonge versus oudere (eind reproductieve leeftijd) WHR (Hoofdstuk 6). De jonge (n=20) en oudere (n=20) ratten waren respectievelijk 10-12 en 40-46 weken oud. Wij zagen dat veroudering gepaard gaat met normale endotheelfunctie, maar dat de endotheel-afhankelijke en -onafhankelijke vaatverwijdende responsen ten gevolge van relaxine blootstelling zijn aangetast.

De derde studie onderzocht de effecten van relaxine in normotensieve WHR (n=20) en spontaan hypertensieve ratten (SHR, n=20) (Hoofdstuk 7). Wij lieten zien dat

primaire hypertensie de relaxine-geïnduceerde verlaging van de myogene reactiviteit en α-adrenerge vasoconstrictieve respons aantast. Dit is onafhankelijk van de relaxine-receptor genexpressie, terwijl relaxine-afhankelijke activatie van de flow-gemedieerde vaatverwijding intact blijft.

In Hoofdstuk 8 bediscussieerden wij onze resultaten en de implicaties hiervan. Daarnaast bespraken wij de mogelijke verklaringen ten aanzien van de vaataanpassingen in ongecompliceerde zwangerschappen en zwangerschappen gecompliceerd door vasculaire aandoeningen. Tevens suggereerden wij mogelijke onderwerpen voor onderzoek in de toekomst. Wij concluderen dat de betrokken mechanismen bij vaataanpassingen ten gevolge van zwangerschap afhankelijk zijn van de onderzochte diersoort, dierstam en zwangerschapsperiode. Dit roept de vraag op welk diermodel het beste de vasculair ongecompliceerde en gecompliceerde zwangerschap bij de mens simuleert. Hoewel het nog niet duidelijk is welke stof of stoffen verantwoordelijk zijn voor de zwangerschapsgeïnduceerde vaatverwijding, lijkt de humorale omgeving een belangrijke rol te hebben. Waarschijnlijk is het niet één hormoon, maar een mix van hormonen met vergelijkbare vaatverwijdende effecten met verschillende gevoeligheid voor de verschillende vaatgebieden. Vasculaire risicofactoren (zoals overgewicht, oudere leeftijd en hypertensie) relateren aan gestoorde vasculaire aanpassingen op relaxine blootstelling. Dit insinueert de aanwezigheid van relaxine-resistentie bij deze risicofactoren en een mogelijk mechanisme van vasculaire maladaptatie en het ontstaan van hypertensieve aandoeningen in de zwangerschap. Het bovenstaande genereert nieuwe inzichten en creëert meerdere hypotheses die nader onderzoek verdienen.

LEGENDS TO ABBREVIATIONS

AC	adenyl cyclase
Akt/PKB	Akt/phospokinase B
$C_{50\%}$	concentration inducing a 50% response
Ca^{2+}	calcium
CGRP	calcitonin gene-related protein
CI	confidence interval
DAG	diaglycerol
$Diam_{max}$	maximal vascular diameter after flow change
EC	endothelium cell
EC_{50}	effective concentration, needed to obtain 50% of maximum effect
ECM	extra-cellular matrix
EDHF	endothelium-dependent hyperpolarizing factor
EFS	electric field stimulation
E_{max}	maximum response
eNOS	endothelial nitric oxide synthase
ET	endothelin
$Flow_{50\%}$	flow rate inducing 50% dilation; flow-sensitivity
GC	guanyl cyclase
GFR	glomerular filtration rate
GP	guinea pigs
hCG	human chorionic gonadotrophin
I^2	heterogeneity
IP_3	inositol 1,4,5-triphospate
K^+	potassium
LER	Long Evans rats
L-NAME	L-Nitro-Arginine Methyl Ester, NO blockade
MAPK	mitogen-activated protein kinase
MD	mean difference
MgSO4	magnesiumsulphate
MMPs	matrix-metallo proteases
MR	myogenic reactivity
MWR	Munich Wistar rats
NO	nitric oxide, stikstofmonoxide
NP	nonpregnant
P	mid-pregnant
PBS	phosphate buffered saline
PGI_2	prostacyclin
PI_3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKG	protein kinase G
PM	pressure myograph

PPM	pressure-perfusion myograph
PSS	physiological salt solution
RAAS	renin-angiotensin-aldosterone system
RBF	renal blood flow
RCT	randomized controlled trial
R_{max}	maximum response
RPF	renal plasma flow
RPFF	renal perfusion flow
RVR	renal vascular resistance
Rxfp1	relaxin family peptide receptor
SDR	Sprague Dawley rats
SHR	spontaneous hypertensive rats
SMC	smooth muscle cell
SMD	standerdized mean difference
SVR	systematic vascular resistance
VC	vasculair compliance
WHR	Wistar Hannover rats
WKY	Wistar Kyoto rats
WM	wire myograph
WOP	whole organ perfusion
WR	Wistar rats

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Na vele jaren is het dan echt zover: mijn proefschrift is af. Terugkijkend was het een mooi avontuur met ups en downs, waarin ik op vele fronten wijzer ben geworden. Dit proefschrift had uiteraard niet tot stand kunnen komen zonder de inzet en medewerking van vele anderen. Een woord van expliciete dank voor enkelen is dan ook zeker op zijn plaats.

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CURRICULUM VITAE

Joris van Drongelen werd op 18 mei 1974 geboren te Delft. Hij groeide op in Ouderkerk aan de IJssel en volgde het basisschool onderwijs aan de Montessori school te Capelle aan de IJssel en te Delft. Hij behaalde zijn eindexamen VWO in 1992 aan het Haags Montessori Lyceum te Den Haag. Vervolgens studeerde hij tussen 1992 en 1996 Biomedische Gezondheidswetenschappen aan de Katholieke Universiteit Nijmegen. Hij studeerde af binnen de afstudeerrichting Epidemiologie, met stages aan de John Hopkins University te Baltimore en het UMC St Radboud te Nijmegen. Vervolgens startte hij met de studie Geneeskunde eveneens aan de Katholieke Universiteit Nijmegen en behaalde zijn artsexamen in 2000. Daarna was hij als poortarts werkzaam in het Bernhoven Ziekenhhuis te Veghel en als assistent gynaecologie niet in opleiding in het Jeroen Bosch Ziekenhuis te 's-Hertogenbosch. In 2002 werd hij aangenomen voor de opleiding tot gynaecoloog, waarvan hij zijn perifere delen in het Jeroen Bosch Ziekenhuis (opleider dr. H.P. Oosterbaan) en het academische deel in het UMC St Radboud (opleider prof. dr. D.D.M. Braat) volbracht. In deze periode startte hij met promotie-onderzoek op het gebied van vaataanpassingen door zwangerschap en relaxine, waarvan dit proefschrift het resultaat is. In 2011 voltooide hij zijn opleiding tot gynaecoloog. In 2013 werd hij, na een opleidingstraject tot subspecialist gevolgd te hebben, perinatoloog in het UMC St Radboud waar hij heden werkzaam is.