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DISSERTATION

Novel insights into the vasodilatory effects of relaxins and supraspinal sympathetic control of murine peripheral arteries

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1. Abstract

English: Total peripheral resistance is invariably increased in all hypertension. The underlying molecular mechanisms are largely unknown, but involve a hyper-contractile state of the arterial vascular smooth muscle cells, reduced endothelial vasodilator capacity and increased arterial stiffness. The aim of the present study was to provide novel insights into the vasodilatory effects of relaxins and supraspinal sympathetic control of peripheral arteries interconnected with increased arterial stiffness. In particular, (1) the vasodilatory effects of relaxins in peripheral arteries, (2) the contribution of renin-angiotensin system (RAS) in supraspinal sympathetic control of peripheral arteries and (3) the role of serum- and glucocorticoid-inducible kinase 1 (SGK1) in vascular calcification were clarified. $G\alpha_{i2}$- (*Gnαi2*$^{-/-}$) and $G\alpha_{i3}$-deficient (*Gnαi3*$^{-/-}$) mice, pharmacological tools and wire myography were used to study G-protein-coupled signaling pathways involved in the vasodilatory effects of relaxins. Angiotensin II (Ang II) type 1a receptor (AT1a) (*Agtr1a*$^{-/-}$) and angiotensin- (1-7) receptor Mas (*Mas*$^{-/-}$) mice were used to study the role of RAS in supraspinal sympathetic control of peripheral arteries after high-level spinal cord injury (SCI). SGK1 deficient (*Sgk1*$^{-/-}$) mice were used to examine the role of SGK1 in the vascular calcification. Relaxin-2 (~50% relaxation at 10^{-11} M) was found to serve as potent vasodilator, which produces endothelium- and NO-dependent relaxation of mesenteric arteries by activation of RXFP1 coupled to G_{i2}-PI3K-eNOS pathway. In SCI mice, the reactivity of wild-type and *Mas*$^{-/-}$ mesenteric arteries was diminished in response to angiotensin II (Ang II), i.e. distal but close to thoracic level T4, but this difference was not observed in the absence of AT1a receptors and all genotype femoral arteries. These results indicate that AT1a receptor signaling underlies increased vascular reactivity in mesenteric arteries in response to Ang II and this may contribute to adaptive changes in regional blood flow. After cholecalciferol treatment, aortic rings from *Sgk1*$^{-/-}$ mice developed less wall tension after ex vivo mechanical stretch as compared with aortic rings from *Sgk1*$^{+/+}$ mice, indicating a higher aortic stiffness in *Sgk1*$^{+/+}$ mice.

Deutsch:

Der gesamte periphere Widerstand ist bei allen Hypertonien unveränderlich erhöht. Die zugrundeliegenden molekularen Mechanismen sind weitgehend unbekannt, beinhalten jedoch einen hyperkontraktilen Zustand der arteriellen Gefäßmuskelzellen, reduzierte endotheliale Vasodilator-Kapazität und erhöhte arterielle Gefäßsteifheit. Das Ziel der vorliegende Versuch war es, neue Einblicke in die vasodilatorischen Effekte von Relaxinen und supraspinaler sympathischer Kontrolle von peripheren Arterien, die mit erhöhter arterieller Steifheit einhergehen, zu gewinnen. Insbesondere (1) die vasodilatorischen Wirkungen von Relaxinen in peripheren Arterien, (2) der Beitrag des Renin-Angiotensin-Systems (RAS) in der supraspinalen sympathischen Kontrolle peripherer Arterien und (3) die Rolle der Serum- und Glucocorticoid-induzierbaren Kinase 1 (SGK1) bei vaskulärer Verkalkung wurden abgeklärt. G_{i2}- (*Gni2* $^{-/-}$) und G_{i3}-defiziente (*Gni3* $^{-/-}$) Mäuse, pharmakologische Hilfsmittel und Gefäß-Myographie wurden verwendet, um G-Protein-gekoppelte Signalwege zu untersuchen, die an den vasodilatorischen Effekten von Relaxinen beteiligt sind. Angiotensin II (Ang II) Typ 1a Rezeptor (AT1a) (*Agtr1a* $^{-/-}$) und Angiotensin (1-7) Rezeptor Mas (*Mas* $^{-/-}$) defiziente Mäuse wurden verwendet, um die Rolle von RAS bei der supraspinalen Sympathikuskontrolle von peripheren Arterien nach hoher Rückenmarksverletzung (SCI) zu untersuchen. SGK1-defiziente (*Sgk1* $^{-/-}$) Mäuse wurden verwendet, um die Rolle von SGK1 bei der Gefäßverkalkung zu untersuchen. Relaxin-2 (~ 50% Relaxation bei 10^{-11} M) erwies sich als potenter Vasodilator, der durch Aktivierung von RXFP1, gekoppelt an den G_{i2}-PI3K-eNOS-Signalweg, eine Endothel- und NO-abhängige Relaxation von Mesenterialarterien bewirkt. Bei SCI-Mäusen war die Reaktivität von Wildtyp- und Mas$^{-/-}$ Mesenterialarterien als Antwort auf Angiotensin II (Ang II) vermindert, nämlich distal, aber nahe dem thorakalen Wirbel T4, wobei dieser Unterschied in Abwesenheit von AT1a-Rezeptoren und in Oberschenkelarterien aller Genotypen nicht beobachtet wurde. Diese Ergebnisse zeigen, dass bei SCI-Mäusen der AT1a-Rezeptorsignalweg als Reaktion auf Ang II zu einer erhöhten vaskulären Reaktivität in Mesenterialarterien führt und dies zu adaptiven Veränderungen des regionalen Blutflusses beitragen kann. Nach der Behandlung mit Cholecalciferol entwickelten Aortenringe von *Sgk1* $^{-/-}$ Mäusen nach ex vivo mechanischer Dehnung weniger Wandspannung als Aortenringe von *Sgk1* $^{+/+}$ Mäusen, was auf eine erhöhte Aortensteifigkeit in *Sgk1* $^{+/+}$ Mäusen hinweist.

2. Introduction

Hypertension is the major risk factor for cardiovascular disease—the most common cause of death worldwide, and the incidence of hypertension is greatest among older adults [1] [2]. Although the etiology of primary (essential) hypertension remains unknown, it is clear that multiple factors contribute to the pathogenesis of hypertension. A hyper-contractile state of the arterial vascular smooth muscle cells, reduced endothelial vasodilator capacity and increased arterial stiffness are important causal contributors to hypertension [3] [2]. On the other hand, dysfunction of peripheral resistance arteries [4] and vascular calcification [5] have been recognized as two complications of long-standing hypertension that integrate long-term adverse effects of elevated blood pressure and other risk factors to chronic vascular disease.

Relaxins are small peptide hormones, structurally related to the insulin hormone superfamily, which are capable of inducing vasodilation in human [6] and rodent microvessels [7]. Given the important contribution of small resistance arteries to systemic vascular resistance and the potential for relaxin to promote vasodilation, it is critical to examine the putative vasodilatory effects and intracellular signaling pathways of relaxins in the microvasculature in order to better understand the systemic haemodynamic effects of relaxins. Cell culture experiments indicate that relaxins could activate endothelial nitric oxide synthase (eNOS) via a pertussis toxin (PTX)-sensitive G_i/phosphoinositide 3-kinase (PI3K) pathway-dependent pathway [8] [9] . However, it is unknown whether this pathway is relevant to vasodilation of intact vessels. It is also unknown which G proteins are involved in this pathway. This is particularly important since there are only few reports that GPCRs are capable to utilize G_i-coupled signaling pathways to cause vasodilation (i.e. for bradykinin, beta2 adrenergic agonists, thrombin) [10] [11]. It is therefore not surprising that current research is focused on the identification of novel compounds and GPCRs which can utilize G_i-signaling pathways to produce potent relaxations.

Moreover, there is a close interrelationship between endothelial dysfunction and autonomic nervous system dysfunction to contribute to atherosclerosis [12]. In hypertension, endothelial dysfunction affects the pathologic process through autonomic nervous pathways, and the pathophysiological process of autonomic neuropathy in diabetes mellitus is closely related with vascular function [13].However, little is known about the role of renin-angiotensin system (RAS) in supraspinal sympathetic control of peripheral arteries and their contribution to arterial stiffness [14]. When supraspinal sympathetic control of the vasculature is lost in high-level spinal cord injury (SCI), activation of RAS is believed to play important roles in keeping and modulating vasoconstriction [15]. Ang II is a hormonal vasoconstrictor acting via the G-

protein- coupled receptors (GPCR) Ang II type 1a (AT1a) and 1b (AT1b) in rodents, which also facilitate peripheral norepinephrine (NE) and adrenal aldosterone release. Ang II can be metabolized to Ang-(1-7) by the angiotensin converting enzyme 2 (ACE2), a homologue of the angiotensin converting enzyme (ACE) and then induces signaling through activation of the GPCR Mas [16] [17]. Ang-(1-7)/Mas has been reported to produce vascular relaxation and to act antagonistic to the AT1 receptor [18]. However, the relative relevance of these two components of the RAS axis in the cardiovascular consequences of high thoracic or cervical SCI is unknown.

It has been known for decades that vascular calcification plays a role in the pathogenesis of hypertension and cardiovascular disease [19]. Vitamin D deficiency is associated with increased arterial stiffness. Recent studies showed that changes in autonomic nervous system activity could underlie an association between 25 hydroxy vitamin D and arterial stiffness [20], which sparked considerable interest in understanding basic mechanisms that produce stiffening of the arterial wall. Our focus is on serum- and glucocorticoid-inducible kinase 1 (SGK1) [21], which is known to be capable of mediating deleterious effects of aldosterone and glucocorticoids in the cardiovascular system [22] [23] and which may represent a novel therapeutic target to limit the progression of vascular calcification.

3. Aims

Firstly (Project 1), the present study aimed to examine the vasodilatory effects of relaxins in the murine peripheral vasculature. The putative vasodilatory effects of relaxins were examined and the hypothesis was tested that the NO-dependent vasodilatory effects of relaxins are mediated by the activation of endothelial relaxin family peptide (RXFP) receptors 1, which are coupled to vasodilatory G_{i2}-PI3K-eNOS signaling pathways.

Secondly (Project 2), the present study aimed to examine the effects of RAS in supraspinal sympathetic control of peripheral arteries. The hypothesis that AT1 and Mas receptors play important roles in regulating the hemodynamic response and regional peripheral vascular reactivity following high-level SCI.

Finally (Project 3), the present study aimed to reveal novel mechanisms of vascular calcification. The hypothesis was tested that SGK1 is upregulated in vascular smooth muscle cells under calcifying conditions and serves as a key regulator of vascular calcification in the murine vasculature.

4. Methods

4.1 Mice

Experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocols were previously approved by the local Animal Care and Use Committee from Berlin LAGeSo (G0132/14). Animal experiments used 10- to 14-wk-old mice of either sex and were housed in groups of 4–6 animals in cages with nesting material, mouse lodges and open access to water and feed, at 23 °C with a 12 h/12 h circadian cycle. Experiments were performed using WT C57BL/6 mice, female $G\alpha_{i2}$-deficient ($Gn\alpha i2^{-/-}$) and $G\alpha_{i3}$-deficient ($Gn\alpha i3^{-/-}$) mice and respective control WT C57Bl/6 mice (Project 1) [24] [25]; Mas deficient ($Mas^{-/-}$), AT1a deficient mice (B6.129P2-Agtr1atm1Unc/J from the Jackson Laboratory) ($Agtr1a^{-/-}$) and respective control WT C57B1/6 mice (Project 2); Sgk1-deficient ($Sgk1^{-/-}$) and its control WT C57Bl/6 mice (Project 3), weighing 20 to 25 g, and at about 3 months of age.

4.2 Preparation of animals

4.2.1 PTX-treated animals

Male wild-type C57BL/6 mice (20-25 g, 8-12 weeks) were maintained according to national guidelines for animal care at the animal facility. Mice were injected intraperitoneally with 150 µg/kg body weight pertussis toxin (PTX) or NaCl solution (0.9 %) as vehicle control 48 hours before use [26].

4.2.2 Spinal cord injury (SCI)

All operative interventions were done under intraperitoneal Ketamine (10 mg/kg) - Xylazine (100 mg/kg) anaesthesia in combination with Isoflurane (1.5-1.8 %) inhalation. A dorsal midline incision was made in the superficial muscle overlying the C8-T3 vertebrae. The dura was opened at the T2-T3 intervertebral gap and the spinal cord was completely transected using microscissors. Complete transection was confirmed by pulling a needle twice between the rostral and caudal spinal cord stumps. Gelfoam was placed above spinal cord to achieve hemostasis. The muscle and skin were closed with absorbable sutures (Vicryl, 4-0, Ethicon GmbH). Animals received warmed saline (1 ml, s.c.), recovered and were kept in the heated cages (30°C). For analgesia mice were treated with Carprofen (4mg/kg, s.c.) directly after operation and next day with 12-hour interval if necessary longer. The bladder was manually emptied three times daily for the whole duration of the experiment, as the bladder function did

not recover.

4.2.3 Cholecalciferol-treated animals

Sgk1-deficient and corresponding WT mice were injected s.c. with 400,000 IU/kg body weight of cholecalciferol or vehicle for 3 days as previously described [27].

