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I. Preface

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1. Summary (English / German)

1.1 Summary

Hypertension, a prevalent global health issue, is primarily driven by increased peripheral vascular resistance, substantially contributing to cardiovascular morbidity and mortality. Among the molecular players, Kv7 channels, particularly Kv7.4 and Kv7.5 subtypes, are recognized as critical regulators of vascular smooth muscle cell (VSMC) membrane potential and vascular tone. This study systematically investigates the vasodilatory properties and Kv7 subtype specificity of two novel activators, KWu183 and KWu176, employing optimized ex vivo vascular models.

A refined isolated perfused kidney (IPK) model and pressurized mesenteric artery system were utilized, providing robust platforms for assessing vascular reactivity under physiologically relevant conditions. To elucidate mechanisms of action, genetically modified mouse models (*Kcnq4*^{-/-}, *Kcnq5*^{-/-}, and *Kcnq4*^{-/-}*Kcnq5*^{-/-}) were combined with endothelial inhibition assays, enabling a clear delineation of endothelial-dependent and -independent pathways.

The results demonstrated that both KWu183 and KWu176 induce dose-dependent vasodilation at nanomolar concentrations, effectively reducing vascular resistance. KWu183 exhibited high specificity for Kv7.5 channels, partially mediated via endothelial mechanisms, whereas KWu176 acted on both Kv7.4 and Kv7.5 subtypes, primarily through endothelial-independent pathways. The involvement of Kv7 channels in these effects was confirmed through pharmacological blockade with XE991. Furthermore, the study highlights the advantages of the optimized IPK model in replicating physiological vascular conditions, reinforcing its value in vascular research.

This investigation establishes KWu183 and KWu176 as promising candidates for therapeutic development targeting Kv7 channels, offering distinct mechanistic insights and subtype selectivity. These findings contribute significantly to the understanding of vascular regulation and lay the groundwork for future clinical exploration of Kv7 modulators in hypertension and vascular disease management.

1.2 Zusammenfassung

Hypertonie, ein weit verbreitetes globales Gesundheitsproblem, wird hauptsächlich durch erhöhten peripheren Gefäßwiderstand verursacht und trägt erheblich zu kardiovaskulärer Morbidität und Mortalität bei. Spannungsgesteuerte Kaliumkanäle der Kv7 Familie, insbesondere die Subtypen Kv7.4 und Kv7.5, sind entscheidend für die Stabilisierung des Membranpotenzials von Gefäßmuskelzellen und die Regulierung des Gefäßtonus. Diese Studie untersucht systematisch die vasodilatierenden Eigenschaften und die Kv7-Subtypspezifität von zwei neuartigen Aktivatoren, KWu183 und KWu176, unter Verwendung optimierter ex vivo Gefäßmodelle.

Ein verfeinertes isoliertes perfundiertes Nierenmodell (IPK) und ein pressurisiertes Mesenterialarteriensystem wurden eingesetzt, um die Gefäßreaktivität unter physiologisch relevanten Bedingungen präzise zu bewerten. Zur Aufklärung der Wirkmechanismen wurden genetisch veränderte Mausmodelle (*Kcnq4*^{-/-}, *Kcnq5*^{-/-} und *Kcnq4*^{-/-}*Kcnq5*^{-/-}) mit Endothelinhibitionsassays kombiniert, um endothelabhängige und -unabhängige Wege klar zu unterscheiden.

Die Ergebnisse zeigten, dass sowohl KWu183 als auch KWu176 dosisabhängige Vasodilatation bei nanomolaren Konzentrationen induzieren und den Gefäßwiderstand effektiv reduzieren. KWu183 wies eine hohe Spezifität für Kv7.5-Kanäle auf, teilweise vermittelt durch endotheliale Mechanismen, während KWu176 sowohl auf Kv7.4- als auch Kv7.5-Subtypen wirkte, hauptsächlich über endothelunabhängige Wege. Die Beteiligung von Kv7-Kanälen an diesen Effekten wurde durch pharmakologische Blockade mit XE991 bestätigt. Darüber hinaus unterstreicht die Studie die Vorteile des optimierten IPK-Modells bei der Replikation physiologischer Gefäßbedingungen und betont dessen Wert in der Gefäßforschung.

Diese Untersuchung identifiziert KWu183 und KWu176 als vielversprechende Kandidaten für die therapeutische Entwicklung, die auf Kv7-Kanäle abzielen, und bietet differenzierte mechanistische Einblicke sowie Subtypspezifität. Diese Erkenntnisse tragen wesentlich zum Verständnis der Gefäßregulation bei und bilden die Grundlage für zukünftige klinische Untersuchungen von Kv7-Modulatoren im Management von Hypertonie und Gefäßerkrankungen.

2 Introduction

2.1. Increased Peripheral Vascular Resistance and Hypertension

Increased peripheral vascular resistance is a key contributor to the development of hypertension, which is one of the most significant risk factors for cardiovascular mortality worldwide (Ercu, Marko, et al., 2020; Olsen et al., 2016; Zhou et al., 2021). Hypertension affects over one billion adults globally, with its prevalence steadily rising (Carey et al., 2022; Dzau & Balatbat, 2019). The substantial burden of hypertension-related cardiovascular complications underscores the urgent need for effective therapeutic interventions targeting the underlying mechanisms of vascular tone regulation. Hypertension not only significantly increases the risk of cardiovascular diseases but also contributes to renal and cerebrovascular disorders, highlighting the systemic impact of elevated blood pressure on multiple organs (Poulter et al., 2015). In addition, the pathophysiology of hypertension involves complex interactions among genetic, environmental, and behavioral factors, presenting challenges in designing targeted therapeutic approaches. Recent advances in understanding the molecular and cellular mechanisms of vascular resistance, particularly the role of ion channels in smooth muscle function, have opened new prospects for hypertension treatment (Daghbouche-Rubio et al., 2022; Joseph et al., 2013).

2.2. Physiological Functions and Pathological Implications of Potassium Voltage-Gated Channel Subfamily Q (Kv7) Channels

The pathogenesis of hypertension is closely associated with peripheral vascular resistance, and alterations in Kv7 channel function constitute a key factor. Therefore, this section will further examine the role of Kv7 channels in vascular physiology and pathology.

2.2.1. Physiological Roles of Kv7 Channels

Kv7 channels play a dual role in both physiological regulation and pathological conditions, notably in vascular systems, where they help maintain normal vascular function and contribute to disease mechanisms. Potassium ion channels are essential for maintaining cell membrane potential stability and regulating excitability, supporting

processes like electrical signaling and osmotic balance (Liu et al., 2023; Oh et al., 2023). Among them, Kv7 channels, encoded by the potassium voltage-gated channel, subfamily Q (KCNQ) gene family, stand out for their role in excitable tissues like smooth muscle. These channels enable repolarization through outward K^+ currents, thereby reducing smooth muscle excitability and contraction (Greenwood & Ohya, 2009; Mackie & Byron, 2008). This unique function highlights their importance as both physiological regulators and therapeutic targets in vascular pathologies.

2.2.2. Kv7 Dysfunction in Vascular Diseases

Kv7 dysfunction is implicated in various vascular diseases, such as hypertension, pulmonary arterial hypertension (PAH), and coronary artery disease. In hypertension, downregulation of Kv7.4 in vascular smooth muscle cells (VSMCs) reduces hyperpolarization and increases vascular resistance, contributing to elevated blood pressure. This is particularly evident in spontaneously hypertensive rats (SHR), where microRNA-153 mediates the post-transcriptional regulation of Kv7.4 (Carr et al., 2016). Kv7 activators, which enhance perivascular adipose tissue (PVAT)-mediated vasorelaxation, have shown promise in hypertension therapy (Tano et al., 2014).

In PAH, impaired Kv7 function leads to increased pulmonary vascular resistance and right ventricular hypertrophy. Kv7.2-5 activators, by relaxing pulmonary arteries, alleviate symptoms such as cardiac hypertrophy (Joshi et al., 2009; Morecroft et al., 2009; Sedivy et al., 2015). Similarly, in coronary artery disease, Kv7 activators induce coronary vasodilation, while blockers reduce coronary flow under hypoxic conditions, emphasizing their protective role during ischemic episodes (Hedegaard et al., 2016; Hedegaard et al., 2014). Additionally, Kv7 modulation may address hypotension in septic shock by countering excessive vasodilation caused by sepsis-associated metabolites (Sakakibara et al., 2015). Other vascular diseases may also involve Kv7 dysfunction, though further research is needed.

These findings underscore the therapeutic potential of Kv7 channels, particularly in conditions such as hypertension, PAH, and septic shock, paving the way for the development of targeted treatments for vascular diseases.

2.3. Structural Features and Composition of Kv7 Channels

The KCNQ gene family consists of five members (KCNQ1-5), located on chromosomal regions 11p15, 20q13, 8q24, 1p34, and 6q13, respectively (Schwake et al., 2003). These genes encode the α -subunits (Kv7.1-5), which assemble to form tetrameric K⁺ channels (Wiener et al., 2008). These tetramers can be homomeric or heteromeric (Jentsch, 2000; Oliveras et al., 2014; Yeung, Pucovsky, et al., 2007). Some potassium channel subunits do not directly participate in voltage sensing or pore formation. These subunits are referred to as auxiliary or β -subunits. These subunits can interact with α -subunits to modulate the biophysical properties of potassium channels, including current magnitude, activation and inactivation kinetics, and pharmacological characteristics (Barhanin et al., 1996; Dedek & Waldegger, 2001; Sanguinetti et al., 1996; Schroeder et al., 2000; Tinel et al., 2000).

Additionally, certain subunits can contribute to the local structure of the channel. For instance, the minK protein from the potassium voltage-gated channel subfamily E regulatory subunit (KCNE) family plays a regulatory role (Kaczmarek & Blumenthal, 1997). Specific residues within these subunits have also been identified as forming parts of the channel pore wall (Tai & Goldstein, 1998; Wang et al., 1996).

2.4 Regulation of Kv7 Channels in Vascular Physiology

2.4.1. Tissue-Specific Expression of Kv7 Channels

Kv7 channels exhibit tissue-specific expression and are critical to various physiological systems, including the heart, central nervous system (CNS), cochlea, and smooth muscle. Kv7.1 is predominantly expressed in the heart, where it forms KCNQ1/KCNE1 heterotetramers responsible for conducting the slow delayed rectifier potassium current (IKs), which is essential for cardiac repolarization (Barhanin et al., 1996; Sanguinetti et al., 1996). In the CNS, Kv7.2/Kv7.3 heteromers are the main contributors to the M-current, which regulates neuronal excitability, while Kv7.5 plays a smaller role (Brown & Adams, 1980; Brown & Passmore, 2009; Wang et al., 1998). In the cochlea, Kv7.4 channels, expressed in outer hair cells, are essential for normal hearing (Kharkovets et al., 2000; Kubisch et al., 1999). In smooth muscle, including vascular and intestinal

types, Kv7.1, Kv7.4, and Kv7.5 are the predominant subtypes (Greenwood & Ohya, 2009). Kv7 channel expression may change during maturation and be influenced by external factors, such as vasoconstrictive agonists, which can alter protein levels without affecting mRNA expression.

2.4.2. Regulation by Membrane Voltage and Mechanical Stimuli

Kv7 channels are regulated by multiple factors, including membrane voltage (V_m), mechanical stimuli, and biochemical signals. V_m is a primary regulator, with activation thresholds (V_{50}) varying among Kv7 subtypes (-20 mV for Kv7.1/4, -30 mV for Kv7.2, and -40 mV for Kv7.3/5). Single-channel conductance for vascular Kv7.1/4/5 channels is ~1-4 pS, and activation/deactivation kinetics vary with V_m , with Kv7.1 generally slower than Kv7.4/5 at different voltages (Barrese, Stott, & Greenwood, 2018). Kv7 channels also mediate responses to mechanical stimuli, such as transmural pressure, via mechanisms involving angiotensin II receptor type 1 (AT1R) (Schleifenbaum, Kassmann, Szijártó, et al., 2014).

2.4.3. Biochemical Regulation and Signaling Pathways

Biochemically, Kv7 channels are influenced by vasoconstrictive pathways involving voltage-operated calcium channels (VOCC), Phosphatidylinositol 4,5-bisphosphate (PIP2), and Gq/11-coupled receptors, as well as vasodilative pathways mediated by cyclic guanosine monophosphate (cGMP) and cGMP-dependent protein kinase (PKG), or cyclic adenosine monophosphate (cAMP) and cAMP-dependent protein kinase (PKA) (Tykocki et al., 2017). PIP2 stabilizes Kv7 channels in the open state, increasing their activation efficiency, particularly in Kv7.1 and Kv7.4 (Brown et al., 2007; Zaydman & Cui, 2014). Gq/11-coupled receptor activation reduces Kv7 currents via PKC-dependent mechanisms, though contradictory findings exist at the whole-vessel level (Mani et al., 2013; Yeung, Pucovsky, et al., 2007). Additionally, long-term angiotensin II exposure may decrease Kv7.4 protein expression via heat shock protein 90 (HSP90), independent of protein kinase C (PKC) (Barrese, Stott, Figueiredo, et al., 2018).

Kv7 channels are also involved in vasodilation via cGMP/PKG and cAMP/PKA pathways. For instance, atrial natriuretic peptide (ANP) induces cGMP-mediated Kv7-dependent

relaxation in rat arteries (Stott et al., 2015), while cAMP increases Kv7 currents through PKA phosphorylation, as seen with β -adrenoceptor agonists (Chadha et al., 2012; Mani et al., 2016; Stott et al., 2016). Notably, Kv7.5 exhibits greater cAMP sensitivity than Kv7.4, highlighting subtype-specific responses (Morales-Cano et al., 2015).

Additional modulators include hydrogen sulfide (H₂S), bradykinin, and oxygen, all of which enhance Kv7 activity or reduce vasoconstriction through Kv7-dependent mechanisms (Hedegaard et al., 2016; Martelli et al., 2013; Zavaritskaya et al., 2013). These findings underscore Kv7 channels' complex and context-dependent regulation in vascular function.

2.5. Function of Kv7 Channels in VSMCs

2.5.1. Smooth Muscle Cells and Membrane Potential

Smooth muscle cells are essential components of involuntary physiological processes, including blood pressure regulation, gastrointestinal motility, and reproductive functions. These cells can be mechanically quiescent (e.g., in arteries) or exhibit spontaneous contractions driven by depolarizing slow waves or rapid action potentials (e.g., in the bladder) (Barrese, Stott, & Greenwood, 2018).

The membrane potential of arterial smooth muscle cells is a key determinant of vascular tone, typically maintained between -40 and -60 mV under physiological conditions. At this range, VOCCs remain partially activated, meaning even minor changes in membrane potential can significantly alter calcium influx and arterial diameter (Nelson, Huang, et al., 1990; Neyroud et al., 1997).

2.5.2. Kv7 Channels as Regulators of Vascular Tone

Kv7 channels are key regulators of this membrane potential in VSMCs, primarily by controlling potassium ion efflux. Their activation induces hyperpolarization, which inhibits VOCCs, reduces calcium influx, and promotes vasodilation, thereby lowering blood pressure. Conversely, the closure of Kv7 channels leads to depolarization, increased VOCC activity, higher intracellular calcium levels, and vascular contraction (Byron & Brueggemann, 2018; Gollasch et al., 2018; Wang et al., 2021). This

mechanism makes Kv7 channels crucial for regulating vascular tension and systemic blood pressure (Nelson, Patlak, et al., 1990).

2.5.3. Kv7 Channels in Complex Regulatory Networks

Kv7 channel-induced hyperpolarization directly modulates the contractile state of VSMCs by suppressing Voltage-gated calcium channel, L-type, alpha 1C subunit (Cav1.2) activity, reducing intracellular calcium levels, and thereby lowering vascular tone (Nelson, Patlak, et al., 1990). Additionally, Kv7 channels form part of a complex regulatory network by interacting with other ion channels and signaling molecules. For instance, Kv7 channels modulate transient receptor potential (TRP) channels, such as transient receptor potential melastatin 4 (TRPM4), which sense mechanical stress and contribute to myogenic responses in arteries. These interactions integrate Kv7-mediated hyperpolarization with mechanical signaling, enabling fine-tuning of vascular tone under various physiological conditions (Schleifenbaum, Kassmann, Szijarto, et al., 2014; Tsvetkov et al., 2024).

