WNK1 Regulates Vasoconstriction and Blood Pressure Response to $\alpha_1$-Adrenergic Stimulation in Mice

Sonia Bergaya, Sébastien Faure, Véronique Baudrie, Marc Rio, Brigitte Escoubet, Philippe Bonnin, Daniel Henrion, Gervaise Loirand, Jean-Michel Achard, Xavier Jeunemaître, Juliette Hadchouel

Abstract—Gain-of-function mutations in the human WNK1 (with-no-lysine[K]) gene are responsible for a monogenic form of arterial hypertension, and WNK1 polymorphisms have been associated with common essential hypertension. The role of WNK1 in renal ionic reabsorption has been established, but no investigation of its possible influence on vascular tone, an essential determinant of blood pressure, has been performed until now. WNK1 complete inactivation in the mouse is embryonically lethal. We, thus, examined in Wnk1$^{+/−}$ haploinsufficient adult mice whether WNK1 could regulate in vivo vascular tone and whether this was correlated with blood pressure variation. Wnk1$^{+/−}$ mice displayed a pronounced decrease in blood pressure responses in vivo and in vascular contractions ex vivo following $\alpha_1$-adrenergic receptor activation with no change in basal blood pressure and renal function. We also observed a major loss of the pressure-induced contractile (myogenic) response in Wnk1$^{+/−}$ arteries associated with a specific alteration of the smooth muscle cell contractile function. These alterations in vascular tone were associated with a decreased phosphorylation level of the WNK1 substrate SPAK (STE20/SPS1-related proline/alanine-rich kinase) and its target NKCC1 (Na$^{+}$−K$^{+}$−2Cl$^{−}$ cotransporter 1) in Wnk1$^{+/−}$ arteries. Our study identifies a novel and major role for WNK1 in maintaining in vivo blood pressure and vasoconstriction responses specific to $\alpha_1$-adrenergic receptor activation. Our findings uncover a vascular signaling pathway linking $\alpha_1$-adrenergic receptors and pressure to WNK1, SPAK, and NKCC1 and may, thus, significantly broaden the comprehension of the regulatory mechanisms of vascular tone in arterial hypertension. (Hypertension. 2011;58:439-445.) ● Online Data Supplement

Key Words: blood pressure  ▪ vasoconstriction  ▪ conductance and resistive arteries  ▪ vascular smooth muscle cell  ▪ $\alpha_1$ adrenergic receptors  ▪ myogenic response

Familial Hyperkalemic Hypertension, or Gordon syndrome, is a rare mendelian form of arterial hypertension caused by gain-of-function mutations in the human WNK1 (with-no-lysine[K]) gene. In addition, several common polymorphisms at the WNK1 locus have been associated with essential hypertension. Because Familial Hyperkalemic Hypertension is associated with renal dysfunction, the role of this novel serine-threonine kinase has been extensively studied in renal ion homeostasis. This allowed to demonstrate that WNK1 is part of a novel signaling pathway involved in the complex regulation of sodium, potassium, and chloride balance in the kidney (for review see Reference$^4$).

WNK1 gives rise to 2 different isoforms with different expression patterns. The kidney-specific isoform of WNK1 (KS-WNK1) is specifically expressed in the distal nephron, whereas the long-WNK1 (L-WNK1) isoform is expressed ubiquitously.$^5$ Investigations of the mechanisms by which L-WNK1 could participate in the control of blood pressure (BP) have been mostly limited to in vitro models of renal transport, and its mechanism of BP regulation in vivo has not yet been elucidated. Indeed, in vivo WNK1 regulation of BP does not seem to be exclusively mediated by its renal action. Lexicon Pharmaceuticals, Inc, generated mice constitutively lacking WNK1 using a gene-trap strategy.$^7$ This mouse model thus represents the mirror image of the human disease for which hypertension has been shown to be caused by gain-of-function mutations of the WNK1 gene leading to overexpression of WNK1.$^3$ Wnk1$^{-/-}$ homozygous embryos died before day 13 of gestation, and WNK1 heterozygous (Wnk1$^{+/−}$) adult mice displayed a decreased systolic BP when compared with wild-type mice (Wnk1$^{+/+}$) despite a lack of alteration in kidney function.$^7$ The surprising absence of any renal dys-

Received February 28, 2011; first decision April 4, 2011; revision accepted June 19, 2011.