4.3 Wire myography

The second branches of mesenteric arteries or right and left femoral arteries or abdominal aortae were isolated from mice sacrificed under general anaesthesia with Rompun/Ketamin, 10 mg/Kg and 100 mg/kg pro bodyweight, respectively. The vessels were then quickly transferred to cold (4°C), oxygenated (95% O_2/5% CO_2) physiological salt solution (PSS) containing (in mmol/L): 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11.1 glucose and 1.6 $CaCl_2$. After cleaning the connective tissue and perivascular adipose tissue under dissecting microscope with scissors without damaging the adventitia, the arteries were dissected into 2 mm rings. Each ring was positioned between two stainless steel wires (diameter 0.0394 mm) in a 5-mL organ bath of a Small Vessel Myograph (DMT 610M, Danish Myo Technology, Aarhus, Denmark) [28].The organ bath was filled with PSS. The bath solution (pH 7.4) was continuously oxygenated (95% O2/5% CO2), and kept at 37°C. The rings were placed under a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mmHg (DMT Normalization module by CHART software) [28]. The software Chart5 (AD Instruments Ltd. Spechbach, Germany) was used for data acquisition and display.

4.3.1 Measurement of vascular reactivity

The rings were pre-contracted and equilibrated for 30 minutes until a stable resting tension was acquired. In some vessels, the endothelium war removed by mechanically disrupted by a whisker or an air bubble [28]. Endothelium integrity or functional removal was confirmed by the presence or absence, respectively, of the relaxant response to 1 µM acetylcholine (ACh) on phenylephrine (PE 1 µM) -pre-contracted arteries. Following PSS wash, the pharmacological drugs were applied. After a waiting period of 30 minutes, PE, or subsequently relaxins or AngII were added to the bath solution. Relaxations induced by relaxins were expressed as percentage relaxations obtained with ACh (100%) or as percentage relaxations of PE contractions. Contractions induced by PE were expressed as percentage tension obtained with KCl-PSS (100%) containing (in mmol/L): 63.7 NaCl, 60 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 Mg_2SO_4 , 11.1 glucose, and 1.6 $CaCl_2$. Contractions induced by AngII were expressed as percentage

tension obtained with KCl (100%). During the experiments, relaxin was applied for at least 5 min and data was recorded to ensure that relaxins achieved their maximal effects [29]. All drugs were added to the bath solution (PSS).

4.3.2 Measurement of aortic stiffness

The vessels were allowed to equilibrate for 30 minutes in the absence of tension. For length/tension analysis, aortae were stretched to an internal circumference [30] that reproduces the wall force exerted on the vessel at a resting transmural pressure of 100 mmHg (DMT Normalization module by CHART software). Thereafter, wall tension was measured by sequential increasing of the distance (stretch length) between the wires in 50-µm increments using the software Chart5 (AD Instruments Ltd.). Wall tension was measured for 2 minutes per aorta after the stretch length was increased [28].

4.4 RNA sequencing of murine mesenteric arteries

For this analysis separate group of mice were operated. Four weeks post-T4-SCI or sham-OP mice were sacrificed and the mesenteric arterial arcade, excluding the superior mesenteric artery, was dissected, cleaned of fat, and snap frozen. For RNA isolation with Trizol and FastPrep beads, the manufacturer's instructions were followed. Reverse transcription was performed with total RNA digested by DNase I (Roche). Total RNA was stored in a 1.5 ml Safe Lock LoBind Tubes. RNA samples were quantified on a spectrophotometer (NanoDrop ND-1000; Thermo Scientific) and quality-analyzed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, US). RNA sequencing was performed at the Scientific Genomics Platform located at the Max Delbrück Center for Molecular Medicine (MDC). mRNA-Seq libraries were generated from 3 sham and 3 T4-SCI mesenteric artery samples using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA) as per the manufacturer's protocol. Data was de-multiplexed to generate the sequencing reads in FastQ format files. The final list of differentially expressed genes was used to compute the enrichment of biological pathways separately for the upregulated and downregulated genes using DAVID [31].

4.5 Real-time qPCR

To evaluate the expression levels of selected genes by RT-PCR, 1 µg of DNA-free total RNA isolated from murine mesenteric arteries was used for first strand cDNA synthesis with using oligo(dT) and random primers. Quantitative polymerase chain reaction (qPCR) analysis was performed using Fast SYBR Green PCR Master Mix (Applied Biosystem) and Quant Studio 5

real-time PCR system (Thermo Fischer). Each cDNA sample was tested in triplicate, and the expression level of each gene was normalized to the hypoxanthine-guanine phosphoribosyl transferase (Hprt) level. Following primers were used: Agtr1a forward: 5'-CCATTGTCCACCCGATGAAG-3', Agtr1a reverse: 5'-TGCAGGTGACTTTGGCCAC-3', Agtr1b forward: 5′-TGGCTTGGCTAGTTTGCCG-3′ Agtr1b reverse: 5′-ACCCAGTCCAATGGGGAGT-3′, Hprt forward: 5′- GTA ATG ATC AGT CAA CGG GGG AC - 3′, Hprt reverse: 5′- CCA GCA AGC TTG CAA CCT TAA CCA - 3′. The fold change was determined using the $2-\Delta\Delta C(t)$ method [32].

4.6 Materials

FR900359 was isolated as described previously [33]. Salts and drugs were purchased from Sigma-Aldrich (Germany) with the exception of PTX and TGX 221, which were obtained from Merck Millipore (Calbiochem) (Germany) and PI-103 from Enzo Life Sciences. The PI3K inhibitors TGX-221, AS-252424 and PI-103 were administered at submaximal concentrations to achieve biological effects [34] [35].

4.7 Statistical analyses

Results were presented as mean ± SEM. Data were analyzed statistically using the GraphPad Prism 7 software (GraphPad Software, CA, USA). Unpaired Student's t-tests or ANOVA were used where appropriate. A value of P less than 0.05 was considered statistically significant; n represents the number of arteries tested.

5. Results

5.1 Relaxins

In this project (Project 1), isolated mesenteric artery rings were pre-contracted by phenylephrine (PE 1 µM) and exposed to acetylcholine (ACh 1 µM) for control. After wash-out of these substances, the vessels were re-exposed to PE and subsequently incubated with 10^{-10} M relaxin-2. Compared to ACh, relaxations in response to relaxin-2 were slow and delayed. Removal of the endothelium abolished both ACh and relaxin- 2 relaxations. These data indicate that relaxin-2 are powerful peptide hormone to produce relaxations, probably utilizing an endothelium-dependent mechanism distinct from ACh.

The $G\alpha_q$ protein inhibitor FR900359 (100 nM) [33] inhibited concentration-dependent relaxations by ACh, but had no effect on relaxin-2- dependent relaxations. In contrast, the eNOS

inhibitor L-NAME (100 μM) inhibited both relaxin-2- and ACh-induced relaxations. Together, the data indicate that both relaxin-2 and ACh produce relaxation by an endothelial- dependent mechanism involving eNOS/NO release. However, whereas ACh utilizes an eNOS/NO signaling pathway involving muscarinic receptors coupled to G_q proteins, relaxin- 2 stimulates RXFP1 receptors coupled to G proteins other than G_q to produce eNOS/NO– dependent relaxation.

G_q-dependent relaxations by ACh were preserved in mesenteric arteries treated with PTX. However, relaxation in response to relaxin- 2 were abolished in mesenteric arteries from mice pre-treated with PTX compared to control arteries. The $G\alpha_{i2}$-deficient (*Gnαi2*$^{-/-}$) and $G\alpha_{i3}$-deficient (*Gnai3*$^{-/-}$) mice were further used to determine which $G\alpha_i$ isoforms are involved in relaxin-2 mediated relaxations. The study shows that relaxin-2 induced relaxations were reduced in $G\alpha_{i2}$-deficient arteries but not in $G\alpha_{i3}$- deficient arteries. These data indicate that $G\alpha_{i2}$ plays an important role in RXFP1 mediated relaxations.

The RXFP1 blocker simazine (100 nM) inhibited relaxations induced by relaxin-2. Relaxin-2-induced relaxations were inhibited by L-NAME. The PI3Kβ inhibitor TGX-221 and the PI3Kγ inhibitor AS-252424 had similar inhibitory effects on stimulation by relaxin-2. However, relaxin-2-induced relaxations were not affected by the PI3Kα inhibitor PI-103. Together, the data suggest that PI3K isoforms β and γ are essential intermediate signaling components in eNOS/NO-dependent relaxation by relaxin-2 which are controlled by relaxin-2 that act on RXFP1 receptors coupled to G_{i2} proteins.

For more details, please, see the publication attached.

5.2 Supraspinal sympathetic control

In this project (Project 2), vessels were isolated from SCI *Agtr1a*$^{+/+}$, sham operated *Agtr1a*$^{+/+}$, T4- SCI *Agtr1a*$^{-/-}$, sham-operated *Agtr1a*$^{-/-}$, T4-SCI *Mas*$^{+/+}$, sham-operated *Mas*$^{+/+}$, T4-SCI *Mas*$^{-/-}$ and sham-operated *Mas*$^{-/-}$ mice. It was found that SCI decreased the reactivity of *Agtr1a*$^{+/+}$, *Mas*$^{+/+}$ and *Mas*$^{-/-}$ mesenteric arteries in response to Ang II. This response was reduced and no change was observed after SCI in *Agtr1a*$^{-/-}$ mesenteric arteries. For comparison, femoral arteries were studied and the results show that SCI did not affect the reactivity of femoral arteries in response to angiotensin II in all genotypes.

Furthermore, real-time qPCR experiments were performed to analyze mRNA expression in

mesenteric arteries isolated from T4-SCI and sham-operated *Agtr1a*$^{+/+}$ and *Mas*$^{+/+}$ mice. The data show that there were no significant differences in the expression of AT1a and AT1b receptors at the mRNA levels in the mesenteric arteries in all genotypes following T4-SCI. These data show that downstream mechanisms of AT1a receptor activation are likely involved in decreased reactivity of mesenteric arteries in response to angiotensin II following T4-SCI. 80 genes were identified (1.5 fold) to be regulated after SCI, 20 of them were up-regulated. Analysis of the biological processes in which these genes are involved using over-representation analysis of biological processes by DAVID revealed developmental and immune processes being enriched.

For more details, please, see the publication attached.

5.3 *Calcification*

In this project (Project 3), after cholecalciferol treatment, aortic rings from *Sgk1*$^{-/-}$ mice developed less wall tension after ex vivo mechanical stretch as compared with aortic rings from *Sgk1*$^{+/+}$ mice, indicative of higher aortic stiffness in *Sgk1*$^{+/+}$ mice. No differences from control-treated mice were observed.

For more details, please, see the publication attached.

6. Discussion

These studies focused on two vascular factors or contributors which are novel candidates to influence the progress of hypertension and arterial stiffness by influencing the function of peripheral arterial vasculature and vascular calcification. In the first two parts of the project, the function of peripheral vasculature either under physiological conditions by relaxin-2 or under pathological conditions of SCI were investigated; in the third part, the role of SGK1 was studied in the vascular calcification. It was fund that relaxin-2 is an extremely potent (in the low Pico molar range) endothelium- dependent vasodilator in mouse mesenteric arteries, which produces eNOS/NO-dependent relaxation most likely due to activation of RXFP1 coupled to a G_{i2}-PI3kβ/PI3kγ pathway. T4-SCI diminished the reactivity of wild-type and *Mas*$^{-/-}$ but not *AT1a* $^{-/-}$ mesenteric arteries to angiotensin II, indicated that AT1a signaling contributes to increased vascular reactivity in mesenteric arteries in response to angiotensin II and this response may contribute to decreased peripheral vasculature blood flow in high-level SCI. In mouse aorta, SGK1 was observed to be dynamically upregulated in the vasculature under calcifying conditions, and to contribute to vascular calcification.

Relaxins

Relaxin-encoding genes are present in all mammals and responsible for the production of the relaxin peptides that have been initially found in circulating blood during pregnancy. However, more recent studies have observed that relaxins are produced in many tissues in mammals as paracrine or autocrine factors to exert a number of different physiological roles in the vasculature, which may exhibit protective effects in cardiovascular disease [36]. Relaxin-2 has been identified as the most important member of the relaxin family and major circulating form of relaxin peptides in humans [37]. In this study on WT mouse mesenteric arteries, the vasoactive function of relaxin-2 has been tested. It was found that low concentration of relaxin-2 can produce potent relaxations. In mouse mesenteric arteries, the endothelium is necessary for the vasodilation by relaxin-2, and removal of the endothelium or treatment with L-NAME inhibited the relaxation by relaxin-2. However, in contrast to ACh, which produces a rapid eNOS/NO-dependent relaxation, relaxin-2 relaxations were slow and delayed, indicating different underlying signaling mechanisms between ACh and relaxins to cause the activation of eNOS **(Figure 1**).

Relaxin produces its major effects via specific G-protein-coupled receptors (GPCRs), i.e. RXFP 1-4 [7] [38]. Among them, RXFP1 has the highest binding potency for relaxin-2 within the several members of the relaxin peptide family [36; 39]. RXFP1 is predominantly expressed in endothelial cells of mesenteric arteries and veins, but also expressed in the aorta and vena cava [40] [8]. In the experiments of this study, the RXFP1 antagonist simazine [41] largely inhibited the endothelium-dependent relaxation induced by relaxin-2, which supports the idea that relaxin-2 produces relaxation primarily through binding to and activation of RXFP1 in the endothelium (**Figure 1)**.