2.5.4. Kv7.4 and Kv7.5 Channels in Regulating Vascular Tone and Blood Pressure

The composition of Kv7 channels varies significantly across cell types and species. KCNQ1, KCNQ4, and KCNQ5 are expressed in mouse, rat, and human vasculature, with KCNQ4 being the most abundant in mice and rats, while KCNQ1, KCNQ3, KCNQ4, and KCNQ5 are more evenly expressed in human arteries (Chadha et al., 2012; Joshi et al., 2009; Ng et al., 2011; Yeung, Pucovský, et al., 2007). Pharmacological and electrophysiological studies have identified Kv7.4 and Kv7.5 as the primary functional voltage-activated potassium channel subtypes in vascular smooth muscle cells (VSMCs), playing a central role in regulating vascular tone (Joshi et al., 2009; Mackie et al., 2008; Ng et al., 2011; Yeung, Pucovský, et al., 2007).

Kv7.4 and Kv7.5 can form both homotetramers and heterotetramers, with the heterotetrameric configuration supported by proximity ligation experiments (Brueggemann et al., 2011; Fan & Byron, 2000). Their negative voltage dependence enables activation during mild depolarization, facilitating K⁺ efflux and membrane hyperpolarization, which reduces calcium influx via voltage-gated calcium channels.

This mechanism promotes vasodilation by attenuating vasoconstrictive stimuli (Barrese, Stott, & Greenwood, 2018; Tsvetkov et al., 2024). These channels are critical for maintaining VSMC resting membrane potential and systemic vascular resistance, thereby supporting cardiovascular homeostasis (van der Horst et al., 2021).

Kv7.4 and Kv7.5 dysfunction is closely linked to vascular diseases such as hypertension, where reduced channel activity leads to enhanced vasoconstriction and elevated blood pressure (Tsvetkov et al., 2024; Wang et al., 2021). Thus, these channels are considered important therapeutic targets. Enhancing their function could improve vasodilation and effectively control blood pressure, providing potential strategies for treating hypertension and other cardiovascular diseases.

2.6. Kv7 Channels as Therapeutic Targets in Cardiovascular Disease

In recent years, targeting Kv7 channels expressed in VSMCs has emerged as a promising approach in the treatment of cardiovascular disease (Schleifenbaum, Kassmann, Szijarto, et al., 2014; Stott et al., 2014; Yeung, Pucovsky, et al., 2007). Kv7 channels are not only critical regulators of vascular tone but also exhibit distinct subtype-specific expression patterns across various tissues, enabling selective therapeutic interventions with minimal off-target effects (Miceli et al., 2008).

These channels are expressed in multiple organs, and the differential expression of Kv7 subtypes across tissues suggests potential for targeted therapeutic interventions (Jepps et al., 2021; Miceli et al., 2008; Yeung, Pucovsky, et al., 2007). For instance, Kv7.4 and Kv7.5 are predominantly expressed in vascular smooth muscle, where they regulate resting membrane potential and vascular tone, making them attractive targets for managing hypertension (Barrese, Stott, & Greenwood, 2018). The specificity of Kv7 channels in vascular smooth muscle distinguishes them from other ion channels that are ubiquitously expressed, presenting a novel and targeted approach to managing hypertension and vascular disorders.

Unlike traditional antihypertensive therapies that often act on broader systems, Kv7 channel modulators offer a more localized mechanism of action, thereby reducing the

risk of systemic side effects such as excessive hypotension or reflex tachycardia (Wurm et al., 2022).

2.7. Development of Selective Kv7 Channel Modulators

2.7.1 Challenges and Advances in Kv7 Channel Openers

Kv7 channels openers have significant potential in regulating neuronal and vascular smooth muscle excitability, making them promising for treating epilepsy, pain, and hypertension (Battefeld et al., 2014; Wulff et al., 2009). Flupirtine was the first approved Kv7.2/3 channel opener, widely used as a non-opioid analgesic for over 30 years. However, its metabolism potentially generates reactive azaquinone diimine metabolites, which are believed to trigger immune-mediated idiosyncratic hepatotoxicity through a hapten mechanism. This issue led to its market withdrawal in 2013 (Andrade et al., 2019; Methling et al., 2009; Michel et al., 2012; Nicoletti et al., 2016; Puls et al., 2011; Scheuch et al., 2015; Siegmund et al., 2015). Retigabine, approved in 2011 as an adjunctive therapy for epilepsy, showed a stable non-oxidative metabolic pathway but was associated with the formation of quinone diimine metabolites in melanin-containing tissues, causing blue discoloration of the skin and ocular tissues. This side effect, along with declining usage, resulted in its discontinuation in 2017 (Ciliberto et al., 2012; Garin Shkolnik et al., 2014; Groseclose & Castellino, 2019; Hempel et al., 1999). Addressing these challenges, the development of metabolically stable and safer Kv7 channel openers has become a key research focus, with potential applications not only in neurological disorders but also in hypertension management (Surur et al., 2019; Zhang et al., 2021).

2.7.2 KWu183 and KWu176: Novel Kv7 Channel-Selective Activators

KWu183 and KWu176, novel derivatives of retigabine, are considered promising Kv7 channel-selective activators and have demonstrated potent vasodilatory effects in vascular smooth muscle cells (VSMCs). KWu183 introduces a tetrafluorophenyl group, which substantially increases its selectivity for Kv7 channels and reduces metabolic instability and toxicity. In contrast, KWu176 incorporates a fluorine atom and an ethyl ester group, significantly improving the compound's lipophilicity and membrane permeability while enhancing binding stability with Kv7 channels. These structural

optimizations enable both compounds to exhibit unique pharmacological advantages in vascular regulation (Wurm et al., 2022).

However, the specific selectivity and mechanisms of action of KWu183 and KWu176 on the primary functional Kv7 channel subtypes in vascular smooth muscle remain unclear. Investigating their binding modes, activation effects, and roles under physiological and pathological conditions will not only provide critical insights into their molecular mechanisms but also guide the development of more targeted Kv7 channel activators. Moreover, such studies hold significant potential for advancing the development of novel therapeutic agents for vascular diseases, including hypertension and arterial stiffness.

To further validate the efficacy of these novel Kv7 activators at the whole-organ level, particularly their impact on the renal vascular bed, we employed the IPK model. This approach enables a physiologically relevant assessment of drug effects under near-native conditions, setting the stage for the experiments detailed in the following section.

2.8. The Isolated Perfused Kidney Model: Advantages and Innovations

2.8.1. Importance and Advantages of the IPK Model

Given the potential of selective Kv7 channel activators, robust experimental models are essential for validating their efficacy and specificity. The IPK model provides a physiologically relevant platform for such evaluations.

The IPK model has been a fundamental tool for investigating vascular smooth muscle function in a physiologically relevant context. Compared to methods such as wire myography, this model enables the study of vascular responses under native pressure conditions, maintaining the integrity of the organ's architecture and functionality (Maack, 1980 Feb; Nizet, 1975). The significance of this model lies in its ability to more accurately replicate *in vivo* conditions, thereby providing insights into the mechanisms of vascular regulation in both health and disease.

2.8.2. Challenges and Methodological Advancements

However, the use of IPK in small animal models, such as mice, has posed substantial technical challenges due to the diminutive size of the renal vasculature and the

associated difficulties in surgical preparation and perfusion (Rahgozar et al., 2004). Given the involvement of renal vasculature in systemic blood pressure regulation, the IPK model provides a unique advantage in studying Kv7 modulators within a physiologically relevant setting. My previously published work introduced key methodological advancements, including streamlined protocols for renal artery isolation and high-throughput pressure monitoring, which have enhanced the reliability and efficiency of this model. These innovations have addressed critical limitations in existing methods and allowed for the detailed characterization of vascular tone and drug responses in genetically modified mouse models. These methodological improvements not only overcame prior limitations but also facilitated detailed characterization of vascular tone and drug responses in genetically modified mouse models, ensuring robust and reproducible outcomes (Chu et al., 2024).

2.9. Objectives of This Dissertation

This dissertation builds upon these foundational advancements to further explore the therapeutic potential of Kv7 channel modulators, KWu183 and KWu176, using the optimized IPK model. By integrating novel pharmacological agents and advanced ex vivo techniques, the research aims to provide a deeper understanding of vascular physiology and develop targeted interventions for hypertension.

3. Hypothesis

Specifically, the following hypotheses were tested:

Hypothesis 1: The optimized isolated mouse kidney perfusion method can effectively evaluate vascular function.

Hypothesis 2: KWu183 and KWu176, as Kv7 channel openers, induce vasodilation, and their effects may be modulated by endothelial function.

Hypothesis 3: KWu183 and KWu176 display specificity towards different sub-types of Kv7 channels (Kv7.4 vs Kv7.5).

4. Materials and Methods

4.1. Animal Models

In this study, a series of genetically modified mouse models targeting Kv7.4 and Kv7.5 genes were utilized to investigate the specific roles of Kv7 channels in vascular physiology. Specifically, *Kcnq4*^{-/-} mice were utilized to clarify the role of Kv7.4 in vascular function. To explore the specific contributions of Kv7.5, *Kcnq5*^{-/-} mice were generated by introducing stop codons into exon 6 (Spitzmaul et al., 2013). Additionally, a dominant negative Kv7.5 knock-in model provided further insights into the functional regulation of Kv7.5, particularly regarding protein localization and impaired transport mechanisms (Fidzinski et al., 2015; Spitzmaul et al., 2013). Finally, *Kcnq4*^{-/-}*Kcnq5*^{-/-} double knockout mice were used to examine potential synergistic interactions between Kv7.4 and Kv7.5 in vascular responses (Tsvetkov et al., 2024).

Wild-type littermate controls, aged 8–12 weeks, were included in the experiments not only as a baseline comparison but also to investigate the pharmacological effects of the test compounds. All animal experiments adhered to the American Physiological Society's guidelines and were approved by local animal welfare committees and relevant institutions (No. OE-017/23, LALLF: Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern / State Office for Agriculture, Food Safety and Fisheries Mecklenburg-Western Pomerania), as previously described (Chu et al., 2024).

4.2. Isolated Perfused Kidney

The method for perfusion of isolated kidneys has been described in detail in previous studies (Chu et al., 2024; Cui et al., 2022). Mouse kidneys were isolated and cannulated through the renal artery, followed by perfusion at 37°C with an oxygenated physiological salt solution (PSS) containing 95% O₂ and 5% CO₂, to maintain optimal vascular function. Perfusion pressure was continuously monitored to evaluate the vasoactive effects of pharmacological agents added to the perfusate. Experimental data were recorded in real time using high-precision pressure sensors (PM-4, Living System Instrumentation, Burlington, VT) and a PowerLab acquisition system (AD Instruments,

Colorado Springs, CO). The data were analyzed using LabChart software (v8.1.22, AD Instruments, Colorado Springs, CO). The use of this system ensured a high level of precision, enabling accurate detection of subtle changes in perfusion pressure induced by pharmacological agents. Perfusion pressure was adjusted to an initial level of 80 mmHg and monitored for subsequent changes induced by pharmacological agents.

4.3. Pressure myography

Pressure myography was performed as previously described (Cui et al., 2022; Ercu, Markó, et al., 2020; Kaßmann et al., 2019; Mederos y Schnitzler et al., 2008; Schleifenbaum, Kassmann, Szijarto, et al., 2014). Mesenteric arteries were mounted on glass cannulas and continuously perfused with oxygenated PSS (95% O₂, 5% CO₂; pH 7.4; 37°C). The vessels were pressurized to 60 mmHg using a pressure control system (Living System Instrumentation, Burlington, VT, USA). The inner diameter of the vessels was measured using a microscope (Nikon Diaphot, Düsseldorf, Germany) and a video camera connected to a personal computer for data acquisition and analysis (HaSoTec, Rostock, Germany). The arteries were equilibrated at 37°C for 30 minutes before starting the experiments.

4.4. Pharmacological Agents

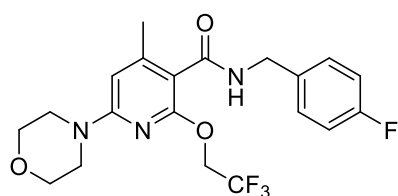
4.4.1. KWu183 and KWu176

KWu183 (C₂₀H₂₁F₄N₃O₃) and KWu176 (C₂₁H₂₆FN₃O₃) are novel Kv7 channel activators developed as carba analogues optimized from the structures of Retigabine and Flupirtine (Wurm et al., 2022). By integrating the core activation structures of Flupirtine and Retigabine, these new compounds retain Kv7 channel activation effects, while introducing key structural modifications to enhance stability and reduce adverse reactions.

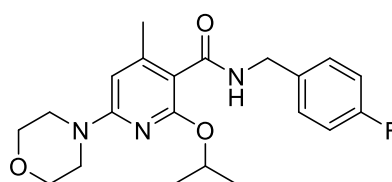
In the molecular structure of KWu183, a tetrafluorophenyl group was introduced, which significantly improves membrane affinity and enhances selectivity for Kv7 channels. The presence of the tetrafluorophenyl group increases the molecule's electron-withdrawing capacity, enabling stronger binding within the hydrophobic regions of the Kv7 channel.

This stable binding characteristic grants KWu183 higher channel activation efficiency and reduces the risk of producing harmful metabolites during oxidative metabolism.

KWu176, on the other hand, incorporates a fluorine atom and an ethyl ester group to increase lipophilicity and membrane permeability, thus improving its absorption in VSMCs and enhancing binding stability with Kv7 channels. These modifications enable KWu176 to penetrate cell membranes more effectively and interact closely with the hydrophobic binding sites of Kv7 channels, thereby enhancing channel activation. These features make KWu176 superior to its precursor compounds in terms of both safety and efficacy.



KWU 183



KWU 176

Both KWu183 and KWu176 were tested in a dose-dependent manner in VSMCs as well as in an isolated kidney perfusion model. A 12-dilution series ranging from 1 nM to 30 μ M was used to characterize the vasodilatory effects of KWu183 and KWu176 for Kv7 channel modulation. In the kidney perfusion experiments, perfusion pressure reductions were observed, showcasing their direct vasodilatory impact on renal vessels. Vessel diameter changes were recorded following each concentration of KWu183 and KWu176 in the VSMC assays, capturing a detailed dose-response profile. The inclusion of both kidney perfusion and VSMC tests provided a comprehensive evaluation of the vasodilatory properties of these compounds across diverse vascular settings.

4.4.2. Flupirtine

Flupirtine, a tri-aminopyridine derivative obtained from Sigma-Aldrich (Cat. No. F8927), was used as a reference compound for Kv7 channel activation. The chemical structure of Flupirtine, 2-amino-3-ethoxy-carbonylamino-6-(4-fluorobenzylamino)-pyridine, was specifically designed to support its functional efficacy. This compound is known to modulate Kv7 channels effectively (Harish et al., 2012). The dose-response profile was

evaluated using a range of concentrations (1 nM, 3nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μ M, 3 μ M, 10 μ M, 30 μ M) in isolated vessel preparations. The recorded data contributed to comparative analysis with KWu183 and KWu176, assessing relative efficacy and potency.

4.4.3. Methoxamine

Methoxamine (ME), a selective α -1 adrenergic receptor agonist, was utilized to induce pre-contraction in isolated vessels at a concentration of 3 μ M (Sigma-Aldrich, Cat. No. M6524). ME activates α -1 adrenergic receptors, initiating the Gq protein-coupled signaling pathway. and subsequent activation of phospholipase C, which generates inositol triphosphate (IP3) and diacylglycerol (DAG). These secondary messengers increase intracellular calcium levels (Chen & Minneman, 2005), resulting in smooth muscle contraction and providing a baseline for subsequent vasodilation assessments. ME is especially suitable for vascular assays due to its limited effects on the central nervous system and its high specificity for α -1 receptors, which reduces the likelihood of off-target interactions (Dean & Reddivari, 2024).

4.4.4. XE991

XE991 was used as a selective Kv7 channel blocker (Jensen et al., 2005; Schröder et al., 2001; Wang et al., 1998; Wright et al., 2021; Yeung, Pucovsky, et al., 2007), at concentrations of 3 μ M and 10 μ M to inhibit Kv7-mediated vasodilation induced by KWu176, KWu183, and Flupirtine. This blocker facilitated the isolation and evaluation of the specific role of Kv7 channels in the observed vasodilatory effects. The tissue was first incubated for 30 minutes, followed by the addition of XE991 and a subsequent 15-minute incubation period to ensure full efficacy of the blocker, enabling precise assessment of Kv7 channel-mediated responses.