From the INSERM (S.B., V.B., X.J., J.H.), U970, Paris Cardiovascular Research Center, Paris, France; Université Paris Descartes (S.B., V.B., X.J., J.H.), Paris, France; Centre National de la Recherche Scientifique UMR 6214 (S.F., D.H.), INSERM U771, Angers University, Angers, France; Assistance Publique-Hôpitaux de Paris (V.B., X.J.), Hôpital Européen Georges-Pompidou, Paris, France; INSERM UMR 915 (M.R., G.L.), CARDIEX Platform, Nantes, France; INSERM U872 (B.E.), CEFI IFR02, Faculté de Médecine Xavier-Bichat, Paris, France; Université Paris Diderot (B.E., P.B.), Faculté de Médecine, Paris, France; Assistance Publique-Hôpitaux de Paris (B.E.), Hôpital Bichat, Paris France; INSERM U965 (P.B.), Paris, France; Assistance Publique-Hôpitaux de Paris (P.B.), Hôpital Lariboisière, Paris, France; Division of Nephrology and Department of Physiology (J.-M.A.), Limoges University Hospital, Limoges, France.

Correspondence to Sonia Bergaya, INSERM U970, Paris Cardiovascular Research Center PARCC, 56 rue Leblanc, 75015 Paris, France. E-mail sonia.bergaya@inserm.fr

© 2011 American Heart Association, Inc.

Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.111.172429
function in these mice thus suggests a more systemic involvement of WNK1 in BP regulation in vivo and points toward a role for L-WNK1 outside the kidney. However, in this model, it was not determined whether L-WNK1 or the kidney-specific isoform of WNK1 was responsible for the BP change and, more importantly, what could be the mechanism responsible for this BP decrease.

L-WNK1 is expressed in the heart and within the whole vascular tree, in both endothelial and vascular smooth muscle cells (VSMCs), during development and adulthood. Because vascular tone is a crucial determinant of BP, we herein investigated the role of L-WNK1 in the physiological regulation of vascular tone in adult mice. Our study reveals that L-WNK1 mediates in vivo BP response and ex vivo vasoconstriction after α1-adrenergic receptor activation and transmural pressure stimulation, in conductance and resistive arteries.

Methods
A detailed Methods section is available in the online Data Supplement at http://hyper.ahajournals.org.

Results
Wnk1+/− Mice as a Model of L-WNK1 Haploinsufficiency
We examined the level of mRNA expression of each WNK1 isoform (Figure S1, please see the online Data Supplement at http://hyper.ahajournals.org). Although L-WNK1 expression was reduced by half in Wnk1+/− mice (≈44% in thoracic aortas and ≈50% in kidneys, \( P < 0.0004 \) and \( P < 0.009 \) versus Wnk1+/+, respectively), there was no difference in KS-WNK1 kidney expression between Wnk1−/− and Wnk1+/+ mice (\( P = 0.77 \)). Accordingly, L-WNK1 protein was decreased by ≈42% in Wnk1−/− aortas (\( P = 0.017 \) versus Wnk1+/+; Figure S1). Wnk1−/− mice thus represent a specific model of L-WNK1 inactivation with no modification in the kidney-specific isoform of WNK1 expression.

Decreased In Vivo BP Response to α1-Adrenergic Receptor Activation in Wnk1+/− Mice
We tested the in vivo BP responses of Wnk1−/− and Wnk1+/+ mice by performing dose-response curves of phenylephrine (Phe), an α1-adrenergic vasoconstrictor agonist, in anesthetized mice (Figure 1). Phe acute pressor responses were significantly attenuated in Wnk1−/− mice when compared with Wnk1+/+ mice (\( P = 0.036 \) from 1 to 30 \( \mu \)g/kg). The ED50 was also significantly higher (≈3 times) in Wnk1−/− mice when compared with Wnk1+/+ mice.