The role of G proteins following RXFP1activation in eNOS/NO-dependent vasodilation by relaxin-2 was further identified. Heterotrimeric G proteins, which mediate signals from cell surface receptors to cellular effectors, are composed of a, b and g subunits, of which Ga defines the class of G proteins [42]. The a subunits that define the basic properties of a heterotrimeric G protein can be divided into four families, namely G_s, G_q/G_{11}, G_i/G_o and $G_{12/13}$ [43]. ACh produces relaxation mainly via the G_q/G_{11}-coupled M3 receptor subtype [44] [44]. In the present study, it was found that ACh-induced relaxations were abolished by FR900359, a selective mammalian G_q/G_{11} signaling inhibitor [33], while the relaxin-2-induced relaxations were not blocked by this drug. The conclusion was drawn that vasodilatory RXFP1-coupled G proteins are distinct from G_q/G_{11} (**Figure 1)**. The data using the pan G_i/G_o inhibitor PTX [42] indicate

that G_i/G_o proteins could play a major role in relaxin-2 mediated vasodilation (**Figure 1**). G_o is particularly abundant in the neuronal and the neuroendocrine system and the G_i-family includes three closely related $G\alpha$ members, $G\alpha_{i1-3}$, which display overlapping expression patterns with $G\alpha_{i2}$ and $G\alpha_{i3}$, abundantly expressed in the cardiovascular system [45]. According to previous studies on cultured cells, RXFP1 has been suggested to couple to Gai3 to release $G\beta\gamma$ dimers to activate the PI3K pathway via Akt phosphorylation and subsequently initiate NOS [9] [46]. The experiments using $G\alpha_{i2}$-deficient ($Gn\alpha i2^{-/-}$) and $G\alpha_{i3}$-deficient ($Gn\alpha i3^{-/-}$) mice failed to implicate an important role of $G\alpha_{i3}$, but revealed a key role of $G\alpha_{i2}$ in RXFP1-eNOS/NO relaxation. In line, relaxin- 2 relaxation was impaired in $Gnai2^{-/-}$ but not in $Gn\alpha i3^{-/-}$ arteries. Although structural similarity between the three G_i subforms suggests that they may exhibit overlapping functions, $G\alpha_{i2}/G\alpha_{i3}$- double-deficient mice cannot be used for myography experiments because they die in utero at early embryonic stages. Nevertheless, present data obtained in mice lacking $G\alpha_{i2}$ or $G\alpha_{i3}$ indicate distinct biological key roles of these two $G\alpha_i$-isoforms [26]. Thus, relaxin- 2 is believed to relax mouse mesenteric arteries primarily via RXFP1 activation and coupling to G_{i2} but not G_{i3} (**Figure 1**). Moreover, since the pan G_i/G_o inhibitor PTX shows a stronger inhibitory effect than the absence of $G\alpha_{i2}$, we should also consider that relaxin-2 could partly act through other G_i proteins.

Following the release of $G\beta\gamma$ from $G\alpha_i$, it was recently suggested that the class I PI3K represents a target for $G_i\beta\gamma$ signaling by relaxins [46]. Accordingly, eNOS activation by relaxin was inhibited by the pan PI3K inhibitors Wortmannin or LY294002 in cultured endothelial cells [47]. Based on the association with non-catalytic binding proteins, catalytic subunits of class I PI3Ks are subdivided into class IA-isoforms (p110α, -β and -δ) or class IB p110γ [48]. In this study, it was found that relaxin-2-RXFP1 relaxation was inhibited by the PI3Kγ and PI3Kβ inhibitors AS-252424 and TGX-221, respectively. The PI3Kα inhibitor PI- 103 had no effects. These data indicate that class I PI3Kγ and PI3Kβ represents likely a target for G_{i2} signaling by relaxins to cause eNOS/NO dependent relaxation. I am not aware of selective PI3kδ inhibitors to determine a possible additional role of PI3Kδ in RXFP1-mediated relaxation. Nevertheless, the data indicate that class I PI3K activation by a target for RXFP/G_i- βγ signaling to cause eNOS activation is not only a cell culture phenomenon [47] [49], but is important for relaxins to produce vascular relaxation (**Figure 1**). Of note, the relaxin-2-induced relaxations were not fully abolished by TGX-221 or AS-252424. This may indicate that both PI3K isoforms or another PI3K isoform are involved in this vasoregulatory pathway. Also, in addition to the slow activation process of the eNOS/NO by relaxins via G_i-PI3K [46], there is also an ultra-slow

mechanism of eNOS/NO stimulation via NFκB–mediated synthesis of the endothelin type B (ETB) receptor upon exposure of cultured endothelial cells to relaxin [50]. Although this putative mechanism cannot be examined by the methodological approach used in the present study, such non-RXFP-mediated effects may contribute to the effects of relaxins in the vasculature in vivo.

In summary, evidence is provided that relaxin-2 is a strong vasodilator in mesenteric arteries, which produces relaxation via activation of endothelial RXFP1 coupled to a G_{i2}- PI3Kβ/γ-eNOS/NO pathway (**Figure 1**). Based on the fact that long- and intermediate-distance conduction of vasodilation is common in the circulation, localized releases of relaxins within a tissue might be able to produce remote vasodilations in regions of reduced blood flow distribution. As a potent vasodilatory $G\alpha_{i2}$-coupled receptor, targeting RXFP1 may represent a promising avenue to study G_i-coupled receptor based drugs in cardiovascular disease that may allow clarifying specific roles for $G\alpha_{i2}$ and $G\alpha_{i3}$ in response to GPCR activation directly in the vasculature.

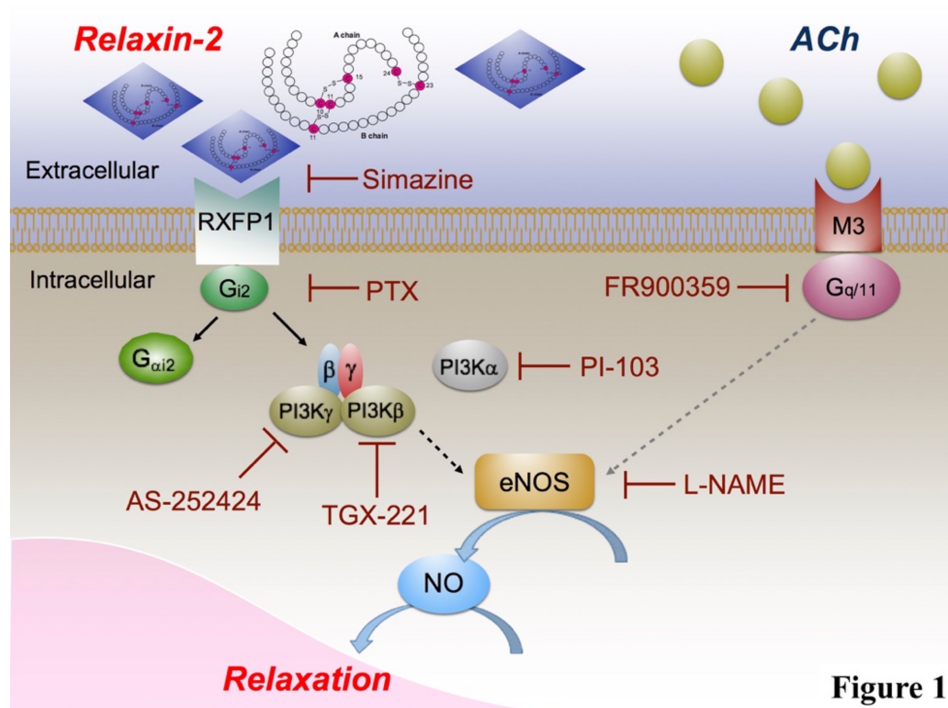

Figure 1: Proposed vasodilatory pathways caused by relaxin-2. Relaxin-2 activates RXFP1 (blocked by simazine), which leads to G_{i2} activation and dissociation of $G\alpha_{i2}$ and βγ subunits (blocked by PTX). Gβγ subunits in turn activate PI3Kβ (blocked by TGX-221) and PI3Kγ (blocked by AS-252424) to initiate eNOS activation and NO release (blocked by L-NAME) to cause relaxation. PI3Kα (blocked by PI-103) seems not to be involved in this

pathway. On the other hand, ACh binds to muscarinic M3 receptors coupled to $G_q/_{11}$ (blocked by FR900359) to produce eNOS/NO dependent arterial relaxation.

Supraspinal sympathetic control

In SCI mouse, the impaired peripheral resistance below the level of SCI is typical [51], and the decreased reactivity of RAAS contributes to the adaptive changes in regional blood flow in SCI. Mesenteric arteries are normally controlled by preganglionic neurons that project from T4 to T13. Femoral arteries are controlled by preganglionic neurons that project from L2 to L4. It is also known that T4-SCI results in a severity-dependent decline in the number of Fluorogold positive neurons in the rostral ventrolateral medulla (RVLM) [52], where it impacts on cardiovascular function and primary composition of C1 adrenaline-synthesizing neurons [53]. After SCI, the sympathetic preganglionic neurons controlling the vasculature abruptly fail to receive signals projected from adrenergic neurons within the RVLM. This process initiates rapid sprouting of remaining sympathetic preganglionic terminals in ganglia to restore transmission to postganglionic neurons, which in the following may be reconnected with the cord below the lesion and can participate at least in simple spinal reflexes and sympathetic neuroeffector transmission on smooth muscle tissues, including the vasculature [51]. Functionally inappropriate reconnections may cause uncontrollable sympathetic outflow and increased nerve-evoked contractions in the presence of Ang II [54] [55], which cannot be reduced by the AT1-receptor antagonist losartan [56]. However, Ang II causes vasoconstriction via AT1 and has been reported to augment vasocontraction through its facilitating effect on norepinephrine (NE)-mediated sympathetic nerve activity [57]. In this study, it was found that SCI decreases the reactivity to Ang II in mesenteric arteries, but not in femoral arteries. Moreover, it was found that this reduced response is absent in mesenteric arteries from AT1a deficient mice, which suggests that this effect requires AT1a receptor signaling. In rodents, the presence of two pharmacologically identical AT1 receptor subtypes have been described, namely AT1a and AT1b receptors, which exhibit different patterns of expression in vasculature. Previous studies have implicated that the AT1a receptor is primarily responsible for the regulation of systemic blood pressure and cardiac function [58], but the regional blood flow mechanisms differ between peripheral systemic and splanchnic vascular beds [59]. In line with other reports [60], the data of this study show that contraction of the femoral arteries to angiotensin II appears to be mediated by AT1b receptors. This may explain why post-SCI did not decrease reactivity of femoral arteries to angiotensin II. Of note, it has been found that AT1b receptors contribute to the regulation of resting blood pressure particularly when AT1a

receptors are absent [61]. Based on our data it is tempting to speculate that the AT1b receptor remains the dominant receptor for vascular Ang II signaling in rodents after the sensitivity of AT1a is diminished in SCI. We are not able to detect changes in AT1a expression at the mRNA level in the vasculature after T4-SCI, although a tendency of increased AT1b expression in mesenteric arteries was observed after SCI. It is therefore likely that reduced sensitivity of AT1a receptors leads to a diminished response of the vasculature to Ang II post SCI. In order to find candidate genes involved in the diminished sensitivity to angiotensin II, we performed RNAseq analysis of mesenteric arteries. The data show dysregulated mRNAs for proteins important for vasorelaxation (e.g., Bdkrb2 and arginase 1) and vascular remodeling (e.g., Hbegf and Hey2). This pathway may contribute to adaptive changes in regional blood flow in SCI.

Calcification

In this part of the study, SGK1 was identified to act as a decisive regulator of vascular calcification and arterial stiffness. SGK1 induces osteo-/chondrogenic reprogramming of vascular smooth muscle cells, at least partly, via NF-κB activation, while SGK1 inhibition blunts or virtually abrogates vascular calcification in vitro and in vivo. SGK1 gene expression is highly dynamic, and strong upregulation of SGK1 is observed in the cardiovascular system under several pathophysiological conditions [10] [62]. SGK1 gene expression is regulated by various clinically important factors contributing to vascular calcification [63] [64]. Together with these observations, it is warranted the further study SGK1 inhibition as novel therapeutic strategy in cardiovascular diseases.

7. References

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8. Anteilserklärung / eidesstattliche Versicherung

Eidesstattliche Versicherung

„Ich, Xiaoming Lian, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: [Novel insights into the vasodilatory effects of relaxins and supraspinal sympathetic control of murine peripheral arteries] selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Meine Anteile an den ausgewählten Publikationen entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst."

____

Datum Unterschrift

<u>Anteilserklärung an den erfolgten Publikationen</u>

Xiaoming Lian hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1:

Lian X, Beer-Hammer S, König GM, Kostenis E, Nürnberg B, Gollasch M. RXFP1 Receptor Activation by Relaxin-2 Induces Vascular Relaxation in Mice *via* a $G\alpha_{i2}$-Protein/PI3Kß/γ/Nitric Oxide-Coupled Pathway. *Front Physiol*. 2018;9:1234.