4.4.5. Acetylcholine

Acetylcholine (ACh, 1 μ M; Sigma-Aldrich, Cat. No. A6625) was used in the experiments as a standard agent to assess endothelial function. ACh activates M3 receptors on endothelial cells, triggering the release of nitric oxide (NO) and subsequent vasodilation (Adapala et al., 2011; Gueret et al., 2016). As this response is endothelium-dependent,

ACh is widely recognized as a reliable marker for evaluating endothelial function. This approach allowed for precise measurement of endothelial-dependent and independent vasodilation effects of the test drugs under controlled conditions, thereby offering deeper insights into their specific mechanisms in vascular responses.

4.4.6. Endothelial Inhibition Cocktail

To selectively inhibit endothelial-mediated responses, a cocktail of 5 μM indomethacin (Sigma-Aldrich, Cat. No. I7378), 300 μM N ω -nitro-L-arginine methyl ester (L-NAME, Merck-Millipore, Cat. No. 483125), 100 nM charybdotoxin (Sigma-Aldrich, Cat. No. C7802), and 100 nM apamin (Sigma-Aldrich, Cat. No. A9459) was employed. This combination effectively blocked endothelium-dependent vasodilation by inhibiting pathways involved in endothelial nitric oxide and prostacyclin production, as well as small- and intermediate-conductance calcium-activated potassium channels (Hercule et al., 2009). This cocktail effectively isolated smooth muscle responses by inhibiting endothelial-derived pathways, enabling precise investigation of vasodilatory mechanisms under endothelial-independent conditions. The concentrations were optimized based on prior studies to ensure effective blockade of endothelial pathways while minimizing off-target effects (Hercule et al., 2009).

4.4.7. Reagents and Solution Preparation

All chemical reagents, including salts and solvents, were sourced from high-quality suppliers (Sigma-Aldrich, Germany; Merck, Germany) to ensure experimental consistency and reproducibility. Drug solutions were freshly prepared prior to each experiment using dimethyl sulfoxide (DMSO) or PSS as solvents. The final DMSO concentration was controlled to remain below 0.5%, minimizing any potential impact on experimental outcomes and ensuring the stability and reliability of results.

5. Statistical Analysis

Data are expressed as mean \pm SEM. Statistical analyses were performed using T-tests and two-way ANOVA, as appropriate, with GraphPad Software (Version 8.0.1; GraphPad Software, San Diego, CA, USA) to analyze drug responses and concentration-response relationships. A $p < 0.05$ was considered statistically significant.

The parameter N indicates the number of mice used in each experiment. Figures were created using CorelDraw Graphics Suite 2020 (Corel Corporation, Ottawa, Canada) for visual data representation.

6. Results

6.1. Evaluation of Optimized Perfusion Method

The optimized isolated mouse kidney perfusion method enabled the effective evaluation of vasoconstrictor and vasodilator effects under controlled conditions. This method features a streamlined renal artery isolation process, reduced procedural complexity, and shorter preparation time while maintaining kidney viability for extended periods. After stabilizing perfusion pressure at 80 mmHg, 3 mM ME was added to activate α -1 adrenergic receptors in VSMCs, resulting in vasoconstriction and increased perfusion pressure. Subsequently, 10 μ M Flupirtine, a Kv7 channel agonist, was added to induce vasodilation by hyperpolarizing VSMCs and reducing intracellular Ca^{2+} levels, which led to decreased perfusion pressure. These effects were reversible upon washout of Flupirtine, demonstrating dynamic modulation of vascular tone. Finally, 60 mM KCl was added to depolarize VSMCs, increasing Voltage-Gated Calcium Channel (VGCC) activity and Ca^{2+} influx, which caused vasoconstriction. This sequential assessment demonstrated the effectiveness of the optimized perfusion method for evaluating vascular function, ensuring consistent and reproducible results. For further methodological details, refer to the published article: "Protocol for assessing myogenic tone and perfusion pressure in isolated mouse kidneys" (Chu et al., 2024).

6.2. KWu183 and KWu176 Reduce Perfusion Pressure in Isolated Perfused Kidneys

Following ME-induced vasoconstriction, KWu183 significantly decreased perfusion pressure in isolated perfused kidneys. The observed reductions in perfusion pressure were dose-dependent, as observed at 1 nM (143.8 ± 15.47 mmHg vs 127.0 ± 12.21 mmHg, $p < 0.01$), 10 nM (154.90 ± 11.45 mmHg vs 114.30 ± 11.50 mmHg, $p < 0.01$), and 100 nM (140.72 ± 7.62 mmHg vs 71.14 ± 5.82 mmHg, $p < 0.001$) (Fig. 1). Similarly, KWu176 decreased perfusion pressure at 1 nM (142.30 ± 5.58 mmHg vs 114.80 ± 5.92

mmHg, $p < 0.05$), 10 nM (124.80 ± 7.97 mmHg vs 101.30 ± 4.67 mmHg, $p < 0.05$), and 100 nM (125.9 ± 7.23 mmHg vs 92.55 ± 9.27 mmHg, $p < 0.01$) (Fig. 2). These effects were reversed upon washout. In contrast, in vessels pre-contracted with 60 mM KCl, neither KWu183 nor KWu176 affected perfusion pressure, suggesting that both compounds act on Kv7 channels in VSMCs rather than on L-type Ca^{2+} channels (Fig. 3). To further confirm Kv7 channel involvement, the pan-Kv7 blocker XE991 (3 μM) was applied, which significantly attenuated the pressure-lowering effects of KWu183 ($64.23 \pm 4.0\%$ vs $95.33 \pm 2.07\%$, $p < 0.0001$) (Fig. 4A, B) and KWu176 ($81.84 \pm 2.44\%$ vs $93.94 \pm 1.11\%$, $p < 0.05$) (Fig. 4C, D). These findings suggest that both KWu183 and KWu176 are potent Kv7 channel activators in ex vivo VSMCs.

6.3. KWu183 and KWu176 Effects Are Mediated by Kv7 Channels

In vessels pre-contracted with 60 mM KCl, neither KWu183 nor KWu176 affected perfusion pressure, suggesting that their vasodilatory effects do not involve L-type Ca^{2+} channels but are likely mediated through potassium channels. Furthermore, XE991, a pan-Kv7 channel blocker, significantly attenuated the pressure-lowering effects of both KWu183 and KWu176 (Fig. 4). These findings provide direct evidence that KWu183 and KWu176 mediate their effects predominantly through Kv7 channels in VSMCs.

6.4. Role of Endothelium in KWu-Mediated Reduction of Perfusion Pressure

To examine the role of endothelium in the KWu183- and KWu176-induced reduction of perfusion pressure, a combination of endothelial blockers—5 μM indomethacin, 300 μM L-NAME, 100 nM charybdotoxin, and 100 nM apamin—was used to inhibit cyclooxygenase, endothelial NO synthase, and intermediate-conductance (IK) and small-conductance (SK) calcium-activated potassium channels, respectively. The effectiveness of this blocking mixture was confirmed using acetylcholine (Fig. 5A, B).

Interestingly, the endothelial blocker mixture attenuated the KWu183-induced decrease in perfusion pressure ($64.23 \pm 4.0\%$ vs $79.15 \pm 3.96\%$, $p < 0.05$) (Fig. 5C, D), while it had no significant effect on the pressure-lowering effect of KWu176 (Fig. 5E, F). These results indicate that both KWu183 and KWu176 are effective Kv7 channel agonists;

however, KWu183 may also activate endothelium-dependent signaling pathways in isolated perfused kidneys, suggesting a distinct mechanism of action relative to KWu176.

6.5. Kv7 Subtype Specificity of KWu183 and KWu176 in Isolated Perfused Kidneys

To determine the specificity of KWu183 and KWu176 towards Kv7.4 and Kv7.5 channels, experiments were conducted using Kv7-deficient mouse models. In *Kcnq5^{-/-}* and *Kcnq4^{-/-}Kcnq5^{-/-}* mice, the perfusion pressure reduction induced by KWu183 was significantly attenuated. For *Kcnq5^{-/-}* mice, the reduction was $80.34 \pm 4.25\%$ compared to $65.90 \pm 4.13\%$ ($p < 0.05$, Fig. 6A, B, E). In *Kcnq4^{-/-}Kcnq5^{-/-}* mice, the reduction decreased from $92.68 \pm 2.12\%$ to $65.90 \pm 4.13\%$ ($p < 0.001$, Fig. 6D, E), whereas KWu183 exhibited normal pressure-lowering effects in kidneys from *Kcnq4^{-/-}* mice (Fig. 6C, E). In *Kcnq4^{-/-}Kcnq5^{-/-}* mice, the reduction in perfusion pressure caused by KWu176 was attenuated ($92.63 \pm 2.0\%$ vs $82.44 \pm 2.87\%$, $p < 0.05$) (Fig. 7A, D, E), while in *Kcnq4^{-/-}* and *Kcnq5^{-/-}* mice, KWu176 led to a normal reduction in perfusion pressure (Fig. 7B, C, E). These results suggest that KWu183 acts as a potent agonist for Kv7.5 channels in isolated perfused mouse kidney models, while KWu176 appears to activate both Kv7.4 and Kv7.5 channels, likely in a heteromeric form.

6.6. KWu183 and KWu176 Produce Vasodilation in Mesenteric Arteries

Using pharmacological and genetic approaches, it was further confirmed that flupirtine-induced vasodilation was reduced in arteries from *Kcnq4^{-/-}Kcnq5^{-/-}* mice (Fig. 8A, B) and in arteries preincubated with XE991 (Fig. 8C, D). Similarly, both KWu183 and KWu176-induced vasodilation were attenuated in pressurized mesenteric arteries from *Kcnq4^{-/-}Kcnq5^{-/-}* mice (Fig. 9), which is consistent with observations in isolated perfused kidneys. As in kidney perfusion experiments, KWu183's vasodilatory effects were attenuated in *Kcnq5^{-/-}* mice, whereas KWu176-induced vasodilation remained unaffected in these animals (Fig. 10).

7. Discussion

7.1. Efficacy of the Optimized Kidney Perfusion Method in Vascular Function Assessment

The optimized isolated mouse kidney perfusion method, as previously described (Chu et al., 2024), proved to be highly effective in evaluating vascular function in this study. Under precise control of perfusion pressure and flow rate, the method effectively evaluated vascular reactivity. Pharmacological interventions with ME, flupirtine, and angiotensin II elicited the expected vasoconstrictive and vasodilatory responses. These results align with the established mechanisms of α -1 adrenergic receptor activation and Kv7 channel-mediated smooth muscle hyperpolarization (Tsvetkov et al., 2024).

The ability to monitor these reactions in a controlled ex vivo setting underscores the robustness of this optimized method for investigating vascular dynamics. Furthermore, the method aligns with foundational studies emphasizing the critical roles of calcium signaling and voltage-gated calcium channels in the regulation of smooth muscle tone (Davis & Hill, 1999; Dietrich & Gudermann, 2007; Pereira-Gonçalves et al., 2018). These findings validate the effectiveness of the optimized isolated kidney perfusion method in assessing vascular function and provide a robust tool for advancing research on the physiological and pharmacological regulation of vascular tone.

7.2. KWu183 and KWu176 Reduce Perfusion Pressure in Isolated Perfused Kidneys

Our study demonstrated that KWu183 and KWu176 effectively reduced perfusion pressure in isolated perfused kidneys under controlled conditions, with a concentration range of 1 to 100 nM. The observed effects aligned with the hypothesis, indicating that these compounds can decrease vascular resistance in the kidney model. These findings reveal the impact of KWu183 and KWu176 on organ-level vascular responses, highlighting their potential as modulators of vascular tone, with certain advantages over existing therapies.

Further analysis showed that KWu183 and KWu176 exhibited dose-dependent reductions in perfusion pressure, with higher concentrations producing more pronounced vasorelaxant effects. These dose-dependent results suggest that KWu183 and KWu176 may have a favorable therapeutic window, which can be optimized through dose adjustments to enhance their vasorelaxant effects while minimizing side effects.

Future research should focus on identifying the precise dose range that balances efficacy and safety.

7.3. KWu183 and KWu176 Induce Vasodilation, Modulated by Endothelial Function

In our study, we evaluated the vascular reactivity of KWu183 and KWu176 in the presence of specific endothelial inhibitors (5 μ M indomethacin, 300 μ M L-NAME, 100 nM charybdotoxin, and 100 nM apamin). The experimental results showed that the vasorelaxant effect of KWu183 was significantly diminished when endothelial inhibitors were used, suggesting that part of its mechanism of action may be modulated by endothelial function. In contrast, KWu176 did not exhibit a significant endothelium-dependent vasodilatory effect, which is consistent with the hypothesis that it acts directly on smooth muscle cells.

Although some studies suggest that Kv7.5 may regulate vascular function through endothelial mechanisms, the data remain controversial. For example, Baldwin et al. demonstrated that Kv7, including Kv7.5, is expressed in the endothelium of rat mesenteric arteries and contributes to NO-induced vasodilation (Baldwin et al., 2020). Similarly, Kv7 channels are involved in endothelium-dependent responses in porcine coronary arteries (Goodwill et al., 2016). However, early studies suggested that retigabine activates Kv7 channels independently of endothelial cells in mice, directly regulating vascular tone through smooth muscle cells (Yeung, Pucovsky, et al., 2007). Our results indicate that KWu183-induced vasodilation is partially endothelium-dependent, while KWu176 does not exhibit such effects. This difference in endothelial dependence between KWu183 and KWu176 may provide insight into their distinct mechanisms of action, warranting further investigation. While endothelial dependence may enhance therapeutic outcomes in conditions with partial endothelial dysfunction, direct smooth muscle action could provide a more consistent effect in severe endothelial damage.

7.4. KWu183 and KWu176 Exhibit Specificity Towards Different Kv7 Channel Subtypes

The results indicate that KWu183 may have specificity for Kv7.5, while KWu176 exhibits specificity for both Kv7.4 and Kv7.5. These conclusions are based on binding affinity studies and functional experiments, demonstrating their subtype-selective effects and supporting the hypothesis.

Currently, the molecular mechanisms underlying the opening of Kv7 voltage-dependent potassium channels are not fully elucidated. Previous research has shown that, compared to carbamate analogs, several amide derivatives of flupirtine and retigabine have superior channel-opening activity (Wurm et al., 2022). This may be due to increased metabolic stability and reduced susceptibility to esterase-mediated cleavage offered by amide derivatives, thereby also reducing hepatotoxicity (Konishi et al., 2018; Wurm et al., 2022). One of the most extensively studied compounds is the Kv7 modulator retigabine. Cryo-EM structures showed that retigabine binds to the transmembrane region of the Kv7.2 subunit (PDB: 7CR2), with only minor conformational differences compared to the apo structure (PDB: 7CR0) (X. Li et al., 2021). Li et al. used cryo-EM to study the Kv7.4 subunit with retigabine and obtained similar results (PDB: 7BYM and 7BYL) (T. Li et al., 2021). Although direct comparison of these conformations does not provide definitive conclusions about the potential opening mechanism, further insights into the action of retigabine have been identified. Additionally, displacement of membrane components in the upper region of the voltage-sensing domain (VSD) may play a role in the opening mechanism, likely leading to a significant increase in VSD mobility.

This is consistent with the recent findings on KWu183 and KWu176, where substitutions at position 2 of the pyridine ring are more likely to interact with membrane components rather than the protein itself in the proposed binding modes (Wurm et al., 2022). In this context, considering the selectivity studies of structurally related retigabine analogs, these modifications could shift selectivity towards Kv7.4 and Kv7.5 channels (Wang et al., 2019). However, the high conservation of amino acids in the retigabine binding site makes it difficult to explain selectivity based solely on specific ligand-protein interactions. Our data provide a foundation for understanding the specificity of KWu183 and KWu176 for Kv7.4 and Kv7.5, respectively, suggesting that modifications in molecular structure can influence both selectivity and efficacy.