Wnk1+/− Mice Exhibit Normal Basal BP and Kidney Function
To assess whether the decreased BP response to Phe was associated with an altered basal BP, systolic BP and diastolic BP were measured by telemetry. No alteration of the basal systolic BP and diastolic BP and of the expected mouse bimodal circadian pattern was observed in Wnk1−/− when compared with Wnk1+/+ mice (Figure S2). Heart rate was also comparable between groups [551.4 ± 21.5 and 558.2 ± 19.7 bpm, respectively, for Wnk1−/− and Wnk1+/+ mice (day period; \( P = 0.64 \)] and 609.7 ± 24.0 and 617.8 ± 16.1 bpm, respectively, for Wnk1+/− and Wnk1+/+ mice (night period; \( P = 0.62 \)]. Measurements of plasma and urine electrolytes showed that basal kidney function was not affected in Wnk1−/− mice (Table S1), as observed previously. Finally, cardiac morphology and function explored by echocardiography were also not altered in Wnk1−/− mice (Table S2), with an exception being made for the left ejection fraction, which was significantly but marginally decreased by ≈8% in Wnk1−/− mice, thus revealing a possible mild systolic dysfunction in these mice. The heart weight/body weight ratio was also similar between Wnk1−/− and Wnk1+/+ mice (5.1 ± 0.3 mg/g, \( n = 7 \), versus 4.6 ± 0.1 mg/g, \( n = 7 \); \( P = 0.15 \)).

Wnk1+/− Mice Exhibit a Strong Hypocontractility Phenotype in Response to α1-Adrenergic Receptor Activation in an NO-Independent Manner
We next examined Phe-induced vasoconstrictions of large conductance and small resistive arteries ex vivo. Amplitudes of the contractile responses to high concentrations of Phe were significantly attenuated in Wnk1−/− thoracic aortic rings and mesenteric arteries when compared with Wnk1+/+ littermates (\( P = 0.032 \) and \( P = 0.037 \), respectively; Figure 2A and 2B). This reduced contractility observed in Wnk1−/− mice remained when these Phe concentration-response curves were performed in presence of Nω-nitro-L-arginine methyl ester (L-NAME; 100 \( \mu \)mol/L), a pharmacological inhibitor of NO synthases, both in thoracic aortas (\( P = 0.016 \)) and mesenteric arteries (\( P = 0.050 \); Figure 2C and 2D). L-NAME did, however, significantly increase Phe-induced vasoconstrictions within each group of mice in aortic rings (\( P < 0.0001 \) and \( P < 0.0001 \), respectively, for Wnk1−/− and Wnk1+/+ mice) and in mesenteric arteries (\( P < 0.0001 \) and \( P = 0.0005 \), respectively, for Wnk1−/− and Wnk1+/+ mice), but to the same extent. The endogenous level of NO cannot, therefore, explain the hyporeactivity phenotype. Indeed, we also showed that endothelial function is preserved in Wnk1+/− mice (Figure S3), as acetylcholine concentration-response curves performed on thoracic aortic rings and mesenteric arteries
induced comparable vasodilatations in both groups of mice (P = 0.57 and P = 0.84, respectively). Similarly, flow-dependent dilatations were comparable between both groups of mice (P = 0.87). Thus, L-WNK1 decreased expression strongly affects vasoconstrictility without altering endothelial function in adult mice.

Specificity to Catecholamines
We performed concentration-response curves to other contracting agents (Figures S4 and S5). Norepinephrine-induced vasoconstrictions (a natural α1-adrenergic agonist) were significantly reduced in Wnk1+/− thoracic aortic rings (P = 0.02 versus Wnk1+/+). Conversely, angiotensin II contractile responses obtained in abdominal aortic rings and mesenteric arteries were comparable between both groups of mice (P = 0.51 and P = 0.89, respectively). Similarly, BP responses to angiotensin II and their associated ED₅₀s were not different between groups (P = 0.39 and P = 0.82, respectively). Finally, we examined vascular contractions induced by potassium chloride (KCl), which are not mediated by vasopressor receptors but rather by a massive entry of calcium in VSMCs. KCl-induced contractions were not affected in Wnk1+/− mesenteric and aortic rings. Thus, Wnk1+/− mice exhibit a significant alteration in BP and vasoconstricting responses specific to α₁-adrenergic vasoconstrictors in both conductance and resistive arteries.