Beitrag im Einzelnen:

Tested and designed the protocol; performed all experiments; analyzed the data and prepared all figures; drafted the manuscript text.

Publikation 2:

Järve, A.,*Todiras, M.,* **Lian, X**.,* Filippelli-Silva, R., Qadri, F., P. Martin, R.,Gollasch, M., Bader, M. Distinct roles of angiotensin receptors in autonomic dysreflexia following high-level spinal cord injury in mice. *Exp Neurol.* 2018;10(311):173-181. (*Contributed equally)

Beitrag im Einzelnen:

Lian, X.: Participated in designing the protocol; performed all myography experiments; analyzed the data of Figures 3, 4, 5; participated in writing the manuscript text.

Järve, A.: Participated in designing the protocol; prapared the mouse models; performed PCR and CRD experiments; analyzed the data of Figures 1, 2, 6; participated in writing the manuscript text.

Todiras, M.: Participated in designing the protocol; prapared the mouse models.

Publikation 3:

Voelkl J, Luong TT, Tuffaha R, Musculus K, Auer T, **Lian X**, Daniel C, Zickler D, Boehme B, Sacherer M, Metzler B, Kuhl D, Gollasch M, Amann K, Muller DN, Pieske B, Lang F, Alesutan I. SGK1 induces vascular smooth muscle cell calcification through NF-κB signaling. *J Clin Invest.* 2018;128(7):3024-3040.

Beitrag im Einzelnen:

Participated in designing the protocol; measured aortic stiffness ex vivo; analyzed the data of Figure 6F

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Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

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Unterschrift des Doktoranden/der Doktorandin

9. Druckexemplare der ausgewählten Publikationen

Publication 1 (Seite 25 - 37):

Lian X, Beer-Hammer S, König GM, Kostenis E, Nürnberg B, Gollasch M. RXFP1 Receptor Activation by Relaxin-2 Induces Vascular Relaxation in Mice *via* a $G\alpha_{i2}$-Protein/PI3Kß/γ/Nitric Oxide-Coupled Pathway. *Front Physiol*. 2018;9:1234.

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Publication 2 (Seite 38 - 46):

Järve, A.,*Todiras, M.,* **Lian, X**.,* Filippelli-Silva, R., Qadri, F., P. Martin, R.,Gollasch, M., Bader, M. Distinct roles of angiotensin receptors in autonomic dysreflexia following high-level spinal cord injury in mice. *Exp Neurol.* 2018;10(311):173-181. (*Contributed equally)

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RXFP1 Receptor Activation by Relaxin-2 Induces Vascular Relaxation in Mice *via* a $G\alpha_{i2}$-Protein/PI3Kß/γ/Nitric Oxide-Coupled Pathway

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Background: Relaxins are small peptide hormones, which are novel candidate molecules that play important roles in cardiometablic syndrome. Relaxins are structurally related to the insulin hormone superfamily, which provide vasodilatory effects by activation of G-protein-coupled relaxin receptors (RXFPs) and stimulation of endogenous nitric oxide (NO) generation. Recently, relaxin could be demonstrated to activate G_i proteins and phosphoinositide 3-kinase (PI3K) pathways in cultured endothelial cells *in vitro*. However, the contribution of the G_i-PI3K pathway and their individual components in relaxin-dependent relaxation of intact arteries remains elusive.

Methods: We used $G\alpha_{i2}$- (*Gnai2*$^{-/-}$) and $G\alpha_{i3}$-deficient (*Gnai3*$^{-/-}$) mice, pharmacological tools and wire myography to study G-protein-coupled signaling pathways involved in relaxation of mouse isolated mesenteric arteries by relaxins. Human relaxin-1, relaxin-2, and relaxin-3 were tested.

Results: Relaxin-2 (∼50% relaxation at 10^{-11} M) was the most potent vasodilatory relaxin in mouse mesenteric arteries, compared to relaxin-1 and relaxin-3. The vasodilatory effects of relaxin-2 were inhibited by removal of the endothelium or treatment of the vessels with *N* (G)-nitro-L-arginine methyl ester (L-NAME, endothelial nitric oxide synthase (eNOS) inhibitor) or simazine (RXFP1 inhibitor). The vasodilatory effects of relaxin-2 were absent in arteries of mice treated with pertussis toxin (PTX). They were also absent in arteries isolated from *Gnai2*$^{-/-}$ mice, but not from *Gnai3*$^{-/-}$ mice. The effects were not affected by FR900359 ($G\alpha_q$ protein inhibitor) or PI-103 (PI3Kα inhibitor), but inhibited by TGX-221 (PI3Kβ inhibitor) or AS-252424 (PI3Kγ inhibitor). Simazine did not influence the anti-contractile effect of perivascular adipose tissue.

Conclusion: Our data indicate that relaxin-2 produces endothelium- and NO-dependent relaxation of mouse mesenteric arteries by activation of RXFP1 coupled to G_{i2}-PI3K-eNOS pathway. Targeting vasodilatory G_i-protein-coupled RXFP1 pathways may provide promising opportunities for drug discovery in endothelial dysfunction and cardiometabolic disease.

Keywords: serelaxin, relaxin-2, endothelial Gα_{i2}, NO, RXFP1 receptor, perivascular-adipose tissue, ADRF

INTRODUCTION

Cardiometabolic syndrome is a combination of metabolic dysfunctions mainly characterized by insulin resistance, impaired glucose tolerance, dyslipidemia, hypertension, and central adiposity. Relaxins were initially viewed as pregnancy hormones because originally identified to be produced by the ovary corpus luteum in pregnant women (Bell et al., 1987). Later relaxins were also found to be produced in the heart, in the mammary gland, in the endometrium (Kakouris et al., 1992; Du et al., 2010), in the placental trophoblast cells and in the prostate (Hansell et al., 1991). Relaxins are structurally related to the insulin hormone superfamily, consisting of two peptide chains derived from a common processor that are linked by two disulfide bridges. They act through different relaxin family peptide receptors (RXFPs) and signaling pathways (Dessauer and Nguyen, 2005). Humans express three forms of relaxins encoded by three separate genes, that is, relaxin-1 (*RLX1*), relaxin-2 (*RLX2*), and relaxin-3 (*RLX3*) genes. Relaxin-1 and relaxin-2 are the major circulating relaxin isoforms in humans and other mammals (Grossman and Frishman, 2010), while relaxin-3 is expressed at high levels in the nucleus incertus in human and rodent brains, where it has been postulated to act locally as a neuropeptide (Goto et al., 2001).

In recent years, there has been increasing interest in the possible role of relaxins in cardiometabolic syndrome. Relaxin treatment reduces food intake in rats (McGowan et al., 2010), reverses insulin resistance and restored endothelial-dependent vasodilatation in high-fat-diet mice (Bonner et al., 2013). Moreover, RXFPs have been suggested to represent potential targets for anti-anxiety and anti-obesity drugs (Halls et al., 2007). Meanwhile, serelaxin (RLX030), the drug represents the recombinant form of human relaxin-2, also shows promising effects in the therapeutic process in patients with hypertension (Papadopoulos et al., 2013), acute heart failure (AHF) (Teerlink et al., 2013), and ischemic heart disease (Parikh et al., 2013), most likely through its vasodilatory, antifibrotic, and antigenic properties (Conrad, 2010).

Relaxins are capable of inducing vasodilation in human (Fisher et al., 2002) and rodent microvessels (Conrad, 2010). The effects are mediated *via* specific G-protein-coupled receptors (GPCRs), the relaxin family peptide receptor (RXFP) 1–4 (Bathgate et al., 2006). RXFP1 is widely expressed in heart, kidney, lung, liver, blood vessels, and various areas of the brain. This GPCR is considered to be the fundamental RXFP receptor to mediate relaxin effects in the cardiovascular system by complex mechanisms and intracellular signaling pathways (Bani-Sacchi et al., 1995; Nistri and Bani, 2003). Cell culture experiments indicate that relaxin could activate endothelial nitric oxide synthase (eNOS) *via* a pertussis toxin (PTX)-sensitive G_i-PI3K-dependent pathway (Halls et al., 2006; Novak et al., 2006; van der Westhuizen et al., 2008). However, it is unknown whether this pathway is relevant to vasodilation of intact vessels. It is also unknown which G proteins are involved in this pathway. RXFP2 activates adenylate cyclase in recombinant systems, but physiological responses are sensitive to pertussis toxin. RXFP3 and RXFP4 resemble more conventional peptide ligand receptors and both inhibit adenylate cyclase, and in addition RXFP3 activates Erk1/2 signaling *in vitro* (Bathgate et al., 2006). Although G_i-proteins have been suggested to play important roles in cardiovascular disease, in particular in ischemia reperfusion injury (Eisen et al., 2004), the involvements of specific G_i isoform(s) [$G\alpha_{i1}$, $G\alpha_{i2}$, and/or $G\alpha_{i3}$] and vasodilatory GPCRs are unknown. This is particularly important since there are only few reports that GPCRs are capable to utilize G_i-coupled signaling pathways to cause vasodilation (i.e., for bradykinin, beta$_2$ adrenergic agonists, thrombin) (Liao and Homcy, 1993; Ciccarelli et al., 2007; Vanhoutte et al., 2017). It is therefore not surprising that current research is focused on the identification of novel compounds and GPCRs which can utilize G_i-signaling pathways to produce potent relaxations. Since relaxins are endogenous hormones, which could exhibit vascular effects *via* G_i protein-coupled pathways (Halls et al., 2006; Novak et al., 2006; van der Westhuizen et al., 2008), our study was aimed to examine the putative vasodilatory effect of relaxins and the involved G- and PI3K-dependent signaling pathways. In this study, we compared the sensitivity of the three human relaxins 1–3 in eliciting relaxation of mouse mesenteric arteries and tested the hypothesis that the NO-dependent vasodilatory effect of relaxins is mediated by the activation of endothelial RXFP1 receptors, which are coupled to vasodilatory G_{i2}-PI3K-eNOS signaling pathways. Finally, we also tested whether the RXFP1 pathway is involved in the periadventitial control of arterial tone by perivascular adipose tissue (PVAT).

MATERIALS AND METHODS

Mice

Experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocols were previously approved by the local Animal Care and Use Committee from Berlin LAGeSo (G0132/14). Animal experiments used 10- to 14-week-old mice of either sex and were housed in groups of four to six animals in cages with nesting material, mouse lodges, and open access to water and feed, at 23°C with a 12 h/12 h circadian

cycle. Most experiments were performed using male wild-type (WT, C57BL/6N) mice. To define $G\alpha_i$ isoforms involved in relaxin effects, we used female $G\alpha_{i2}$-deficient (*Gnai2*$^{-/-}$) and $G\alpha_{i3}$-deficient (*Gnai3*$^{-/-}$) mice and respective littermate (+/+) controls. The generation and basal phenotypic characterization of $G\alpha_{i2}$-deficient and $G\alpha_{i3}$-deficient mice are described elsewhere (Rudolph et al., 1995; Gohla et al., 2007; Ezan et al., 2013; Wiege et al., 2013).

Preparation of PTX-Treated Animals

Male wild-type C57BL/6 mice (20–25 g, 8–12 weeks) were maintained according to national guidelines for animal care at the animal facility. Mice were injected intraperitoneally with 150 μg/kg body weight pertussis toxin (PTX) or NaCl solution (0.9 %) as vehicle control 48 h before use (Köhler et al., 2014).

Measurement of Vascular Reactivity by Wire Myography

The second branches of mesenteric arteries were isolated from mice under inhalation anesthesia with isoflurane and killed by cervical dislocation. The vessels were then quickly transferred to cold (4°C) and oxygenated (95% O_2/5% CO_2) physiological salt solution (PSS) containing (in mmol/L): 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11.1 glucose, 1.6 $CaCl_2$, and then dissected into 2 mm rings whereby perivascular fat and connective tissue were either intact [(+) PVAT or removed (−) PVAT] without damaging the adventitia. Each ring was positioned between two stainless steel wires (diameter 0.0394 mm) in a 5-mL organ bath of a Small Vessel Myograph (DMT 610M, Danish Myo Technology, Aarhus, Denmark) (Fésüs et al., 2007). The software Chart5 (AD Instruments, Ltd., Spechbach, Germany) was used for data acquisition and display. The rings were pre-contracted and equilibrated for 30 min until a stable resting tension has been acquired. In some vessels, the endothelium was removed mechanically by a whisker or an air bubble (Fésüs et al., 2007). Endothelium integrity or functional removal was confirmed by the presence or absence, respectively, of the relaxant response to 1 μM acetylcholine (ACh) on phenylephrine (PE 1 μM) pre-contracted arteries. Following PSS wash, the pharmacological drugs were applied. After a waiting period of 30 min, PE and subsequently relaxin-1 or -2 or -3 or vehicle (PSS) was added to the bath solution. Relaxations induced by relaxins were expressed as percentage relaxations obtained with ACh (100%) or as percentage relaxations of PE contractions. Contractions induced by PE were expressed as percentage tension obtained with KCl-PSS (100%) containing (in mmol/L): 63.7 NaCl, 60 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 Mg_2SO_4, 11.1 glucose, and 1.6 $CaCl_2$. During the experiments, relaxin was applied for at least 5 min, and data were recorded to ensure that relaxins achieved their maximal effects (Willcox et al., 2013). All drugs were added to the bath solution (PSS).