Further pharmacological studies indicate that the selectivity of KWu183 and KWu176 may be related to their binding sites and affinity for different Kv7 channel subtypes. Such studies may include mutagenesis or affinity measurements to further characterize these interactions. A deeper understanding of this subtype selectivity will aid in the development of a new generation of highly selective Kv7 channel openers, optimizing their clinical effectiveness, particularly in the treatment of hypertension and other vascular diseases.

8. Conclusion

This study developed an optimized isolated perfused kidney model to evaluate the effects of the Kv7 activators, KWu183 and KWu176. The findings underscore the distinct selectivity of KWu183 for Kv7.5 and the dual targeting of Kv7.4 and Kv7.5 by KWu176, demonstrating their potential to modulate vascular tone and reduce vascular resistance. These results provide a strong foundation for the future development of Kv7-targeted therapies for hypertension and related vascular disorders. However, further studies are necessary to evaluate their long-term safety and clinical applicability.

9. List of figures

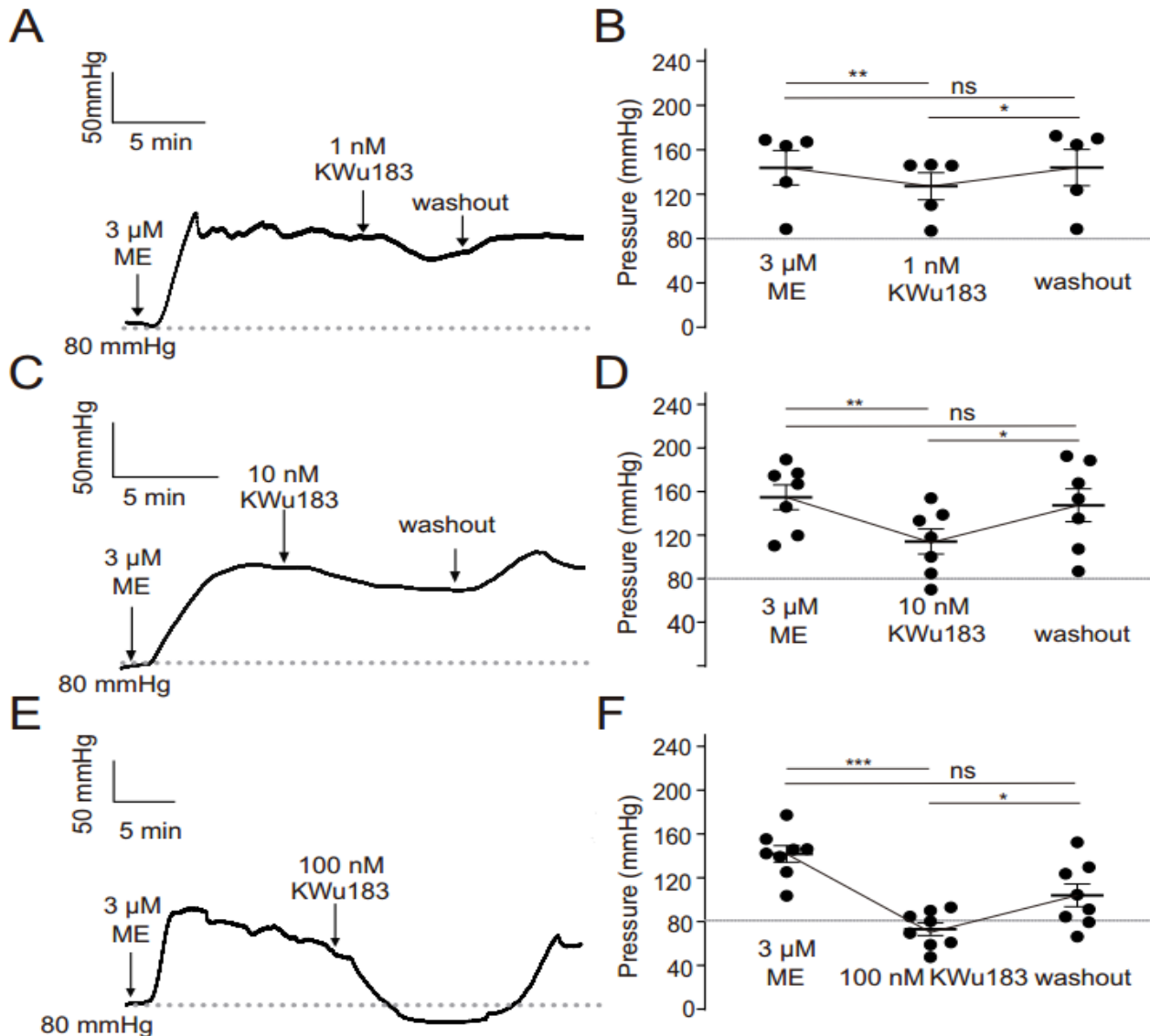


Figure 1: KWu183 reduces perfusion pressure in isolated kidneys pre-contracted with α -1 adrenoreceptor agonist ME

A, Original recordings of perfusion pressure with 3 μ M ME and subsequent 1 nM KWu183 and **B**, The change of pressure (N = 5 mice). **C**, Original recordings of perfusion pressure with ME and subsequent 10 nM KWu183 and **D**, The change of pressure (N = 7 mice). **E**, Original recordings of perfusion pressure with ME and subsequent 100 nM KWu183 and **F**, the change of pressure (N = 8 mice). Data are presented as mean \pm SEM. Statistical significance was assessed using a t-test. ns: not significant; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

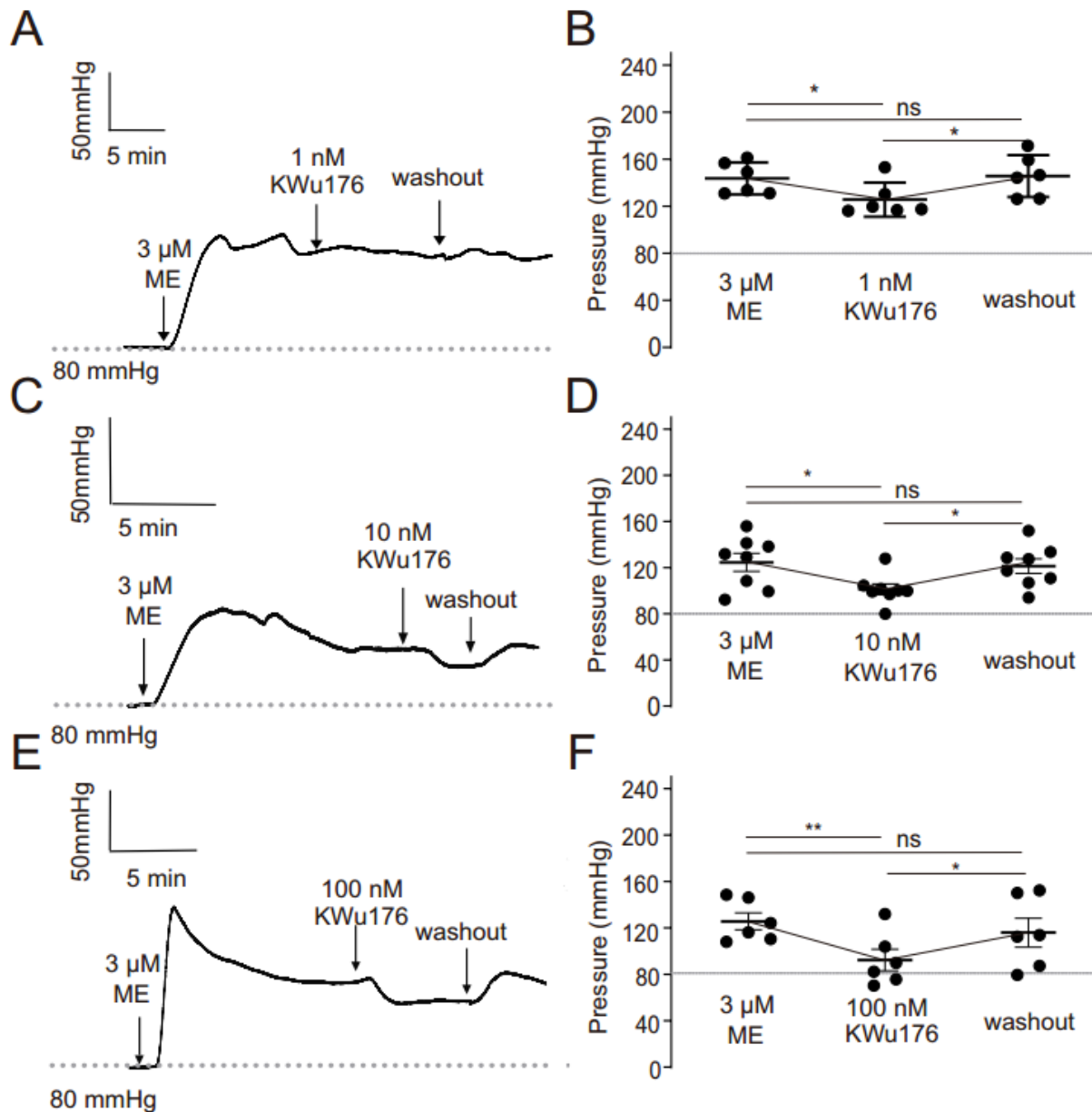


Figure 2: KWu176 reduces perfusion pressure in isolated perfused kidneys pre-contracted with α -1 adrenoreceptor agonist ME

A, Original recordings of perfusion pressure with 3 μ M ME and subsequent 1 nM KWu176 and **B**, The change of pressure (N = 6 mice). **C**, Original recordings of perfusion pressure with ME and subsequent 10 nM KWu176 and **D**, The change of pressure (N = 8 mice). **E**, Original recordings of perfusion pressure with ME and subsequent 100 nM KWu176 and **F**, The change of pressure (N = 6 mice). Data are presented as mean \pm SEM. Statistical significance was assessed using a t-test. ME: methoxamine; ns: not significant; *: $p < 0.05$, **: $p < 0.01$.

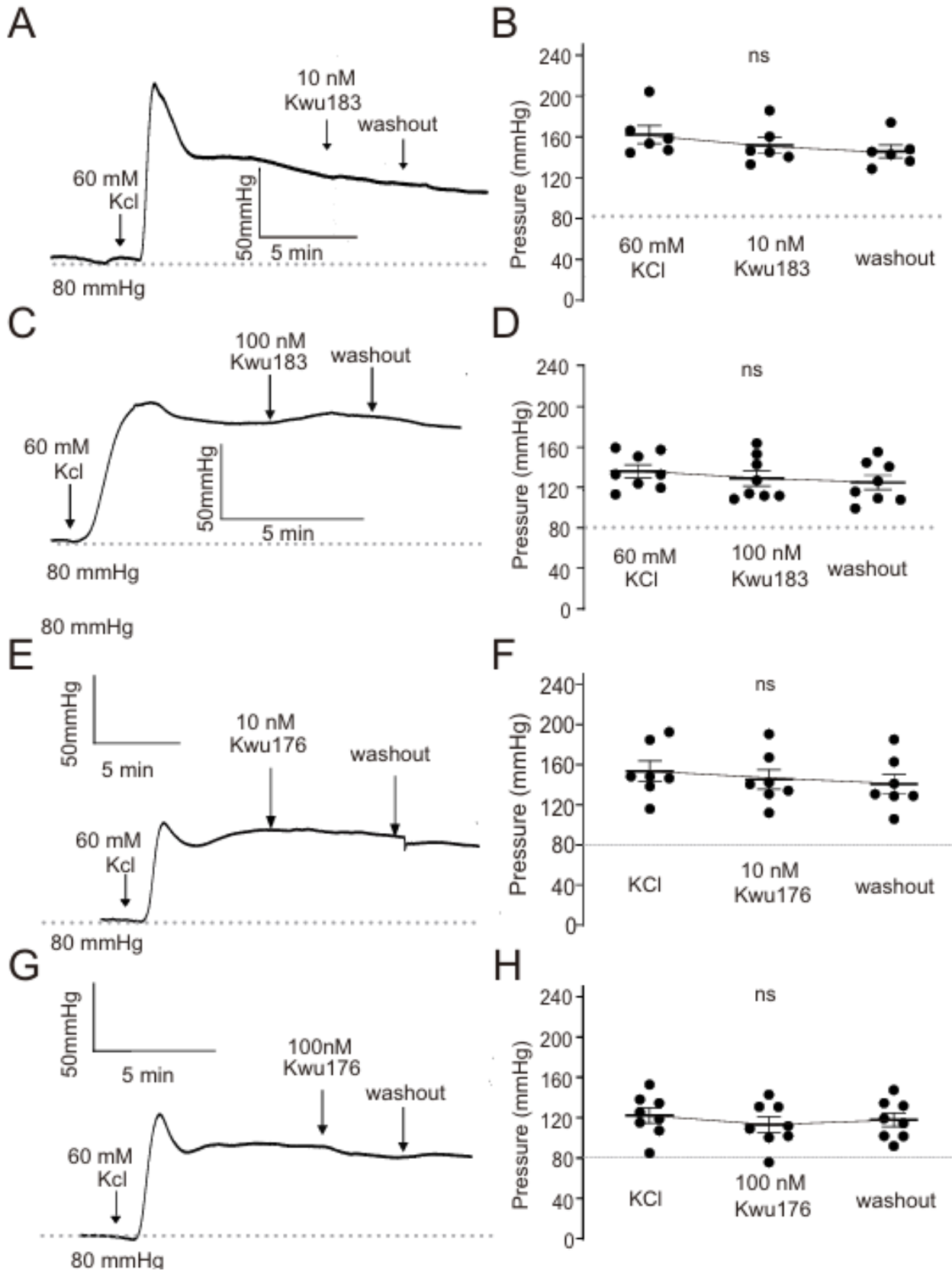


Figure 3: KWu183 and KWu176 had no effects in isolated perfused kidneys pre-contracted with 60 mM KCl

A, Original recordings of perfusion pressure and **B**, The change of pressure with application of 10 nM KWu183 (N = 6 mice). **C**, Original recordings of perfusion pressure and **D**, The change of pressure with application of 100 nM KWu183 (N = 8 mice). **E**, Original recordings of perfusion pressure and **F**, The change of pressure with application of 10 nM KWu176 (N = 7 mice). **G**, Original recordings of perfusion pressure and **H**, The change of pressure with application of 100 nM KWu176 (N = 8 mice). Data are presented as mean \pm SEM. Statistical significance was assessed using a t-test. KCl: Potassium chloride; ns: not significant.

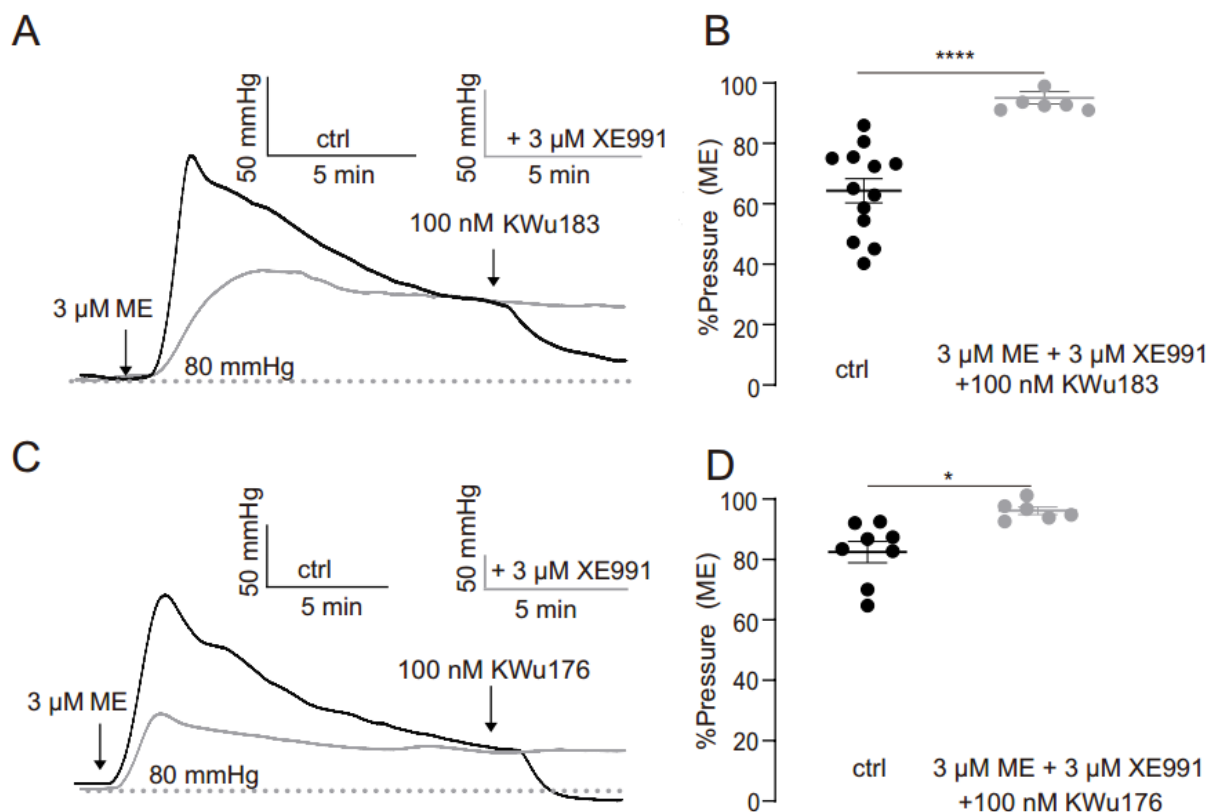


Figure 4: KWu183 and KWu176 induced vasodilation depends on Kv7 channels

A, Original recordings of perfusion pressure with ME and subsequent 100 nM KWu183 in the presence and absence of 3 μM XE991. **B**, The change of pressure (N = 13 and N = 6 mice). **C**, Original recordings of perfusion pressure with ME and subsequent 100 nM KWu176 in the presence and absence of 3 μM XE991. **D**, The change of pressure (N = 8 and N = 6 mice). Data are presented as mean ± SEM. Statistical significance was assessed using a t-test. ME: methoxamine; *: $p < 0.05$, ****: $p < 0.0001$.