Specific Alteration of the VSMC Contractility in Wnk1+/− Mice
We next tested the contractile response in arteries without endothelium (de-endothelialized) to evaluate the specific contribution of VSMCs (Figure S6). Phe-induced contractions were strongly altered in Wnk1+/+ de-endothelialized aortic rings (P = 0.023; 60% decrease at maximal Phe concentration) even more than in normal arteries. Interestingly, KCl contractions, whereas unaffected in intact arteries, were reduced at 60 and 125 mmol/L in Wnk1+/− de-endothelialized aortic rings (P = 0.048 and P = 0.045, respectively). The hypocontractility phenotype observed in Wnk1+/− mice is, thus, attributed to a specific and important alteration of the VSMC contractile signaling.

Major Alteration in Pressure-Induced Myogenic Response in Wnk1+/− Mice Associated With Normal Basal Vascular Resistances
Resistive arteries have a specific inherent property called myogenic response, which corresponds with their ability to vasoconstrict in response to an increase in pressure and which
is known to be mediated by VSMCs. We, thus, examined as another VSMC-mediated vasoconstricting stimulus, the effect of an increase in intra-arterial pressure in second-order mesenteric arteries of Wnk1+/+ and Wnk1−/− mice mounted ex vivo. The myogenic response was calculated for each pressure step as the difference between passive and active diameters (measured in Ca2+-free and in normal physiological salt solution, respectively). The myogenic response obtained in Wnk1−/− mesenteric arteries was markedly blunted when compared with Wnk1+/+ mice (P=0.0003; Figure 3).

We next wanted to ascertain whether this attenuated response to transmural pressure was attributed to a specific defect in VSMC contractile response after pressure stimulation or whether it was the consequence of basal vascular resistance alteration. Similar arterial diameters were observed whatever the level of pressure, as showed by the pressure-diameter curves performed on carotids and mesenteric arteries from Wnk1+/+ and Wnk1−/− mice. Their arterial cross-sectional compliance and cross-sectional distensibility were also comparable between both groups for each pressure step (P>0.05; Figure S7). These results demonstrated that, as for α1-adrenergic mediated contractions, L-WNK1 plays a crucial role in maintaining the pressure-induced contraction of resistive arteries.

**Decreased SPAK and NKCC1 Phosphorylation in Wnk1−/− Arteries**

L-WNK1 is known to regulate several ion channels and transporters in the kidney, and among them is the Na⁺-K⁺-2Cl⁻ cotransporter (NKCC).9,10 Interestingly, NKCC1, the only NKCC isofrom expressed in the vessel wall, regulates BP and vascular tone in rodents11–13 by increasing intracellular [Cl⁻] in VSMCs, thereby initiating depolarization and contraction.14 NKCC1 could thus be a possible end-target of L-WNK1 during vascular tone regulation. WNK1 has been shown in vitro to promote NKCC1 activity through phosphorylation of the STE20/SPS1-related proline/alanine-rich kinase (SPAK), which itself interacts with and phosphorylates NKCC1.9,10 However, there is no evidence yet that L-WNK1 regulates SPAK or NKCC1 phosphorylation in vivo and in vascular tissue. SPAK phosphorylation on Ser373 (WNK1-targeted site) was significantly reduced by ≈40% in Wnk1−/− aortas (P=0.00005 versus Wnk1+/+; n=16 in each group), whereas total SPAK expression was nonsignificantly decreased by ≈20% (P=0.15 versus Wnk1+/+; Figure 4). Phospho-SPAK:total-SPAK ratio was, thus, nonsignificantly decreased by ≈20% in Wnk1−/− aortas (P=0.23 versus Wnk1+/+). The 3 bands observed on the immunoblot have been sequenced previously, and all correspond to SPAK isoforms.16 Similarly, phosphorylation of NKCC1 on the cluster Thr207/Thr212/Thr230 (SPAK-targeted site) was significantly decreased by ≈30% in Wnk1−/− aortas (P=0.046 versus Wnk1+/+; n=8 in each group). Again, total NKCC1 expression was nonsignificantly decreased by ≈20% in Wnk1−/− aortas (P=0.14 versus Wnk1+/+; Figure 4). Phospho-NKCC1:total-NKCC1 ratio thus remained comparable between groups (P=0.32). We, therefore, showed that L-WNK1 expression is necessary to maintain vascular SPAK and NKCC1 phosphorylation and, to a lesser extent, their protein expression level.