Materials

FR900359 was isolated as described previously (Schrage et al., 2015). Salts and drugs have been purchased from Sigma-Aldrich (Germany) with the exception of PTX and TGX 221, which were obtained from Merck Millipore (Calbiochem) (Germany) and PI-103 from Enzo Life Sciences. The PI3K inhibitors TGX-221, AS-252424, and PI-103 were administered at submaximal concentrations to achieve biological effects (Ali et al., 2008; Tsvetkov et al., 2016a). All drugs were freshly dissolved on the day of the experiment according to the material sheet. The relaxin peptides have been purchased from Sigma-Aldrich (Germany). Relaxins were dissolved in water. The following concentrations were used: relaxin-1 at 10 pM or at 100 pM, relaxin-2 at 10 pM or at 100 pM or from 1 pM to 10 nM, relaxin-3 at 10 pM or at 100 pM.

Statistical Analyses

Results are presented as mean ± SEM. Data were analyzed statistically using the GraphPad Prism 7 software (GraphPad Software, San Diego, CA, United States). Unpaired Student's *t*-tests or ANOVA were used where appropriate. A value of P less than 0.05 was considered statistically significant; n represents the number of arteries tested.

RESULTS

Relaxation of Mesenteric Arteries by Relaxin-1, Relaxin-2, and Relaxin-3

We first evaluated the vasoactive properties of the three different relaxins, that is, relaxin-1, relaxin-2, and relaxin-3. Isolated mesenteric artery rings were pre-contracted by phenylephrine (PE 1 μM) and exposed to acetylcholine (ACh 1 μM) for control. After wash-out of these substances, the vessels were re-exposed to PE and subsequently incubated with relaxin-1, relaxin-2, or relaxin-3 (at 10^{-10} M each) in separate vessels (Willcox et al., 2013) (**Figure 1**). All three relaxins produced relaxations (**Figure 2A**). However, relaxin-2, at the same concentration tested, was more effective than relaxin-1 or relaxin-3 in producing vasodilatory effects (**Figure 2A**). Importantly, this increased efficiency for relaxin-2 was even more pronounced at 10-fold lower concentrations (10^{-11} M; **Figure 2B**). Compared to ACh, relaxations in response to relaxin-1, relaxin-2, and relaxin-3 were slow and delayed (**Figure 1**). In order to exclude the possibility that relaxations by relaxins result from a spontaneous loss of the pre-contraction level, we performed control experiments with vehicle (PSS) in arteries with and without endothelium. Our results argue against this possibility (**Figure 2**). Removal of the endothelium abolished both ACh and relaxin-2 relaxations (**Figure 1A**, right). These data indicate that relaxins are powerful peptide hormones to produce relaxations, probably utilizing an endothelium-dependent mechanism distinct from ACh. Since relaxin-2 was the strongest vasodilator, we continued the following mechanistic studies using this relaxin peptide.

Relaxin-2 Causes RXFP-Induced Relaxation via eNOS/NO Signaling Without Involvement of $G\alpha_q$ Proteins

Figure 3 shows that the RXFP1 blocker simazine (100 nM) inhibited relaxations induced by relaxin-2. **Figure 4** shows

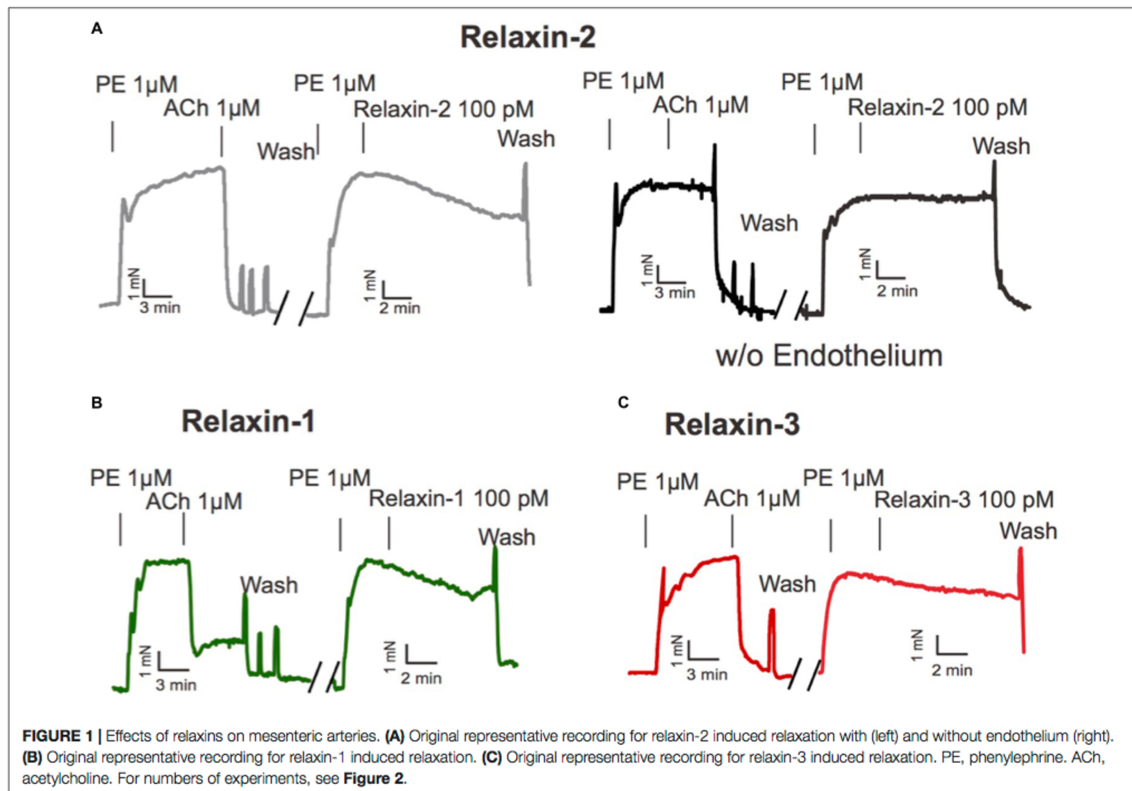

FIGURE 1 | Effects of relaxins on mesenteric arteries. **(A)** Original representative recording for relaxin-2 induced relaxation with (left) and without endothelium (right). **(B)** Original representative recording for relaxin-1 induced relaxation. **(C)** Original representative recording for relaxin-3 induced relaxation. PE, phenylephrine. ACh, acetylcholine. For numbers of experiments, see **Figure 2**.

that the $G\alpha_q$ protein inhibitor FR900359 (100 nM) (Schrage et al., 2015) inhibited concentration-dependent relaxations by ACh (**Figure 4B**), but had no effect on relaxin-2-dependent relaxations (**Figure 4A**). In these experiments, pre-tension was induced by KCl-PSS, which causes membrane depolarization to cause Ca^{2+} influx into vascular smooth muscle cells and hence vasocontraction independently of $G\alpha_q$ protein activation (Wirth et al., 2008). In contrast, the eNOS inhibitor L-NAME (100 μM) inhibited both relaxin-2- and ACh-induced relaxations (**Figures 4A,B**, respectively). Together, the data indicate that both relaxin-2 and ACh produce relaxation by an endothelial-dependent mechanism involving eNOS/NO release. However, whereas ACh utilizes an eNOS/NO signaling pathway involving muscarinic receptors coupled to G_q proteins (Kruse et al., 2012), relaxin-2 stimulates RXFP1 receptors coupled to G proteins other than G_q to produce eNOS/NO-dependent relaxation.

Involvement of G_i Proteins in Relaxin-2-Induced Relaxation

To determine a possible role of G_i proteins in relaxin-2-dependent relaxation, we treated mice with pertussis toxin (PTX), which is used as pan-$G\alpha_i$-inhibitor *in vivo* (Devanathan et al., 2015). Control mice were treated with 0.9% NaCl only. **Figure 5A** shows that G_q-dependent relaxations by ACh were preserved in mesenteric arteries treated with PTX. However, relaxation in response to relaxin-2 were abolished in mesenteric arteries from mice pre-treated with PTX (**Figures 5A,C**) compared to control arteries (**Figures 5B,C**). In contrast, relaxations in response to ACh were not changed by PTX treatment (**Figure 5D**). The G_i-family comprises three closely related Gα members, $G\alpha_{i1-3}$, with $G\alpha_{i2}$ and $G\alpha_{i3}$ abundantly expressed in the cardiovascular system (Hippe et al., 2015). We therefore used $G\alpha_{i2}$-deficient (*Gnai2*$^{-/-}$) and $G\alpha_{i3}$-deficient (*Gnai3*$^{-/-}$) mice to determine which $G\alpha_i$ isoforms are involved in relaxin-2 mediated relaxations. **Figure 6** shows that relaxin-2 induced relaxations were reduced in $G\alpha_{i2}$-deficient arteries (**Figures 6A,C**) but not in $G\alpha_{i3}$-deficient arteries (**Figures 6B,D**). These data indicate that $G\alpha_{i2}$ plays an important role in RXFP1 mediated relaxations.

Involvement of PI3Kβ and PI3Kγ in Relaxin-2-Dependent Relaxations

PI3K signaling has been proposed to mediate slow and sustained activation of eNOS and subsequent NO release in cell culture experiments (Dimmeler et al., 1999). We tested the contribution of different PI3K isoforms in eNOS/NO-dependent RXFP1 receptor-mediated relaxations in comparison to ACh-induced

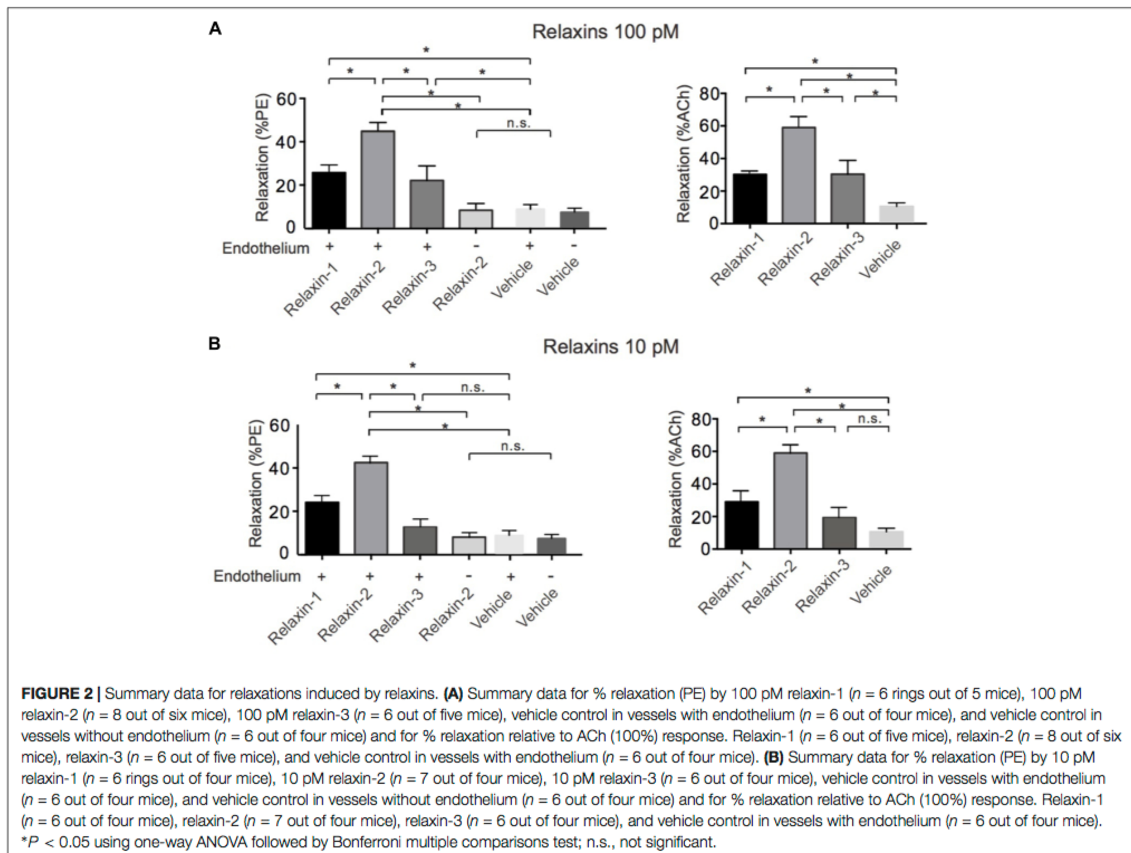

FIGURE 2 | Summary data for relaxations induced by relaxins. **(A)** Summary data for % relaxation (PE) by 100 pM relaxin-1 (n = 6 rings out of 5 mice), 100 pM relaxin-2 (n = 8 out of six mice), 100 pM relaxin-3 (n = 6 out of five mice), vehicle control in vessels with endothelium (n = 6 out of four mice), and vehicle control in vessels without endothelium (n = 6 out of four mice) and for % relaxation relative to ACh (100%) response. Relaxin-1 (n = 6 out of five mice), relaxin-2 (n = 8 out of six mice), relaxin-3 (n = 6 out of five mice), and vehicle control in vessels with endothelium (n = 6 out of four mice). **(B)** Summary data for % relaxation (PE) by 10 pM relaxin-1 (n = 6 rings out of four mice), 10 pM relaxin-2 (n = 7 out of four mice), 10 pM relaxin-3 (n = 6 out of four mice), vehicle control in vessels with endothelium (n = 6 out of four mice), and vehicle control in vessels without endothelium (n = 6 out of four mice) and for % relaxation relative to ACh (100%) response. Relaxin-1 (n = 6 out of four mice), relaxin-2 (n = 7 out of four mice), relaxin-3 (n = 6 out of four mice), and vehicle control in vessels with endothelium (n = 6 out of four mice). *P < 0.05 using one-way ANOVA followed by Bonferroni multiple comparisons test; n.s., not significant.

relaxations. Vessels were pre-contracted with PE, incubated with L-NAME or various PI3K inhibitors and subsequently exposed to relaxin-2 (**Figure 7**). **Figures 7A,B** shows that relaxin-2-induced relaxations were inhibited by L-NAME. The PI3Kβ inhibitor TGX-221 (**Figure 7B**) and the PI3Kγ inhibitor AS-252424 (**Figure 7B**) had similar inhibitory effects on stimulation by relaxin-2. However, relaxin-2-induced relaxations were not affected by the PI3Kα inhibitor PI-103 (**Figure 7B**). Together, the data suggest that PI3K isoforms β and γ are essential intermediate signaling components in eNOS/NO-dependent relaxation by relaxin-2, which are controlled by relaxin-2 that act on RXFP1 receptors coupled to G_{i2} proteins.