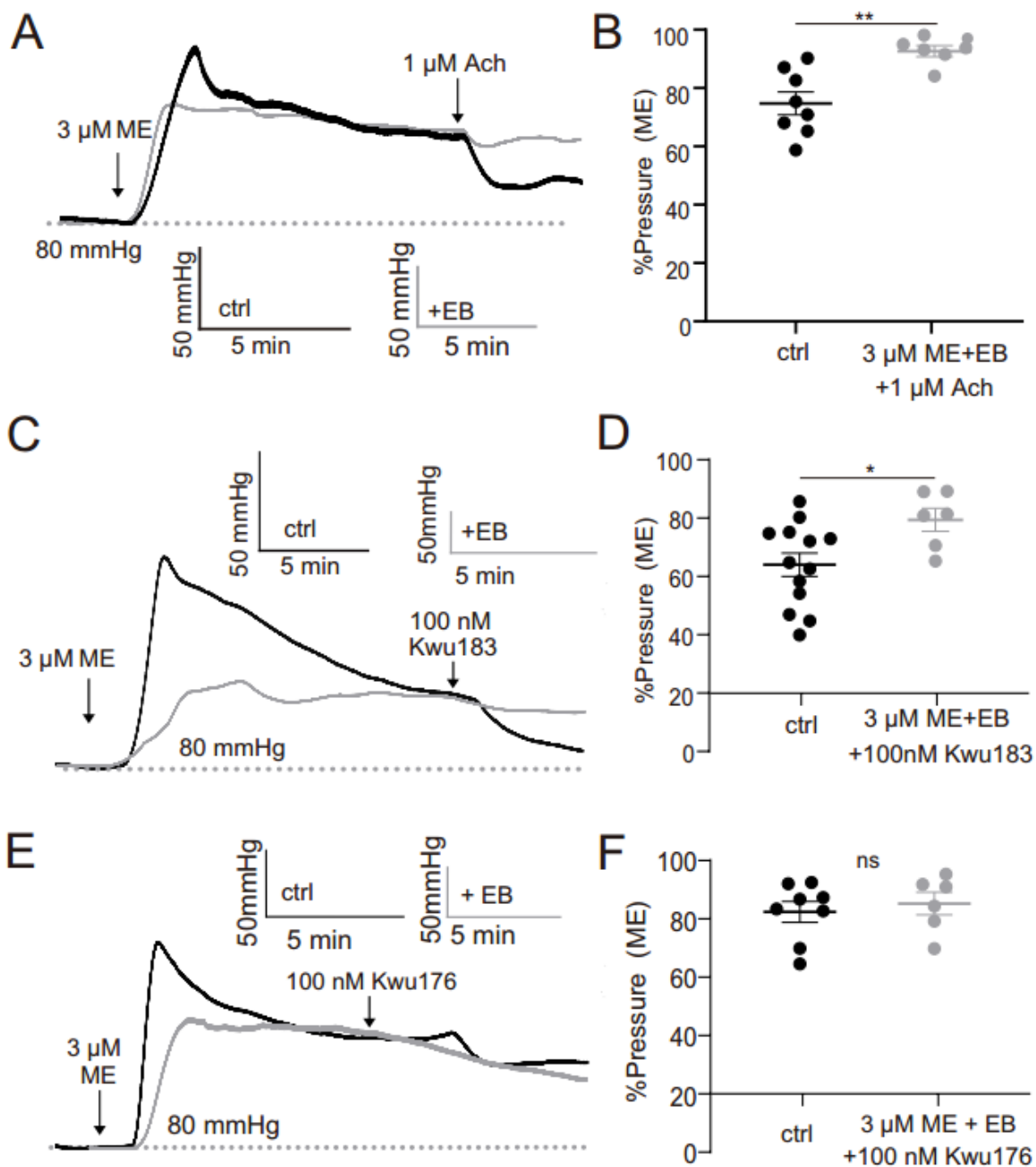


Figure 5: KWu183 and KWu176 induce vasodilation dependent on endothelium in isolated perfused kidneys

A, Original recordings of perfusion pressure. **B**, The change of pressure demonstrating the relaxant effect of 1 μ M ACh in endothelium-intact (black traces, N = 8 mice) and endothelium-blocked (gray traces, N = 7 mice) isolated perfused kidneys.

C, Original recordings of perfusion pressure with ME and subsequent 100 nM KWu183 in endothelium-intact and endothelium-blocked isolated perfused kidneys. **D**, The

change of pressure with application of KWu183 (N = 13 and N = 6 mice). **E**, Original recordings of perfusion pressure with ME and subsequent 100 nM KWu176 in endothelium-intact and endothelium-blocked isolated perfused kidneys. **F**, The change of pressure with application of KWu176 (N = 8 and N = 6 mice). Data are presented as mean \pm SEM. Statistical significance was assessed using a t-test. EB: Endothelium blocker composed of 5 μ M indomethacin, 300 μ M L-NAME, 100 nM charybdotoxin, and 100 nM apamin. ME: methoxamine; ns: not significant; *: $p < 0.05$; **: $p < 0.01$.

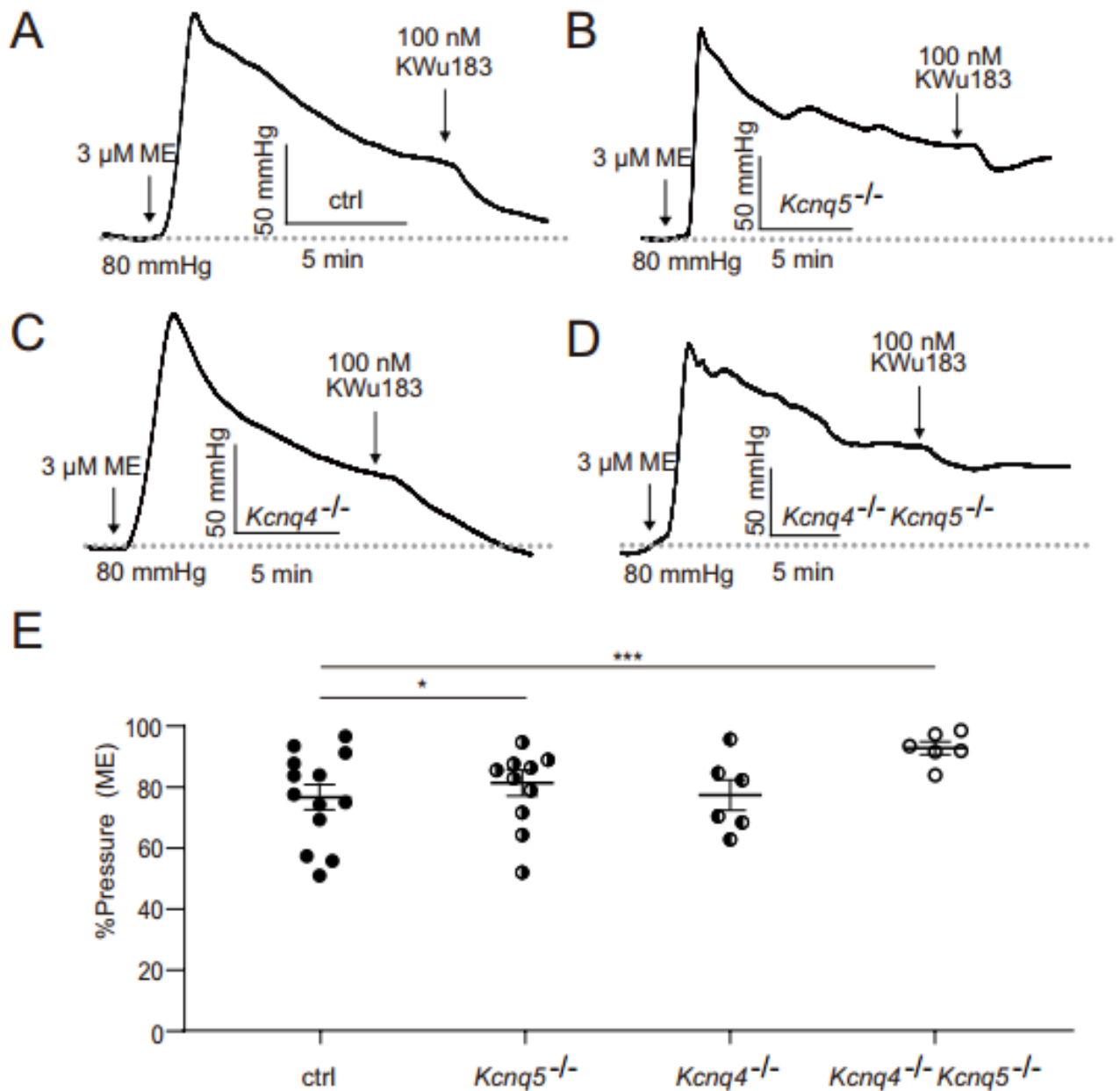


Figure 6: Kv7.5 channel is required for KWu183-mediated relaxant effect in isolated perfused kidneys

Original recordings of perfusion pressure illustrating the relaxant effect of 100 nM KWu183 in **A**, kidneys from wild-type, **B**, kidneys from *Kcnq5*^{-/-} mice, **C**, kidneys from *Kcnq4*^{-/-} mice, and **D**, kidneys from *Kcnq4*^{-/-}*Kcnq5*^{-/-} mice. **E**, Changes in pressure normalized to precontraction with 3 μ M ME. $N \geq 6$ mice. Data are presented as mean \pm SEM. Statistical significance was assessed using a t-test. ME: methoxamine. *: $p < 0.05$, ***: $p < 0.001$.

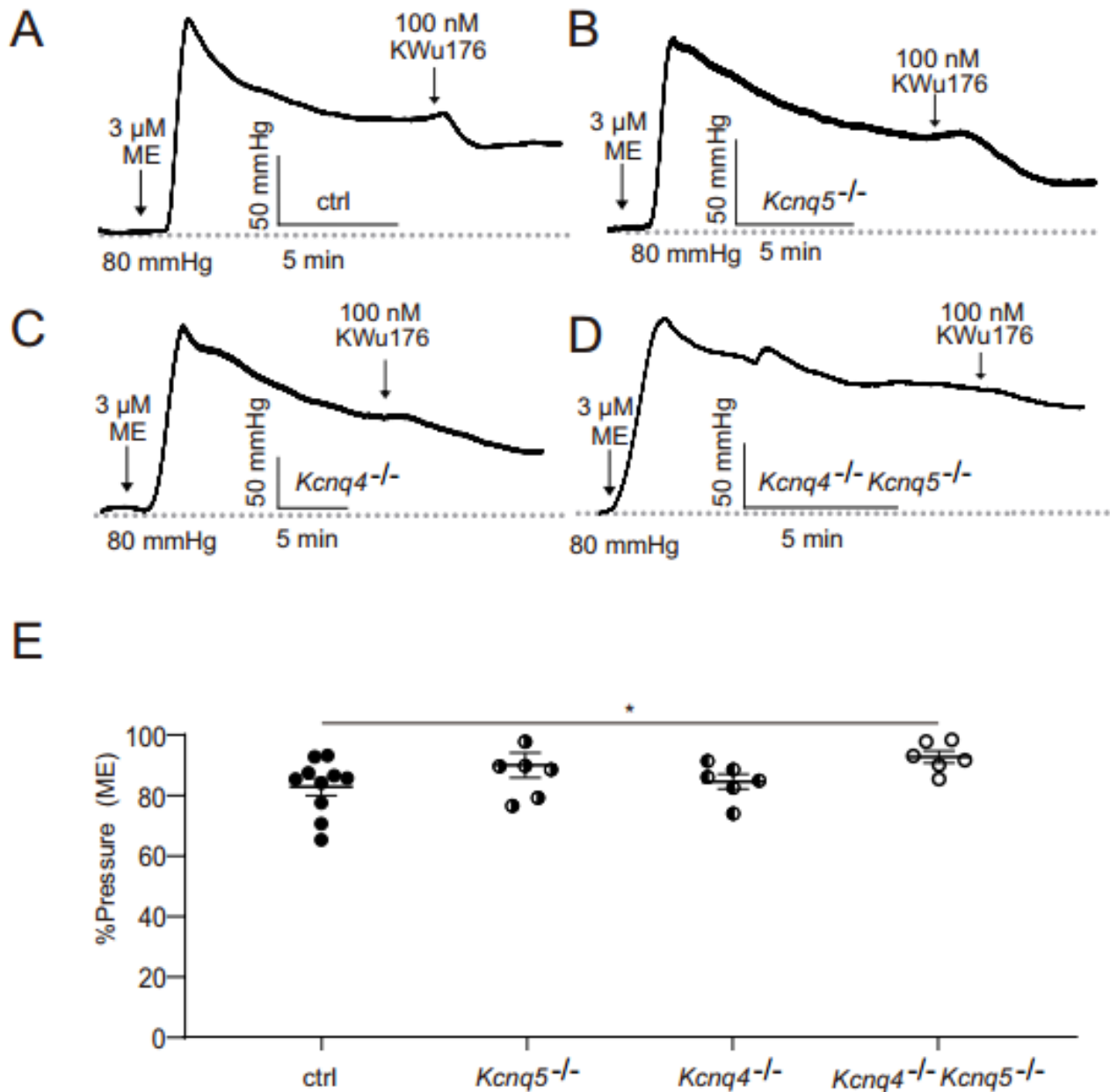


Figure 7: Relaxant effect of KWu176 in Kv7.4 and Kv7.5 deficiency in isolated perfused kidneys

Original recordings of perfusion pressure illustrating the relaxant effect of 100 nM KWu176 in **A**, wild-type kidneys, **B**, kidneys from *Kcnq5*^{-/-} mice, **C**, kidneys from *Kcnq4*^{-/-} mice, and **D**, kidneys from *Kcnq4*^{-/-}*Kcnq5*^{-/-} mice. **E**, Changes in pressure normalized to precontraction with 3 μM ME. N_≥6 mice. Data are presented as mean ± SEM. Statistical significance was assessed using a t-test. ME: methoxamine. *: p < 0.05.

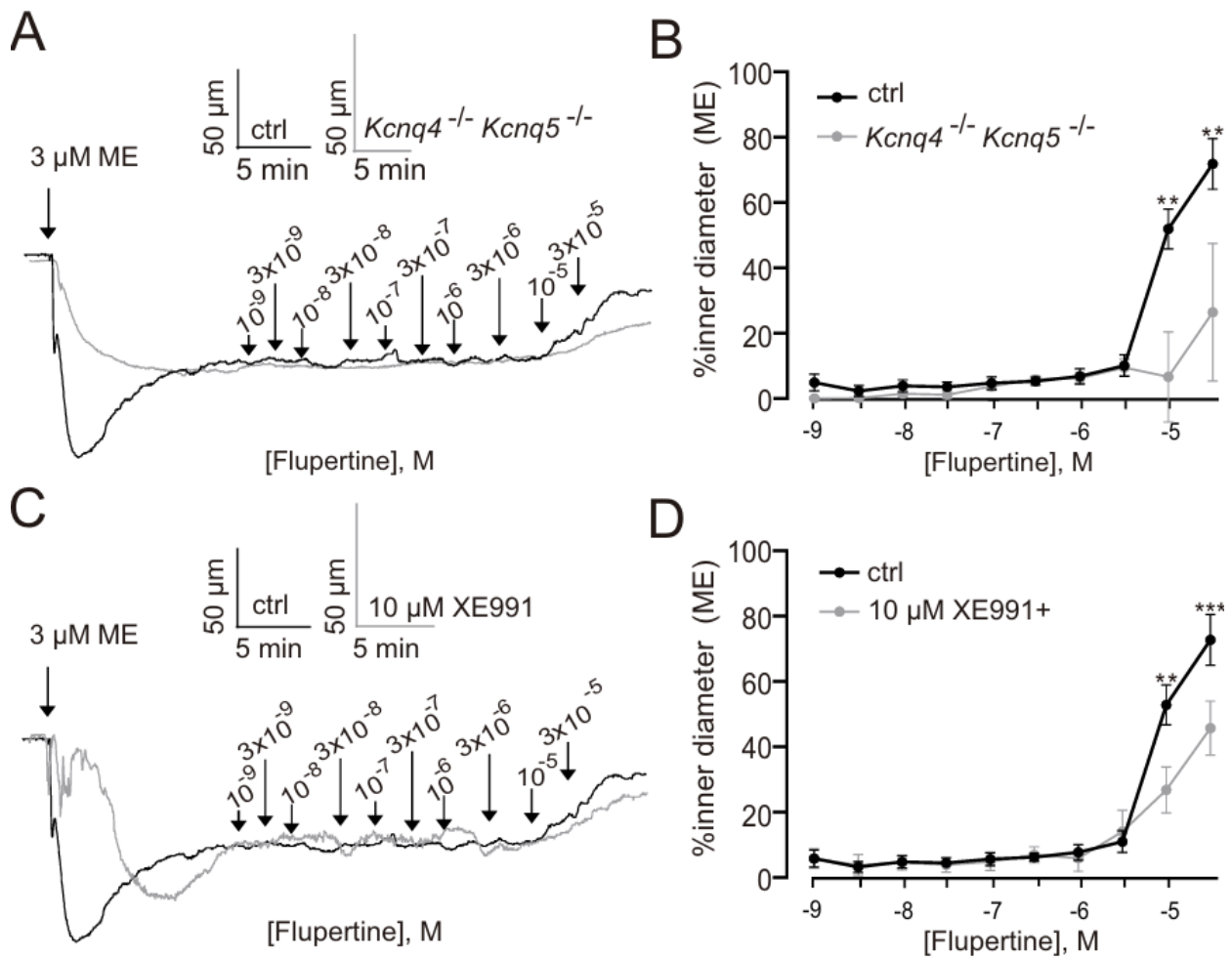


Figure 8: Kv7.4 and Kv7.5 play a role in flupirtine-mediated mesenteric artery relaxation, but not reduced kidney perfusion pressure

A, Original recordings illustrating the relaxant effect of Flu on wild-type (ctrl, black color, N = 5 mice) and *Kcnq4*^{-/-}*Kcnq5*^{-/-} (gray color, N = 4 mice) mesenteric arteries, and **B**, concentration-response relationships for Flu. **C**, Original recordings illustrating the effect of 10 μM XE991 on Flu-dependent relaxations. Mesenteric arteries were preincubated with XE991 before application of 3 μM ME (gray color, N = 8 mice) and without XE991 (ctrl, dark color, N = 5 mice), and **D**, Concentration-response relationships for Flu. Data are presented as mean ± SEM. Statistical significance was assessed using two-way ANOVA. Flu: flupirtine, ME: methoxamine, ns: not significant; **: $p < 0.01$; ***: $p < 0.001$.

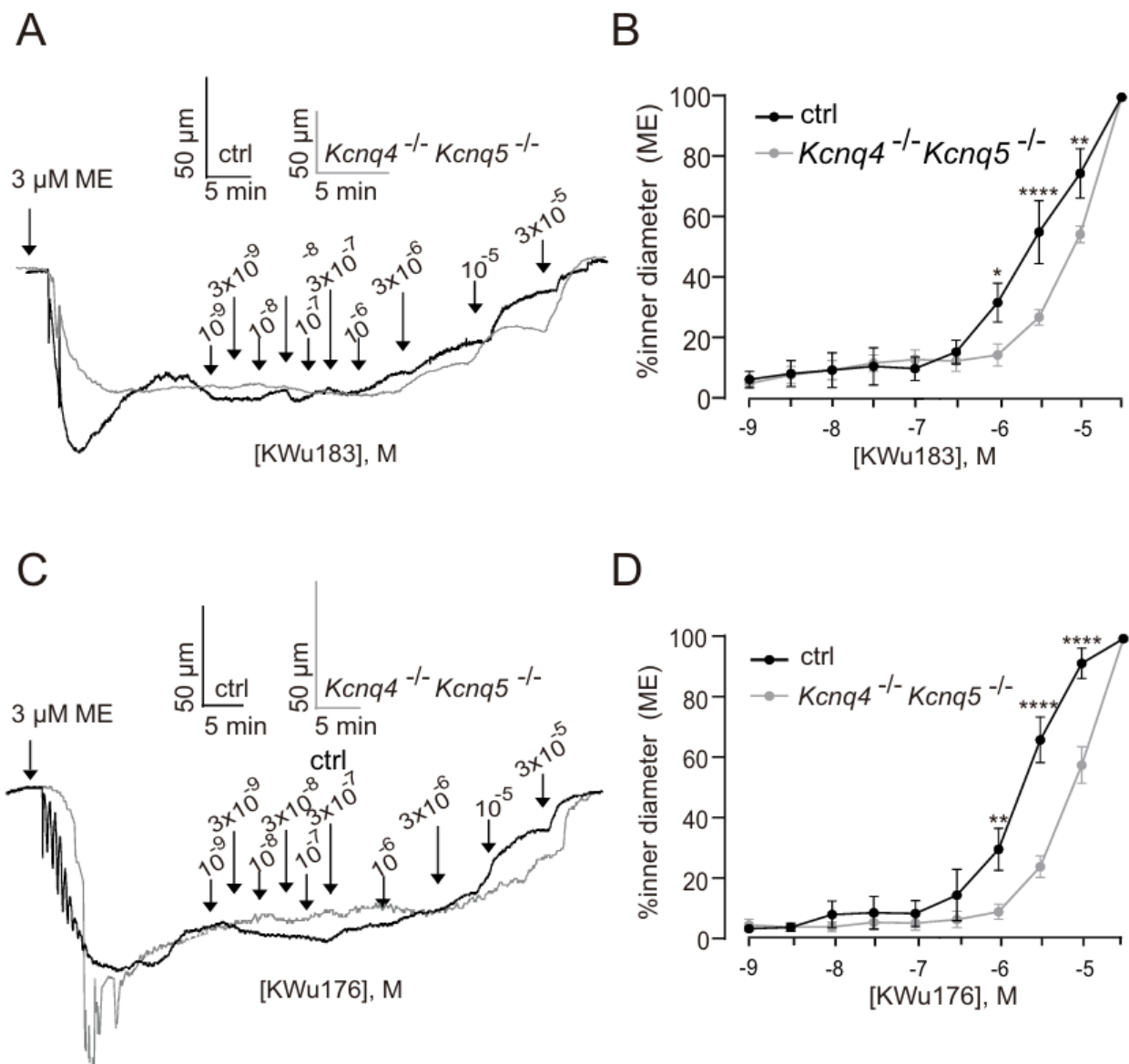


Figure 9: KWu183-mediated mesenteric artery relaxation depends on Kv7.4 and Kv7.5

A, Original recordings illustrating the relaxant effect of KWu183 on wild-type (Ctrl, black color, N = 6 mice) and *Kcnq4*^{-/-}*Kcnq5*^{-/-} (gray color, N = 8 mice) mesenteric arteries, and **B**, concentration-response relationships. **C**, Original recordings illustrating the relaxant effect of KWu176 on wild-type (Ctrl, black color, N = 5 mice) and *Kcnq4*^{-/-}*Kcnq5*^{-/-} (gray color, N = 8 mice) mesenteric arteries and **D**, concentration-response relationships. Data are presented as mean ± SEM. Statistical significance was assessed using two-way ANOVA. ME: methoxamine, *: $p < 0.05$, **: $p < 0.01$ ****: $p < 0.0001$.

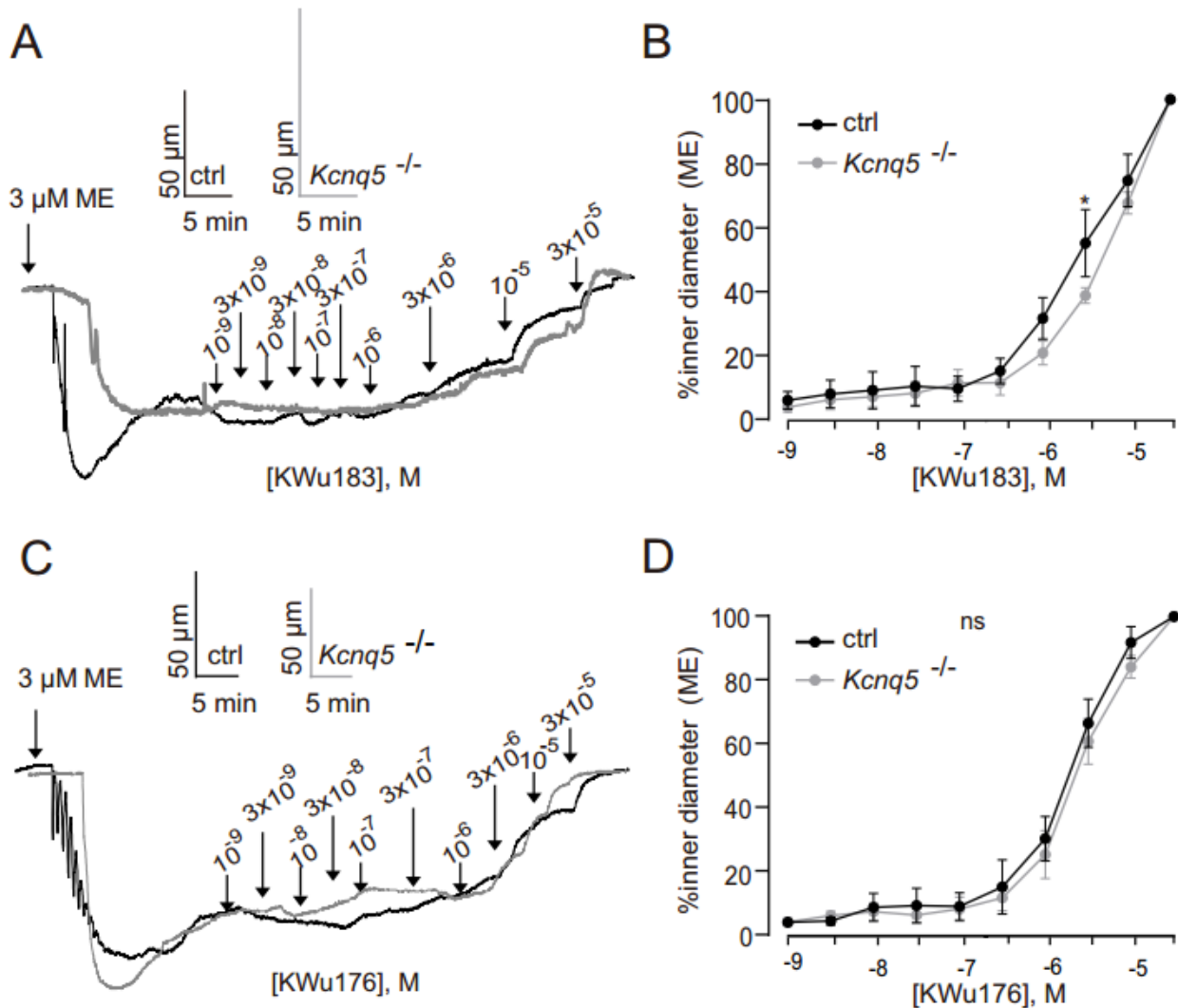


Figure 10: KWu176-mediated mesenteric artery relaxation is independent from Kv7.5

A, Original recordings illustrating the relaxant effect of KWu176 on wild-type (Ctrl, black color, N = 5 mice) and *Kcnq4*^{-/-}*Kcnq5*^{-/-} (gray color, N = 8 mice) mesenteric arteries, and **B**, concentration-response relationships. **C**, Original recordings illustrating the relaxant effect of KWu176 on wild-type (Ctrl, black color, N = 5 mice) and *Kcnq5*^{-/-} (gray color, N = 6 mice) mesenteric arteries, and **D**, concentration-response relationships. Data are presented as mean \pm SEM. Statistical significance was assessed using two-way ANOVA. ME: methoxamine, ns: not significant; *: $P < 0.05$.

10. List of Abbreviations

ACh: Acetylcholine

ANP: Atrial Natriuretic Peptide

Cav1.2: Voltage-Gated Calcium Channel, L-Type, Alpha 1C Subunit

cAMP: Cyclic Adenosine Monophosphate

cGMP: Cyclic Guanosine Monophosphate

CNS: Central Nervous System

DMSO: Dimethyl Sulfoxide

EB: Endothelium Blocker

Flu: Flupirtine

H₂S: Hydrogen Sulfide

IK: Intermediate-Conductance Calcium-Activated Potassium Channel

IKs: Slow Delayed Rectifier Potassium Current

IP₃: Inositol Triphosphate

KCNE: Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit

KCNQ: Potassium Voltage-Gated Channel, Subfamily Q

KV7: Voltage-Gated Potassium Channel Subfamily Q Member

L-NAME: N ω -Nitro-L-arginine Methyl Ester

M-current: Muscarinic-Sensitive Potassium Current (Neuronal)

ME: Methoxamine

NO: Nitric Oxide

PIP₂: Phosphatidylinositol 4,5-bisphosphate

PKC: Protein Kinase C

PKA: cAMP-Dependent Protein Kinase

PKG: cGMP-Dependent Protein Kinase

PSS: Physiological Salt Solution

PVAT: Perivascular Adipose Tissue

SEM: Standard Error of the Mean

SHR: Spontaneously Hypertensive Rats

SK: Small-Conductance Calcium-Activated Potassium Channel

SKCa: Small Conductance Calcium-Activated Potassium Channel

TRP: Transient Receptor Potential

TRPM4: Transient Receptor Potential Melastatin 4

V_m: Membrane Voltage

VOCC: Voltage-Operated Calcium Channels

VSD: Voltage-Sensing Domain

VSMC: Vascular Smooth Muscle Cell

XE991: Selective KV7 Channel Blocker

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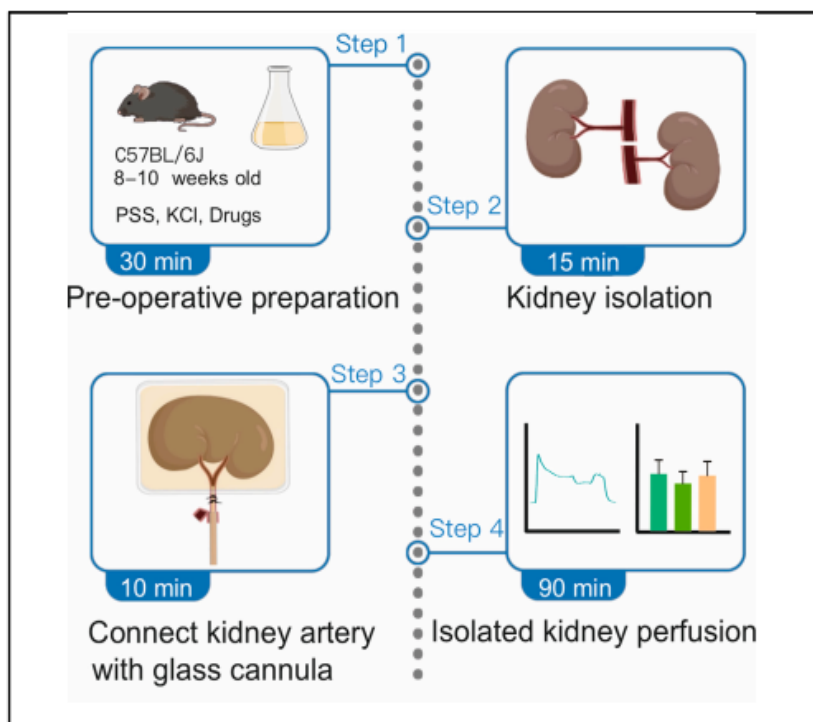
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12. **Appendix:** Chu. et al., Protocol for assessing myogenic tone and perfusion pressure in isolated mouse kidneys (Star Protocols, 2024)

Protocol

Protocol for assessing myogenic tone and perfusion pressure in isolated mouse kidneys



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Highlights

Assessing vascular smooth muscle function in a physiologically relevant context

Recording of four kidneys in parallel for high-throughput data acquisition

Detailed procedures from kidney isolation to data collection

The isolated perfused kidney is a classic *ex vivo* preparation for studying renal physiology in general and vascular function. Here, we present a protocol for assessing myogenic tone in isolated mouse kidneys as well as vasodilatory and vasoconstrictive responses, expressed as perfusion pressure. We describe steps for pre-operative preparation, kidney and renal artery isolation, and connection of renal artery with glass cannula. We then detail how to measure pressure changes in perfused kidneys and the myogenic tone.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

Protocol for assessing myogenic tone and perfusion pressure in isolated mouse kidneys

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<https://doi.org/10.1016/j.xpro.2024.102845>

SUMMARY

The isolated perfused kidney is a classic *ex vivo* preparation for studying renal physiology in general and vascular function. Here, we present a protocol for assessing myogenic tone in isolated mouse kidneys as well as vasodilatory and vasoconstrictive responses, expressed as perfusion pressure. We describe steps for pre-operative preparation, kidney and renal artery isolation, and connection of renal artery with glass cannula. We then detail how to measure pressure changes in perfused kidneys and the myogenic tone.