RXFP1 Activation by Relaxins Is Unlikely Involved in Arterial Tone Regulation by Perivascular Adipose Tissue

Perivascular adipose tissue (PVAT) plays a functional role in regulating the contractile state of arteries by production of numerous vasodilatory substances (Gollasch, 2017). Since relaxin(s) are expressed in adipose tissue (Hausman et al., 2006), these polypeptide hormones might represent an adipose-derived relaxing factor released by PVAT to contribute to anti-contractile effects on arterial tone. Therefore, we studied vascular contractions in response to PE in arterial ring in the presence and absence of PVAT. **Figure 8** shows that in the absence of PVAT phenylephrine was more potent in causing effective contractions of arterial rings than in the presence of PVAT. This anti-contractile effect of PVAT was not influenced by the incubation of the arteries with the RXFP1-receptor antagonist simazine (100 nM) (**Figure 8**). We also performed control experiments on whether simazine or the other drugs used in this study affect contractions caused by PE. The results in **Supplementary Figure S1** show that contractions caused by PE in the absence (first PE application) and presence of the respective drugs (second PE application) were not different. The data demonstrate that neither simazine nor the other drugs used in this study affected PE contractions.

DISCUSSION

Our study shows that relaxins are extremely potent (in the low picomolar range) endothelium-dependent and

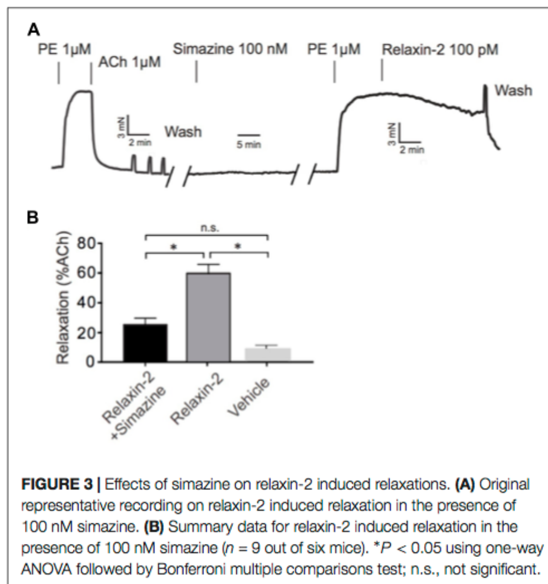

FIGURE 3 | Effects of simazine on relaxin-2 induced relaxations. **(A)** Original representative recording on relaxin-2 induced relaxation in the presence of 100 nM simazine. **(B)** Summary data for relaxin-2 induced relaxation in the presence of 100 nM simazine (n = 9 out of six mice). *P < 0.05 using one-way ANOVA followed by Bonferroni multiple comparisons test; n.s., not significant.

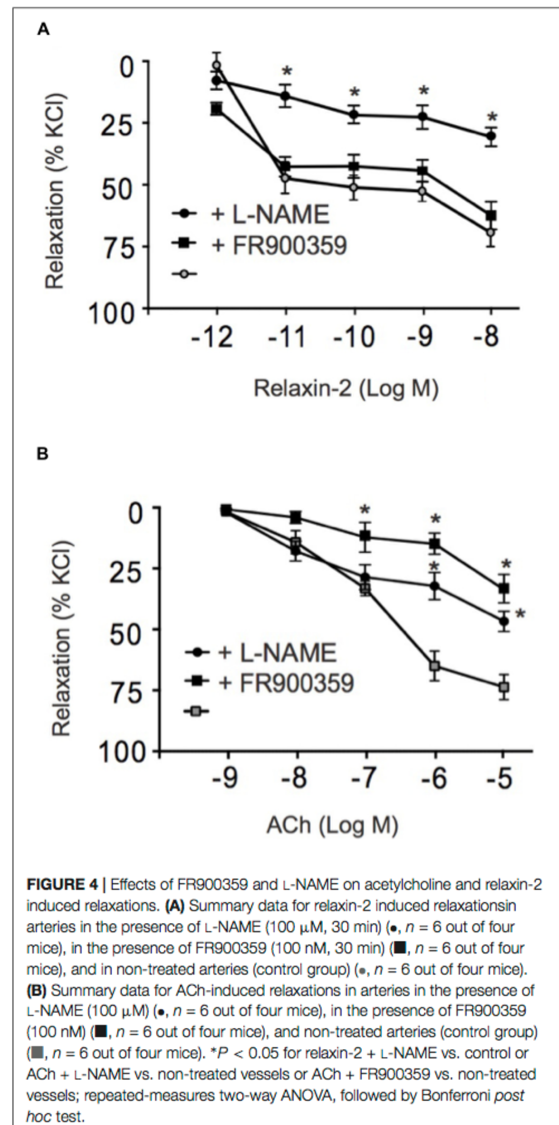

FIGURE 4 | Effects of FR900359 and L-NAME on acetylcholine and relaxin-2 induced relaxations. **(A)** Summary data for relaxin-2 induced relaxationsin arteries in the presence of L-NAME (100 μM, 30 min) (•, n = 6 out of four mice), in the presence of FR900359 (100 nM, 30 min) (■, n = 6 out of four mice), and in non-treated arteries (control group) (•, n = 6 out of four mice). **(B)** Summary data for ACh-induced relaxations in arteries in the presence of L-NAME (100 μM) (•, n = 6 out of four mice), in the presence of FR900359 (100 nM) (■, n = 6 out of four mice), and non-treated arteries (control group) (■, n = 6 out of four mice). *P < 0.05 for relaxin-2 + L-NAME vs. control or ACh + L-NAME vs. non-treated vessels or ACh + FR900359 vs. non-treated vessels; repeated-measures two-way ANOVA, followed by Bonferroni *post hoc* test.

L-NAME-sensitive vasodilators in mouse mesenteric arteries. Similar vasodilatory potencies of relaxins have been observed renal arteries of rats (Novak et al., 2002) and human small gluteal and subcutaneous arteries (Fisher et al., 2002). Among the three human relaxins studied, we identified relaxin-2 as the most effective vasodilator, which produces eNOS/NO-dependent relaxation most likely due to activation of RXFP1 coupled to a G_{i2}-PI3kβ/PI3kγ pathway. Although, we probed human relaxins in a non-human vascular preparation, that is, in isolated arteries from mice, this is the first study suggesting that RXFP1 coupled to a G_{i2}-PI3kβ/PI3kγ pathway is capable of producing vascular relaxation. Furthermore, our data indicate that this pathway does not contribute to PVAT control of arterial tone.

Relaxin Family of Peptides

Relaxin-encoding genes are present in all mammals and responsible for the production of the relaxin peptides that have been initially found in circulating blood during pregnancy. However, more recent studies have observed that relaxins are produced in many tissues in mammals as paracrine or autocrine factors to exert a number of different physiological roles in the vasculature, which may exhibit protective effects in cardiovascular disease (Samuel et al., 2006). The injection of recombinant human relaxins to normotensive rats for 1 to 6 h induced a systemic vasodilatory response (Debrah et al., 2005). This finding suggests that certain vascular beds, for example, in the kidney or mesentery, are able to respond by a vasodilatory response caused by relaxins. Relaxin-2 has been identified as the most important member of the relaxin family and major circulating form of relaxin peptides in humans (Grossman and Frishman, 2010). Relaxin-1 is also believed to exist as circulatory peptide in the circulation (Bathgate et al., 2013), but the function of the relaxin-1 in humans and higher primates is mostly unclear. Relaxin-3 is the most recently identified member of the relaxin family and is primarily expressed in the brain of mammals (Heidari et al., 2018). In our study, we first tested the vasoactive function of all three relaxins in mouse mesenteric arteries. We found that all three relaxins can produce relaxations, with relaxin-2 being the most effective member of the relaxin family. In contrast to ACh, which produces a rapid relaxation, relaxin-1, relaxin-2, and relaxin-3 induced slow and delayed relaxations, indicating different underlying signaling mechanisms between ACh and relaxins to cause endothelium-dependent relaxations.

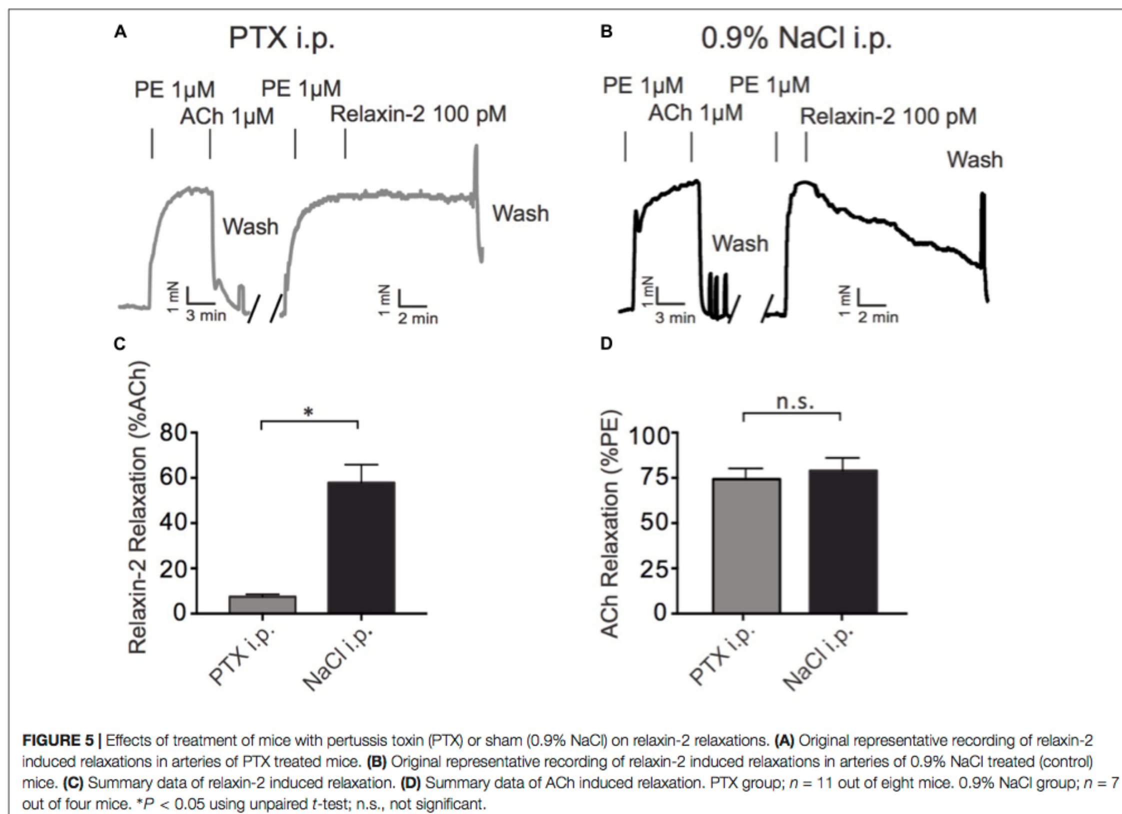

FIGURE 5 | Effects of treatment of mice with pertussis toxin (PTX) or sham (0.9% NaCl) on relaxin-2 relaxations. **(A)** Original representative recording of relaxin-2 induced relaxations in arteries of PTX treated mice. **(B)** Original representative recording of relaxin-2 induced relaxations in arteries of 0.9% NaCl treated (control) mice. **(C)** Summary data of relaxin-2 induced relaxation. **(D)** Summary data of ACh induced relaxation. PTX group; $n = 11$ out of eight mice. 0.9% NaCl group; $n = 7$ out of four mice. $^{*}P < 0.05$ using unpaired t-test; n.s., not significant.