For complete details on the use and execution of this protocol, please refer to Cui et al.¹

BEFORE YOU BEGIN

Cardiovascular disease is the commonest cause of death worldwide² and hypertension is the major driver.³ Since cardiac output remains normal in hypertension, systemic vascular resistance increases.⁴ To determine the underlying pathophysiology, small resistance vessels must be isolated and investigated. The isolated perfused kidney technique allows accessing vascular smooth muscle function in a physiologically relevant context and is superior to other *ex vivo* other methods, such as wire myography. After all, vessels act under pressure *in vivo* and are not stretched between wires.⁵ The isolated perfused kidney technique was first developed for use in large animals, but was successfully transferred to rats. The availability of gene-modified mice made murine models the ideal experimental animal model; however, substantial technical problems arose.^{6–9} Moving from 250 g rats (average kidney weight 2.5 g) to 25 g mice (average kidney weight 0.25 g) mice was a major challenge. Nonetheless, our approach is simple and allows the recording of four kidneys in parallel for high-throughput data acquisition. We describe our method including renal surgical preparation, artery isolation, and equipment assembly in detail (Graphical Abstract).

△ **CRITICAL:** To avoid renal artery damage, a comprehensive understanding of murine renal anatomy is essential.

Note: Compared with the abdominal aorta and renal artery, the renal vein and inferior vena cava are larger, are located superficially, and have thinner walls. By removing the renal vein and underlying connective tissue, the location and shape of the renal artery and abdominal aorta can be clearly identified. The left renal artery typically lacks other major branches,



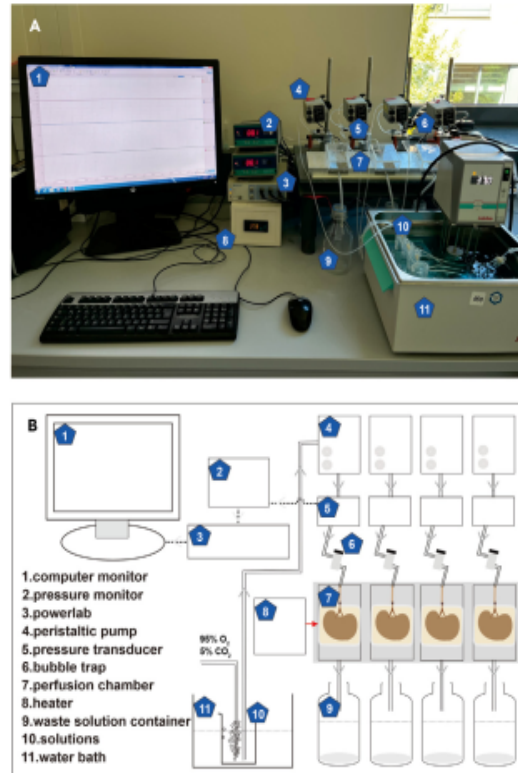


Figure 1. Isolated kidney perfusion setup
(A) Assembled set up.
(B) Schematic representation of the assembled unit.

whereas the right renal artery often has one or more branches. Identifying the branches of renal arteries to avoid their damage is essential.

Institutional permissions

All animal care followed American Physiological Society guidelines, and all protocols were approved by local animal welfare officers and authorities (No. OE-017/23, LALLF: Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg Vorpommern / State Office for Agriculture, Food Safety and Fisheries Mecklenburg-Western Pomerania), confirming that all experiments conform to the relevant regulatory standards. The described methods do not include experiments on live vertebrates or higher invertebrates. Depending on local regulatory rules, permissions from the relevant institutions might be required. There are no ethical concerns.

1. Confirm the strain, sex, age, and weight of the mice.
2. Prepare the setup, and solutions needed according to [Figure 1](#) and [materials and equipment](#).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
NaCl	Sigma-Aldrich	31434-1KG-R
NaHCO ₃	Sigma-Aldrich	S5761-5KG

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
KCl	Sigma-Aldrich	60130-1KG
MgSO ₄ ·7H ₂ O	Merck	105886
D-Glucose	Sigma-Aldrich	G8270-1KG
CaCl ₂ ·2H ₂ O	Roth	5239.1
KH ₂ PO ₄	Roth	3904.1
Flupirtine	Sigma-Aldrich	F8927-5MG
Methoxamine	Sigma-Aldrich	M6524-500G
Ethanol (70%)	Carl Roth	200-578-6
Experimental models: Organisms/strains		
Mice, C57BL/6J strain. 8–12 weeks old, 25 g, female or male. Alternative strains can be used	The Jackson Laboratory	RRID:IMSR_JAX:000664
Other		
Surgical scissors	Fine Science Tools (FST)	15018-10
Forceps	FST	11254-20
Silk thread	RESORBA	6/0 USP, 0.7 metric
Silicon tubes	VWR International	228-5209 228-5208
PowerLab	ADInstruments	ML845 4/25, or PL2604 4/26 series, or PowerLab C or similar alternative data acquisition hardware can be used
Perfusion pressure monitor	Living Systems	PM-4
Glass cannula	Science Products	GB200-8P
Water bath	JULABO	12876
Micropipette puller	Narishige	PB-7 Vertical
Filter	Carl Roth	112A-240
Carbogene (95% O ₂ /5% CO ₂)	Air Liquide	N/A
Peristaltic pump	Instech	Model P720
Replacement pressure transducer	Living Systems	PT-F
Bubble trap	Custom-made, alternatives can be used https://schmidt-haensch.com/product/glass-tube-with-bubble-trap/	N/A
Chamber	Custom-made, plexiglass, alternatives can be used	N/A
Chamber heater	Custom-made, alternatives can be used	N/A
LabChart	ADInstruments	v8.1.22, also lower and higher versions can be used

MATERIALS AND EQUIPMENT

Physiological Salt Solution (PSS)		
Reagent	Final concentration, mM	Amount
NaCl	119	13.91 g
KCl	4.7	0.71 g
KH ₂ PO ₄	1.2	0.327 g
NaHCO ₃	25	4.2 g
MgSO ₄ ·7H ₂ O	1.2	0.592 g
Glucose	11.1	4 g
CaCl ₂ ·2H ₂ O	1.6	0.47 g
H ₂ O	N/A	2000 mL
Total	N/A	2000 mL

Store at +4°C for up to 1 week.



KCl 60 mM		
Reagent	Final concentration, mM	Amount
NaCl	63.7	7.445 g
KCl	60	8.947 g
KH ₂ PO ₄	1.2	0.327 g
NaHCO ₃	25	4.2 g
MgSO ₄ ·7H ₂ O	1.2	0.592 g
Glucose	11.1	4 g
CaCl ₂ ·2H ₂ O	1.6	0.47 g
ddH ₂ O	N/A	2000 mL
Total	N/A	2000 mL

Store at +4°C for up to 1 week.

STEP-BY-STEP METHOD DETAILS

Preparation of glass cannula

⌚ Timing: 5 min

This step describes the preparation of the glass cannula for renal perfusion.

1. Prepare a glass cannula of the indicated shape using a micropipette puller (Figure 2A).
 - a. Install the glass tube into the pulling head of the puller and make adjustments to ensure the proper positioning of the glass material (Figure 2B).
 - b. Set both scales of the puller to the starting position (0). This step allows finer adjustments of the distance during the pulling process that defines the shape of the cannula (Figure 2C).
 - c. Turn on the heater and allow the lower scale descend for 4 mm. Fix the lower part of the tube holder and then turn the heater off (Figure 2D).

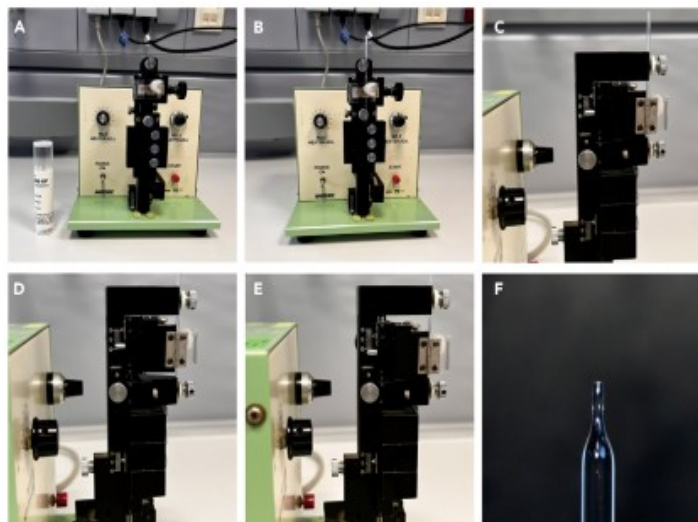


Figure 2. Process of glass cannula

(A) Micropipette puller.

(B) Glass is installed onto the puller.

(C) Two scales of the puller are set to the starting position (0).

(D) Heater is switched on and the lower scale is adjusted to 4 mm. After adjusting, the heater is switched off.

(E) Heater is switched on and the upper scale is adjusted to 3.5 mm. After the cannula is made, the heater is switched off.

(F) Thin part of the glass cannula is removed and the surface is polished.



- d. Adjust the upper scale of the tube holder to 3.5 mm (Figure 2E). Switch on the heater again and gradually pull the glass material until the glass tube is separated into 2 parts.
- e. After completing of the pulling process, remove the longer cannula from the puller. Trim any excess of the tube and polish the surface of the cannula using a smooth metal surface (e.g., forceps) (Figure 2F).
- f. Verify the inner diameter of the tube according to the experimental requirements.

⚠ **CRITICAL:** The inner diameter of the glass cannula tip is crucial. A larger cannula diameter can make the connection with the renal artery unreliable, whereas a smaller diameter might significantly increase the resistance. The optimal inner diameter of glass cannula for mouse isolated kidney perfusion should range between 0.3 mm to 0.4 mm.

Pre-operative preparation

⌚ **Timing:** 30 min

This section describes preoperative preparation procedures.

2. Oxygenate solutions.
 - a. Set the water bath temperature to 37°C.
 - b. Place solutions including physiological salt solution PSS and 60 mM KCl in the water bath.
 - c. Place carbogen (95% O₂ and 5% CO₂) tubes into the solution bottles.
 - d. Place a small bottle of PSS solution on ice and connect it to carbogen.

Note: The solutions need to be oxygenated for at least 15 min before the kidney isolation.

3. Prepare required equipment.
 - a. Turn on the temperature control device of the chambers and set it to 37°C (Figures 1A and 1B: module 8).
 - b. Switch on the pressure monitor (Figures 1A and 1B: module 2) and peristaltic pump (Figures 1A and 1B: module 4).
 - c. The solution must fill the entire tubing system. Then, pause the peristaltic pump and set pressure monitor to 0 mm Hg.
4. Open the LabChart software, set the pressure measurement range, and parameters such as the number of chambers (4 in our case).

Kidney isolation

⌚ **Timing:** 5 min

In next two sections, we describe surgical techniques related to kidney isolation and isolation of renal arteries (Methods video S1).

5. Isolate mouse kidneys.
 - a. Open the abdominal cavity of a sacrificed mouse to expose internal organs such as intestine and liver. Gently displace the intestine and part of the liver towards the left side to visualize both kidneys.
 - b. Carefully transect the connective tissue, aorta, vena cava inferior above the kidneys. (Figure 3A).
 - c. Using forceps, take the lower part of right kidney of the mouse and lift it up. Using scissors, dissect the connective tissue on the dorsal side of the right kidney from bottom to top (Figure 3B).
 - d. Repeat the same procedure with the left kidney (Figure 3C) and move it to the right side (Figure 3D).

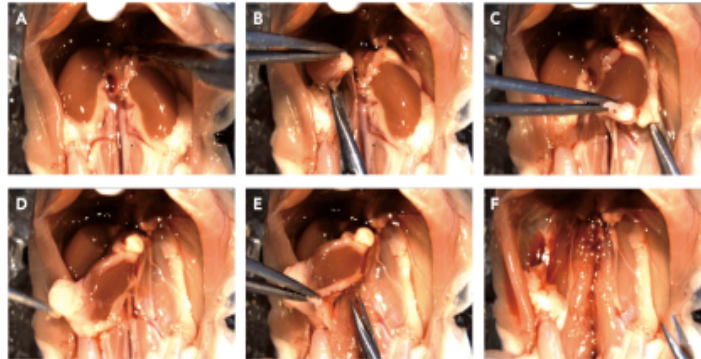


Figure 3. Kidney isolation

(A) Cutting the aorta and vena cava inferior, after the stomach and intestine were removed or moved to the side.
 (B) Separation of the right and left kidney (C) from underlying connective tissue.
 (D) Flipping the left kidney onto the right side.
 (E) Separation of kidneys, abdominal aorta, renal artery, and inferior vena cava from connective tissue and fascia.
 (F) Kidneys are removed.

- e. Once the aorta is exposed, grab the abdominal aorta and inferior vena cava at the level of the lower kidney part and cut them.
- f. Next, delicately separate them from the underlying muscle and connective tissue (Figure 3E).
- g. Once the kidneys are removed (Figure 3F), place them into a plate filled with cold oxygenated PSS solution (see step 1, preoperative preparation).

⚠ **CRITICAL:** During this procedure, there is a risk of damaging renal arteries. A useful technique is to lift the abdominal aorta while dissecting, and to cut as close as possible to the underlying muscle (Methods video S1, 1 min 10–40 s).

Isolation of renal arteries

⌚ Timing: 10 min

6. Isolate renal arteries (Methods video S2).
 - a. Use needles to fix adipose tissue at the upper and lower margins of the kidneys, maintaining slight tension on the renal artery (Figure 4A).
 - b. Remove an excess of abdominal aorta (Figure 4B).
 - c. Make an upward incision along the vena cava inferior towards 11 o'clock (Figure 4C).
 - d. Extend this incision to the right renal vein using scissors to clearly visualize the right renal artery (Figure 4D). Apply same procedure to left kidney vein.

⚠ **CRITICAL:** The renal vein is semitransparent, aim to make incisions within this translucent area to avoid damaging the branches of underlying renal artery.

- e. Use scissors to remove surrounding connective and adipose tissue of renal arteries and aorta (Figure 4E).
- f. Separate two renal arteries from the aorta (Figure 4F).

Connection of renal artery with glass cannula

⌚ Timing: 5 min

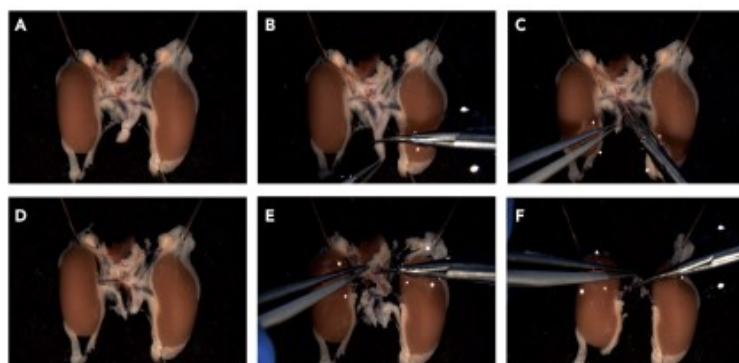


Figure 4. Isolation of renal arteries

- (A) Fixation of the kidneys with needles onto a custom-made Sylgard plate.
 (B) Cutting off the lower part of the aorta, inferior vena cava and surrounding tissue with scissors.
 (C) Incision of the inferior vena cava and (D) subsequent right renal vein with removal of surrounding connective and perivascular adipose tissue.
 (E) Removing excess of abdominal aorta with connective tissue; the part of aorta with both renal arteries is left intact.
 (F) All connective and perivascular adipose tissue are removed; the two renal arteries are separated from the aorta.

This section describes how to connect the mouse renal artery to the glass cannula and the pressure-monitoring device ([Methods video S3](#)).

7. Connect renal arteries with glass cannula.

- a. Secure the glass cannula in the chamber. Make a knot using silk thread and place it over the cannula for future use. Rinse the cannula with PSS to remove air bubbles from the cannula ([Figure 5A](#)).
- b. Place the kidney in the chamber on paper tissue, ensuring that the renal artery is oriented towards the glass cannula ([Figure 5B](#)). Either left or right kidneys can be used.
- c. After adjustment of the position and the angle of the kidney by moving the paper tissue, use forceps to place renal artery on the glass cannula ([Figure 5C](#)).
- d. Use a piece of paper and forceps to adjust the position the renal artery ([Figure 5D](#)) ([Methods video S3](#): 25–35 s).

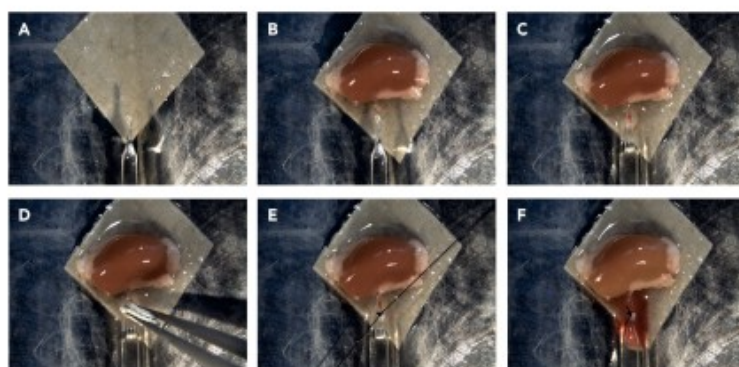


Figure 5. Connection of renal artery with glass cannula

- (A) Purging air from the glass cannula with PSS through a syringe; a part of a paper towel is placed on the platform.
 (B) Placing the kidney on the platform.
 (C) Connecting the renal artery with the glass cannula.
 (D) Using a part of a paper towel to position the renal artery appropriately.
 (E) Securing the renal artery with a surgical knot.
 (F) Condition of the kidney after perfusion with PSS through a syringe and removing the excess of silk material.



△ **CRITICAL:** During this procedure, to avoid grasping the artery with the forceps is crucial, since the renal artery can be damaged.

- e. Move the knot to fix the renal artery to glass cannula, and securely tie it (Figure 5E).
- f. Gently inject cold oxygenated PSS solution into the glass cannula using a syringe. Successful cannula connection to the renal artery and the kidney is indicated by a pale color of the whole kidney and lack of leakage (Figure 5F).

Note: If only part of the kidney appears pale after PSS infusion, the result could indicate that the glass cannula is located in a branch of the renal artery. Adjusting the catheter's position might address this problem.

Pressure changes in isolated perfused kidneys

⌚ Timing: 2 h

In this section, we describe the protocol of measuring the perfusion pressure in isolated kidneys due to pharmacological activation of KCNQ channels (K_v7 family of voltage-gated potassium channels). The background information is provided in the [expected outcomes](#) section.

8. Measure the pressure changes in isolated perfused kidneys.
 - a. Using peristaltic pump, perfuse the kidney with very low flow rate (0.3 mL/min) for 45 min - 1 h.
 - b. Set the flow rate of peristaltic pump to achieve the stable perfusion pressure of 80 mm Hg (~15 min)
 - c. Perfuse the kidney with 3 μM Methoxamine (ME, α1-adrenoreceptor agonist) solution until the pressure reaches the plateau.

Note: It is recommended to wait until stable pressure - plateau is reached (ca. 5 min).

- d. Perfuse the kidney with 3 μM ME and drug of interest (e.g., KCNQ opener, 10 μM Flupirtine)
- e. Wash out the KCNQ opener by perfusing the kidney with 3 μM ME
- f. Wash out the ME by perfusing the kidney with PSS
- g. Perfuse the kidney with 60 mM KCl.

Myogenic tone

⌚ Timing: 2 h

In this section, we describe the protocol of measuring the myogenic tone. The background information is provided in the [expected outcomes](#) section.

9. Measure the myogenic tone.
 - a. Perfuse the kidney with very low flow rate (0.3 mL/min) for 45 min - 1 h.
 - b. Stepwise increase the flow rate (0.7; 1.3; 1.9 mL/min). The duration of each step can vary and depends on reaching the plateau (ca. 3-5 min).
 - c. Reduce the flow rate to 0.5 mL/min until the pressure reaches a plateau (~10 min).
 - d. Perfuse the kidney with 10 nM Ang II until the pressure reaches a plateau (~5 min)
 - e. Wash out the Ang II by perfusing the kidney with PSS.
 - f. Perfuse the kidney with Ca²⁺ free PSS until the pressure reaches a plateau (~10 min).
 - g. Wash out Ca²⁺ free PSS with PSS until the pressure reaches a plateau (~10 min).
 - h. Perfuse the kidney with 60 mM KCl until the pressure reaches a plateau (~5 min).

EXPECTED OUTCOMES

Pressure changes in isolated perfused kidneys

Small-artery and resistance-vessel regulation is the key element regulating blood pressure and blood flow.^{10,11} Systemic vascular resistance in hypertension “is not everything; it is the only thing”. Vasoconstriction is mediated by increased intracellular Ca^{2+} concentration in vascular smooth muscle cells (VSMCs). This result is achieved by promoting the entry of extracellular calcium ions into the cells and releasing calcium ions stored within the cells.^{10,12–17}

Once the perfusion pressure has stabilized at the level of 80 mm Hg, 3 μM Methoxamine (ME) is added to perfusate (Figure 6, part a). Methoxamine binds α_1 -adrenergic receptors in vascular smooth muscle cells, activating Gq signaling pathway. Although the detailed mechanism is a matter of debate, this effect is expected to cause vasoconstriction and increase the perfusion pressure (Figure 6, part a).¹⁸ After reaching a plateau, 10 μM Flupirtine (KCNQ channel agonist) is added to perfusate. This result is expected to increase open probability of KCNQ channels and enhance efflux of potassium ions (K^+) from the cells causing VSMCs hyperpolarization. The hyperpolarized cell membrane closes VGCCs, reducing intracellular calcium ions (Ca^{2+}) concentration. Therefore, it is expected to cause vasodilation and a decrease in peripheral vascular resistance, reducing the perfusion pressure. (Figure 6, part b). If the binding to KCNQ channel is reversible, after Flupirtine is washed out perfusion pressure should increase again. Finally, 60 mM KCl is added to perfusate (Figure 6, part c).

High extracellular K^+ concentration results in membrane depolarization and an increased opening probability of VGCCs. Intracellular calcium ion (Ca^{2+}) level rises, which is followed by smooth muscle contraction. As consequence, vasoconstriction and an increase in peripheral vascular resistance occurs, thereby the increasing of perfusion pressure is expected. This step helps to ensure that there is no leakage between glass cannula and renal artery and the VSMCs are fully functional. The last steps (5–7) in the protocol 1 are optional. Difference in pressure can be calculated and represented as shown (Figure 6B).

Myogenic tone

Elevation of intravascular pressure causes constriction (myogenic tone) of small arteries and arterioles. The smooth muscle of both large arteries and small arterioles constrict in response to increased pressure and dilate in response to decreased pressure, a phenomenon known as the “Bayliss effect”.¹⁹ This effect and has been observed in various microvascular beds.²⁰ In the initial stage, the kidney is perfused at a relatively low-flow rate to achieve equilibration. Subsequently, the flow rate is gradually increased (0.3; 0.7; 1.3; 1.9), leading to an increase in the amount of fluid passing through the blood vessels per unit time (Figures 7A and 7B).¹ The flow rate is then adjusted to 0.5 mL/min, causing the perfusion pressure to decrease. Next, angiotensin II (Ang II) is applied. The peptide binds to angiotensin II type 1 receptor, which activates Gq/11-dependent signaling

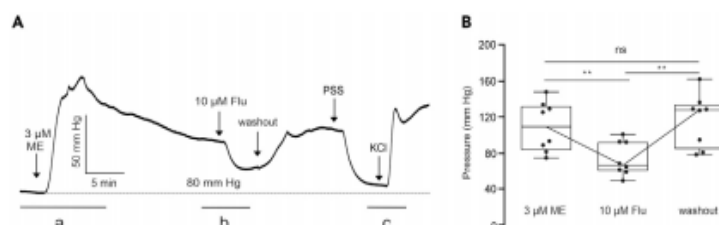


Figure 6. Expected results: impact of methoxamine (ME), flupirtine (Flu), and KCl on renal perfusion pressure

(A) Original recordings of the perfusion pressure in kidneys using 3 μM ME, 10 μM Flu, subsequent washout, and 60 mM KCl.

(B) Changes in the perfusion pressure induced by ME (increase), Flu (decrease) and subsequent washout procedure (increase). **P < 0.01; n.s., not significant; PSS, physiological saline solution.

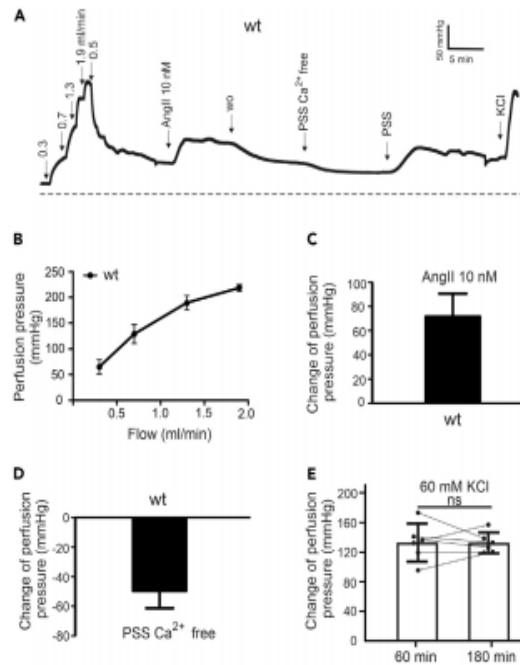


Figure 7. Myogenic tone

(A-C) Original recordings of the perfusion pressure in kidneys (A). Increase in perfusion pressure induced by increased flow rate (B) and 10 nmol/L Ang II (C). (B and C) Data are mean \pm SEM.

(D) Change of pressure in response to Ca^{2+} free PSS. Data are mean \pm SEM.

(E) Increase in perfusion pressure induced by 60 mM KCl at 60 min and 180 min $n = 6$, kidneys from $N = 6$ mice, n.s., not significant as determined by paired t-test. Error bars represent 95% confidence interval (CI) of mean.

pathway, thereby increasing intracellular Ca^{2+} concentration in vascular smooth muscle cells via the entry of extracellular calcium ions into the cells and the release of calcium ions stored within the cells. The increased calcium concentration stimulates the binding of actin and myosin, leading to subsequent vasoconstriction, resulting in an elevation of perfusion pressure. When changing to Ca^{2+} free PSS, no extracellular Ca^{2+} ions are available, which explains the decrease in perfusion pressure. This difference gives a measure of myogenic tone and helps to differentiate these state-of-the-affairs from passive dilation. Difference in pressure can be calculated and represented (Figures 7C and 7D).

LIMITATIONS

In summary with our protocol, whole kidneys can be studied in a (murine) rodent vascular model. Our interests are directed at small resistance vessels and not at basic renal physiology. Neither venous collection, nor urine collection are addressed using this technique, although these should be possible. Potential limitations of isolated kidney perfusion experiments include the prolonged use of colloid-free and cell-free solutions. Repetitive use of PSS may result in tissue edema, affecting hemodynamics, and renal function. These confounding variables could influence the accuracy of perfusion pressure measurements. Therefore, to ensure the reliability and accuracy of the results, awareness and control of these potential limitations is crucial. Fresh solutions should be prepared. Moreover, the composition of the perfusion fluid (PSS) may potentially influence the physiological state and vascular reactivity of the kidneys. Using PSS as a substitute for whole blood may not fully replicate *in vivo* physiology, leading to inaccuracies in assessing vascular responsiveness.²¹ In isolated rat kidney perfusion, removing the renal capsule during preparation may reduce the impact of tissue edema.²² Adding a erythrocyte concentrate or albumin solution could help to overcome these issues.²³ However according to our experience, acute experiments of 3 h duration did not



cause substantial problems regarding the vascular resistance, since the vascular response to 60 mM KCl remained unchanged (Figure 7F).

TROUBLESHOOTING

Problem 1

Fluctuations in perfusion pressure (increase).

Potential solution

- Due to small size of capillaries within the kidney, dust particles in the perfusion solution can block the capillaries. This effect increases perfusion pressure. Before the experiment, perfusion solutions should be filtered using filter-paper. To avoid possible binding of drugs to filter paper and to ensure the correct drug concentrations, we suggest filtering PSS solutions and using the filtrate for further drug dilutions.
- In addition to dust, air bubbles may increase pressure. In this case, we recommend adjusting the solution level in the bubble trap (Figure 1).

Problem 2

Fluctuations in perfusion pressure (decrease).

Potential solution

- Most often, a leakage causes the pressure instability. Familiarize yourself with the anatomy of the mouse blood vessels. Avoid damaging the arteries when dissecting the surrounding adipose and connective tissues. Often, it is possible to position the knot in order to close the leakage in the corresponding small branch of the renal artery.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dmitry Tsvetkov (dmitry.tsvetkov@med.uni-greifswald.de).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Dmitry Tsvetkov (dmitry.tsvetkov@med.uni-greifswald.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Additional data are available from the [lead contact](#) upon reasonable request. This study did not generate new codes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2024.102845>.

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AUTHOR CONTRIBUTIONS

All authors planned and designed experimental studies. Z.C. performed the experiments. Z.C. and D.T. drafted the article, and all authors contributed to its completion.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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13. Curriculum Vitae

The curriculum vitae is not included in the submitted version.

14. Statutory declaration

Hiermit erkläre ich, dass ich die vorliegende Dissertation eigenständig verfasst und dabei ausschließlich die angegebenen Hilfsmittel (einschließlich Werkzeuge der Künstlichen Intelligenz) verwendet habe.

Ich habe ChatGPT-4 für die Überprüfung von Grammatik und Zeichensetzung verwendet.

Die Dissertation wurde bislang keiner anderen Fakultät oder wissenschaftlichen Einrichtung zur Begutachtung vorgelegt.

Ich versichere, dass ich bisher kein Promotionsverfahren erfolglos abgeschlossen habe und dass keine Aberkennung eines bereits erlangten Doktorgrades vorliegt.

Datum 01. 10. 2025

Unterschrift Zhugang Chu

15. Acknowledgment

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