Relaxin-2 Exerts Relaxation by Activating RXFP1 in Endothelium

Studies on isolated vessels have shown that the endothelium is necessary for vasodilation by relaxin in renal and human subcutaneous arteries (McGuane et al., 2011). In our study on mouse mesenteric arteries, we also found that removal of the endothelium or treatment with L-NAME inhibited relaxation by relaxin-2. Our data support previous findings indicating that relaxins cause vasodilation primarily by an eNOS/NO-dependent mechanism (McGuane et al., 2011; Ng et al., 2015; Leo et al., 2016). Relaxin produces its major effects *via* specific G-protein-coupled receptors (GPCRs), that is, RXFP 1-4 (Hsu et al., 2002; Bathgate et al., 2006). Among them, RXFP1 was the first to be identified and remains in focus of interest because of its crucial role in the cardiovascular system (Bathgate et al., 2013). RXFP2 receptors are mainly activated by insulin-like (INSL) 3 in the gubernaculum to facilitate testicular descent into the scrotum, and RXFP3 and RXFP4 receptors are activated by relaxin-3 and INSL5, respectively (van der Westhuizen et al., 2008). RXFP1 has the highest binding potency for relaxin-2 within the several members of the relaxin peptide family (Samuel et al., 2006; Nistri et al., 2007). RXFP1 is predominantly expressed in endothelial cells of mesenteric arteries and veins, but also expressed in the aorta and vena cava (Novak et al., 2006; Jelinic et al., 2014). In our experiments, the RXFP1 antagonist simazine (Park et al., 2016) largely inhibited the endothelium-dependent relaxation induced by relaxin-2, which supports the idea that relaxin-2 produces relaxation primarily through binding to and activation of RXFP1 in the endothelium (**Figure 9**). The remaining relaxation of about 25% in simazine-treated vessels may represent spontaneous relaxation or caused by RXFP activation distinct from RXFP1 in the vasculature or both of them.

Relaxin-2 Induces eNOS/NO-Dependent Vasodilation Through a G_{i2}-PI3Kβ/PI3Kγ Pathway

We aimed to identify the G proteins coupled with RXFP1 to cause eNOS/NO-dependent vasodilation by relaxin-2. Heterotrimeric G proteins, which mediate signals from cell surface receptors to cellular effectors, are composed of α, β, and γ subunits, of which Gα defines the class of G proteins (Simon et al., 1991). The α subunits that define the basic properties of a heterotrimeric G protein can be divided into four families, namely G_s, G_q/G_{11}, G_i/G_o, and $G_{12/13}$ (Kaziro et al., 1988; Wettschureck and Offermanns, 2005). ACh produces relaxation

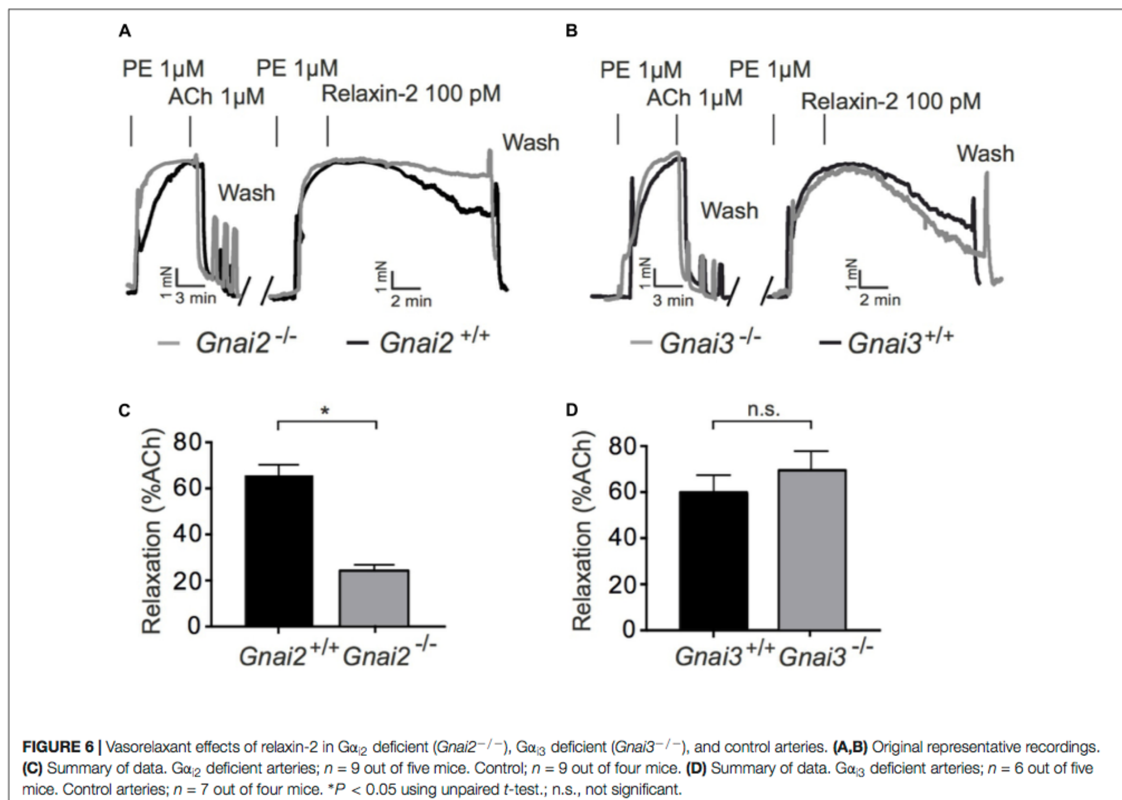

FIGURE 6 | Vasorelaxant effects of relaxin-2 in $G\alpha_{i2}$ deficient (*Gnai2*$^{-/-}$), $G\alpha_{i3}$ deficient (*Gnai3*$^{-/-}$), and control arteries. **(A,B)** Original representative recordings. **(C)** Summary of data. $G\alpha_{i2}$ deficient arteries; $n = 9$ out of five mice. Control; $n = 9$ out of four mice. **(D)** Summary of data. $G\alpha_{i3}$ deficient arteries; $n = 6$ out of five mice. Control arteries; $n = 7$ out of four mice. $^*P < 0.05$ using unpaired *t*-test.; n.s., not significant.

mainly *via* the G_q/G_{11}-coupled M3 receptor subtype (**Figure 9**) (Jaiswal et al., 1991; Kruse et al., 2012). In our study, we found that ACh-induced relaxations were abolished by FR900359, a selective mammalian G_q/G_{11} signaling inhibitor (Schrage et al., 2015), while the relaxin-2-induced relaxations were not blocked by this drug. We conclude that vasodilatory RXFP1-coupled G proteins are distinct from G_q/G_{11} (**Figure 9**). Our data using the pan G_i/G_o inhibitor PTX (Simon et al., 1991) indicate that G_i/G_o proteins could play a major role in relaxin-2 mediated vasodilation. G_0 is particularly abundant in the neuronal and the neuroendocrine system and the G_i-family includes three closely related Gα members, $G\alpha_{i1-3}$, which display overlapping expression patterns with $G\alpha_{i2}$ and $G\alpha_{i3,}$ abundantly expressed in the cardiovascular system (Hippe et al., 2015). According to previous studies on cultured cells, RXFP1 has been suggested to couple to $G\alpha_{i3}$ to release Gβγ dimers to activate the PI3K pathway *via* Akt phosphorylation and subsequently initiate NOS (Halls et al., 2006; McGuane et al., 2011). Our experiments using $G\alpha_{i2}$-deficient (*Gnai2*$^{-/-}$) and $G\alpha_{i3}$-deficient (*Gnai3*$^{-/-}$) mice failed to implicate an important role of $G\alpha_{i3}$, but revealed a key role of $G\alpha_{i2}$ in RXFP1-eNOS/NO relaxation. We found that relaxin-2 induced relaxation was impaired in arteries from *Gnai2*$^{-/-}$, but not from *Gnai3*$^{-/-}$ mice. Although structural similarity between the three G_i subforms suggests that they may exhibit overlapping functions, $G\alpha_{i2}/G\alpha_{i3}$-double-deficient mice cannot be used for myography experiments because they die *in utero* at early embryonic stages. Nevertheless, present data obtained in mice lacking $G\alpha_{i2}$ or $G\alpha_{i3}$ indicate distinct biological key roles of these two $G\alpha_i$-isoforms (Köhler et al., 2014). Thus, we believe that relaxin-2 relaxes mouse mesenteric arteries primarily *via* RXFP1 activation and coupling to G_{i2} but not G_{i3} (**Figure 9**). Moreover, since the pan G_i/G_o inhibitor PTX shows a stronger inhibitory effect than the absence of $G\alpha_{i2}$, we should also consider that relaxin-2 could partly act through other G_i proteins.

Following the release of Gβγ from $G\alpha_i$, it was recently suggested that the class I PI3K represents a target for $G_i\beta\gamma$ signaling by relaxins (McGuane et al., 2011). Accordingly, we found that eNOS activation by relaxin was inhibited by the pan PI3K inhibitors Wortmannin or LY294002 in cultured endothelial cells (Dessauer and Nguyen, 2005). Based on the association with non-catalytic binding proteins, catalytic subunits of class I PI3Ks are subdivided into class IA-isoforms (p110α, -β, and -δ) or class IB p110γ (Hennessy et al., 2005). In this study, we found that relaxin-2-RXFP1 relaxation was inhibited by the PI3Kγ and PI3Kβ inhibitors AS-252424 and TGX-221, respectively. The PI3Kα inhibitor PI-103 had no effects. These data indicate that class I PI3Kγ and PI3Kβ

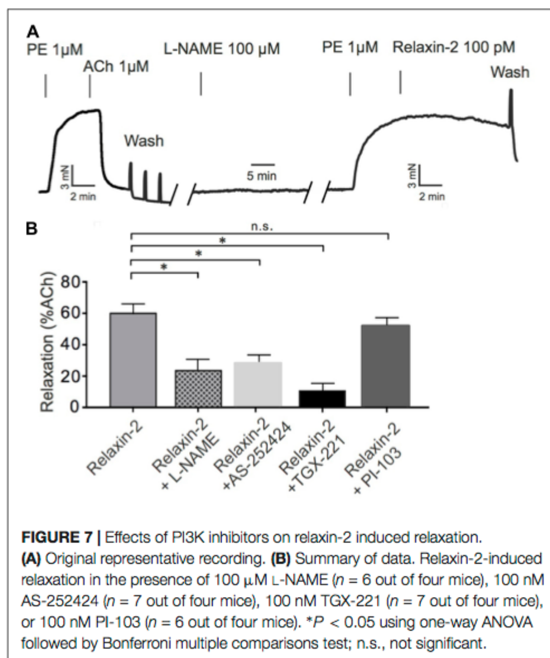

FIGURE 7 | Effects of PI3K inhibitors on relaxin-2 induced relaxation. **(A)** Original representative recording. **(B)** Summary of data. Relaxin-2-induced relaxation in the presence of 100 μM L-NAME (*n* = 6 out of four mice), 100 nM AS-252424 (*n* = 7 out of four mice), 100 nM TGX-221 (*n* = 7 out of four mice), or 100 nM PI-103 (*n* = 6 out of four mice). **P* < 0.05 using one-way ANOVA followed by Bonferroni multiple comparisons test; n.s., not significant.

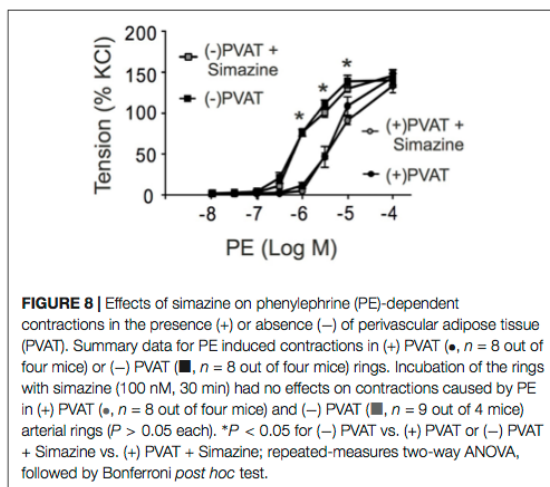

FIGURE 8 | Effects of simazine on phenylephrine (PE)-dependent contractions in the presence (+) or absence (–) of perivascular adipose tissue (PVAT). Summary data for PE induced contractions in (+) PVAT (•, *n* = 8 out of four mice) or (–) PVAT (■, *n* = 8 out of four mice) rings. Incubation of the rings with simazine (100 nM, 30 min) had no effects on contractions caused by PE in (+) PVAT (•, *n* = 8 out of four mice) and (–) PVAT (■, *n* = 9 out of 4 mice) arterial rings (*P* > 0.05 each). **P* < 0.05 for (–) PVAT vs. (+) PVAT or (–) PVAT + Simazine vs. (+) PVAT + Simazine; repeated-measures two-way ANOVA, followed by Bonferroni *post hoc* test.

represents likely a target for G_{i2} signaling by relaxins to cause eNOS/NO dependent relaxation. We are not aware of selective PI3kδ inhibitors to determine a possible additional role of PI3Kδ in RXFP1-mediated relaxation. Nevertheless, our data indicate that class I PI3K activation by a target for RXFP/Gi-βγ signaling to cause eNOS activation is not only a cell culture phenomenon (Dimmeler et al., 1999; Dessauer and Nguyen, 2005), but is important for relaxins to produce vascular relaxation (**Figure 9**). Of note, the relaxin-2-induced relaxations were not fully abolished by TGX-221 or AS-252424. This may indicate that both PI3K isoforms or another PI3K isoform are involved in this vasoregulatory pathway. Also, in addition to the slow activation process of the eNOS/NO by relaxins *via* G_i-PI3K (McGuane et al., 2011), there is also an ultra-slow mechanism of eNOS/NO stimulation *via* κ upon exposure of cultured endothelial cells to relaxin (Dschietzig et al., 2003). Although this putative mechanism cannot be examined by the methodological approach used in our study, such non-RXFP-mediated effects may contribute to the effects of relaxins in the vasculature *in vivo*.

Microarray studies showed expression of relaxin in pig adipose tissue (Hausman et al., 2006). According to our previous studies, PVAT inhibits vessel contraction and produces endothelium-independent relaxation by releasing adipocyte-derived relaxing factor (ADRF) (Löhn et al., 2002; Dubrovska et al., 2004; Verlohren et al., 2004; Tsvetkov et al., 2016b). PVAT dysfunction is characterized by disturbed secretion of various adipokines, which, together with endothelial dysfunction, contribute to hypertension and cardiovascular risk (Lian and Gollasch, 2016). With our interest, we aimed to unravel whether relaxin(s) acting through RXFP1 receptors may represent an ADRF. Our data showed that the RXFP1 antagonist simazine does not influence the anti-contractile effects of PVAT, which indicates that it is unlikely that relaxin(s) acting though RXFP1 receptors contributes to PVAT control of arterial tone, at least in mouse mesenteric arteries.

Limitations

There are a number of limitations in the present study. First, we studied human relaxins rather than mouse relaxins to identify G_i protein-dependent vasodilatory pathways in the murine vasculature. Utilizing the murine vasculature enabled us to use advance of transgenic mouse models. Since serelaxin (RLX030) represents the recombinant form of human relaxin-2, which shows quite reliable therapies in cardiovascular diseases (Papadopoulos et al., 2013; Parikh et al., 2013; Teerlink et al., 2013), we are aimed to identify the function and mechanisms of RXFP1signaling pathways utilized by human relaxins in the vasculature. Of note, human relaxin-2 (H2 relaxin) is the counterpart of mouse relaxin-1 (M1 relaxin) within the structurally related insulin/relaxin superfamily, and mouse RXFP1 shows 89% identity to human RXFP1 (Sherwood, 2004; Kong et al., 2010). Binding of relaxins to RXFP1 is mediated *via* high-affinity binding to extracellular domain of RXFP1 and an additional binding site in the transmembrane (TM) exoloops (Sherwood, 2004). A recent study has shown that specific residues in the center of the H2 relaxin A-chain are necessary for ligand activity at RXFP1 (Park et al., 2008). Importantly, modeling of the ligand–receptor interaction for different RXFP receptors suggests that once the B-chains of the ligands are bound to the primary binding site in a large ectodomain with 10 leucine-rich repeats (LRRs) that the A-chain is presented in a favorable orientation for interaction with the TM exoloops (Hartley et al., 2009). Hence, it is likely that human and murine relaxins utilize similar common

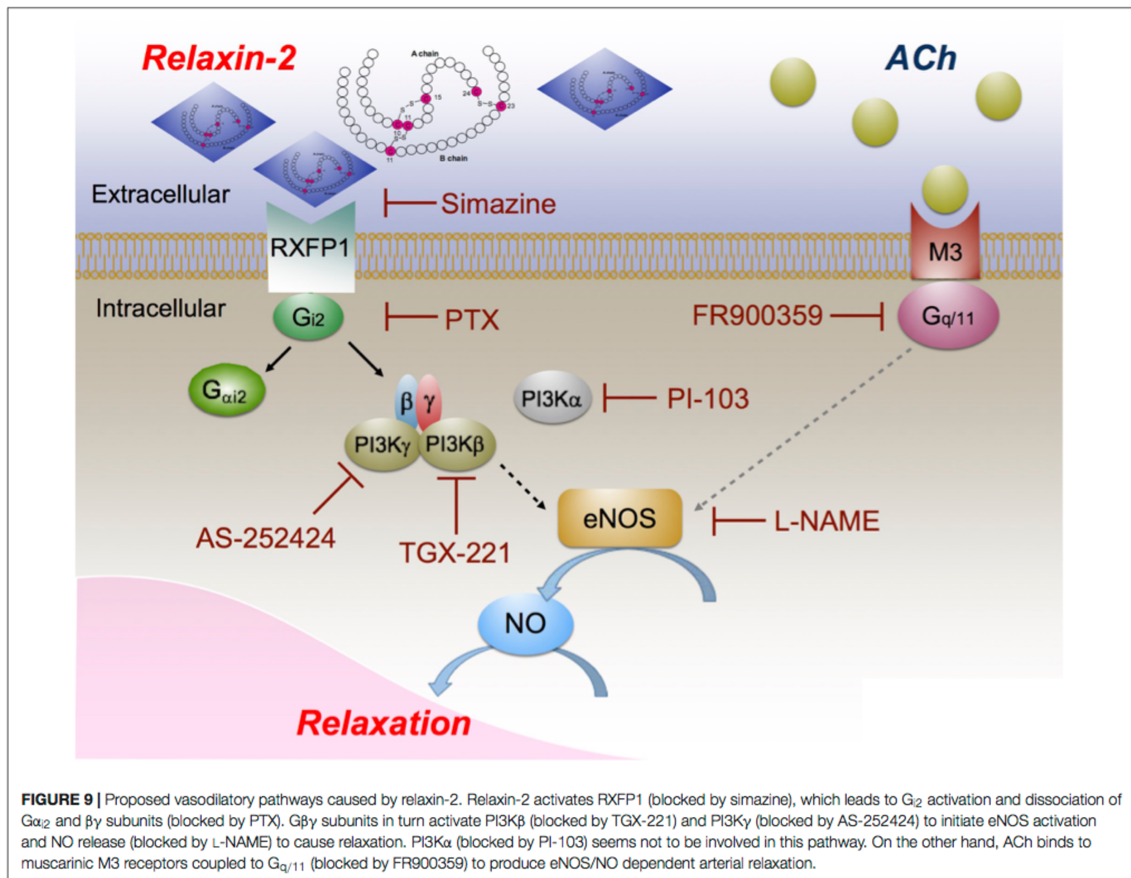

FIGURE 9 | Proposed vasodilatory pathways caused by relaxin-2. Relaxin-2 activates RXFP1 (blocked by simazine), which leads to G_{i2} activation and dissociation of $G\alpha_{i2}$ and βγ subunits (blocked by PTX). Gβγ subunits in turn activate PI3Kβ (blocked by TGX-221) and PI3Kγ (blocked by AS-252424) to initiate eNOS activation and NO release (blocked by L-NAME) to cause relaxation. PI3Kα (blocked by PI-103) seems not to be involved in this pathway. On the other hand, ACh binds to muscarinic M3 receptors coupled to $G_{q/11}$ (blocked by FR900359) to produce eNOS/NO dependent arterial relaxation.

mechanisms to activate RXFP1 receptors, although there might be species-dependent differences in the mode of interaction between the individual relaxins, extracellular RXFP1 domains and the TM exoloops of the individual receptors. Second, we used female instead of male *Gnai2*$^{-/-}$ and *Gnai3*$^{-/-}$ mice and respective littermate controls. Because of the estrous cycle in females, there could be gender-related differences of the vascular reactivity and endothelial function (Sader and Celermajer, 2002), which should be considered. Nevertheless, our data clearly demonstrate a genotype-dependent inhibition of relaxin-2 relaxations, that is, lack of relaxin-2 relaxation in arteries from *Gnai2*$^{-/-}$, but not from *Gnai3*$^{-/-}$ mice. Lastly, considering that inhibition of endothelium may have influence on PE pre-contraction levels, for example, by mitogen-activated protein kinase kinase/extracellular signal-regulated kinase-dependent mechanisms (Molnar et al., 2008), we compared relaxin relaxations with ACh relaxations also in KCl-precontracted vessels. Our data show relaxin-2 relaxations involve an RXFP1-G_i protein pathway in both conditions. The G_q inhibitor FR900359 did not inhibit relaxin-2 relaxations and PE contractions were not affected the drugs used in our study (**Supplementary Figure S1**). Nevertheless, removal of the endothelium may stabilize the level of pre-contraction to play an additional role in eliminating endothelium-dependent RXFP1 relaxations.

CONCLUSION

In summary, we provide evidence that all three relaxins, that is, relaxin-1, relaxin-2, and relaxin-3, are potent vasodilators in mesenteric arteries of mice. Among them, relaxin-2 is the strongest vasodilator, which produces relaxation *via* activation of endothelial RXFP1 coupled to a G_{i2}-PI3Kγ/β-eNOS/NO pathway. Based on the fact that long- and intermediate-distance conduction of vasodilation is common in the circulation, localized releases of relaxins within a tissue might be able to produce remote vasodilations in regions of reduced blood flow distribution. As a potent vasodilatory $G\alpha_{i2}$-coupled receptor, targeting RXFP1 may represent a promising avenue to study G_i-coupled receptor based drugs in cardiovascular disease that may allow clarifying specific roles for $G\alpha_{i2}$

and $G\alpha_{i3}$ in response to GPCR activation directly in the vasculature.

AUTHOR CONTRIBUTIONS

XL performed the wire myography experiments. XL and MG drafted the article. All authors planned and designed the experimental studies and contributed to its completion.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01234/full#supplementary-material

FIGURE S1 | Contractions induced by phenylephrine (PE). Summary data of contractions induced by PE in the absence (first application) and presence (second) application of vehicle (control), simazine, L-NAME, AS252424, TGX-221, or PI-103. For concentrations and number of rings, see other figure legends. w/o endothelium; effects of vehicle in the absence of endothelium. In all other experiments, the endothelium was intact. n.s., not significant.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publication 2: Page 38-46

Järve, A.,*Todiras, M.,* **Lian, X.**,* Filippelli-Silva, R., Qadri, F., P. Martin, R.,Gollasch, M., Bader, M. Distinct roles of angiotensin receptors in autonomic dysreflexia following high-level spinal cord injury in mice. *Exp Neurol.* 2018;10(311):173-181. (*Contributed equally)

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Publication 3: Page 47-63

Voelkl J, Luong TT, Tuffaha R, Musculus K, Auer T, **Lian X**, Daniel C, Zickler D, Boehme B, Sacherer M, Metzler B, Kuhl D, Gollasch M, Amann K, Muller DN, Pieske B, Lang F, Alesutan I. SGK1 induces vascular smooth muscle cell calcification through NF-κB signaling. *J Clin Invest.* 2018;128(7):3024-3040.
https://doi.org/10.1172/JCI96477

10. Lebenslauf (Curriculum Vitae)

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

11.Komplette Publikationsliste

11.1 Manuskriptes

1. **Lian X**, Gollasch M. A Clinical Perspective: Contribution of Dysfunctional Perivascular Adipose Tissue (PVAT) to Cardiovascular Risk. *Curr Hypertens*. 2016;8(11):82.

2. **Lian X**, Beer-Hammer S, König GM, Kostenis E, Nürnberg B, Gollasch M. RXFP1 Receptor Activation by Relaxin-2 Induces Vascular Relaxation in Mice *via* a $G\alpha_{i2}$-Protein/PI3Kß/γ/Nitric Oxide-Coupled Pathway. *Front Physiol*. 2018;9:1234.

3. Järve, A.,*Todiras, M.,* **Lian, X**.,* Filippelli-Silva, R., Qadri, F., P. Martin, R.,Gollasch, M., Bader, M. Distinct roles of angiotensin receptors in autonomic dysreflexia following high-level spinal cord injury in mice. *Exp Neurol.* 2018;10(311):173-181. (*Contributed equally)

4. Voelkl J, Luong TT, Tuffaha R, Musculus K, Auer T, **Lian X**, Daniel C, Zickler D, Boehme B, Sacherer M, Metzler B, Kuhl D, Gollasch M, Amann K, Muller DN, Pieske B, Lang F, Alesutan I. SGK1 induces vascular smooth muscle cell calcification through NF-κB signaling. *J Clin Invest.* 2018;128(7):3024-3040.

11.2 Presentations and Posters

Oral presentation: “Spinal Cord Injury and Reactivity of Mouse Arteries to Humoral Vasoagonists”, Smooth Muscle Meeting, Lübeck, 2017

Oral presentation: “RXFP1 receptor activation by relaxin-2 induces vascular relaxation in mice via a $G\alpha_{i2}$-protein/ PI3Kß/γ/nitric oxide-coupled pathway”, The 8th Scientific Meeting of Asian Society of Vascular Biology, Shanghai, 2018 (sponsored by an awarded Reisestipendium of the Charité – University Medicine)

Poster presentation: “RXFP1 receptor activation by relaxin-2 induces vascular relaxation in mice via a $G\alpha_{i2}$-protein/ PI3Kß/γ/nitric oxide-coupled pathway”, 10th Jahrestagung der Deutschen Gesellschaft für Nephrologie, Berlin, 2018

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