**Discussion**

This study reports a novel role for L-WNK1 in BP response to α1-adrenergic receptor activation in vivo and in maintaining pressure and catecholamine-induced vasoconstriction in conductance and resistive arteries. These results bring impor-
Figure 4. Phospho- and total-SPAK and NKCC1 protein expression in abdominal aorta. Immunoblots show a significant decrease in NKCC1 and SPAK phosphorylation associated with a nonsignificant decrease in total NKCC1 and SPAK proteins in Wnk1+/− mice when compared with Wnk1+/+ mice. Quantitative histograms represent phospho-protein:actin, total-protein:actin, and phospho:total ratios for SPAK (n=16 mice in each group) and NKCC1 (n=8 mice in each group), which respectively correspond with the mean of 4 and 2 independent experiments (†P<0.05 vs Wnk1+/+; ‡P<0.01 vs Wnk1+/+).

tant new insights into the mechanisms by which L-WNK1 could participate in the regulation of BP in adults, since no investigation of the role of L-WNK1 in the adult cardiovascular function had been performed until now.

In vivo BP increases to α1-adrenergic receptors activation were significantly attenuated in L-WNK1–haploinsufficient mice. This was confirmed on isolated arteries in which a ∼30% and ∼50% decrease in Phe maximal contraction was observed, in thoracic aortas and mesenteric arteries, respectively. Interestingly, these reduced vasoconstrictions were observed with different catecholamines but not with angiotensin II. This suggests that L-WNK1 belongs to a signaling pathway triggered specifically by α1-adrenergic receptors, which signal only through Gαq/11 proteins and which might be, in Wnk1+/− mice, compensated for or silenced by other activated signaling pathways during angiotensin II activation, which involves not only Gαq/11 proteins but also Gα12/13 and Gi/o proteins, as well as activation of G protein–independent signaling pathways. Importantly, the decreased vasoconstrictions were not correlated with endothelial dysfunction but rather with a significant alteration of VSMC function. Indeed, L-WNK1–haploinsufficiency led to a more pronounced fractional decrease in Phe contraction in de-endothelialized arteries than in intact ones. In addition, KCl-induced vasoconstrictions, whereas unaffected in Wnk1+/− intact arteries, became significantly reduced in Wnk1+/− de-endothelialized arteries. This suggested that the endothelium could mask or compensate for the alteration in VSMC contractility observed in Wnk1+/− arteries.

Another major finding was the marked attenuation of the myogenic response developed by mesenteric arteries in Wnk1+/− mice (a 76% decrease at 75 mm Hg). This autoregulatory response regulates tissue perfusion and protects the downstream capillary bed from variations in systemic BP. We, therefore, uncovered a major role for L-WNK1 in a fundamental regulation mechanism. Myogenic response is known to be initially mediated by several stretch-activated ion channels at the plasma membrane, as well as by other mechanosensor elements, such as integrins, caveolae, and the cytoskeleton.21,22 It can also be directly mediated by the Gαq/11 heterotrimeric GTP-binding protein–coupled receptor family (which comprises the vascular α1-adrenergic receptors), which can act as mechanosensors in VSMCs in a ligand-independent manner,23 suggesting that L-WNK1 could belong to a signaling pathway triggered by both α1-adrenergic receptors and transmural pressure.

SPAK is, to date, one of the best characterized substrates of the WNKs. WNK1 has been shown to interact with and phosphorylate SPAK in several in vitro models.10,15 We demonstrate here for the first time that, in vascular tissue, L-WNK1 regulates in vivo SPAK phosphorylation and, to a lesser extent, its protein expression. Recently, reduced vasoconstrictions in response to high molar Phe concentrations were reported in aortas from SPAK+/− and SPAK−/− mice.24
Thus, a haploinsufficiency in either L-WNK1 or SPAK led to very similar hypocontractility phenotypes. We propose that L-WNK1 maintains contractile tone possibly through the regulation of SPAK phosphorylation and/or protein expression in arteries. SPAK also interacts with and phosphorylates NKCC1 in vitro, thereby promoting its activity. We further showed that NKCC1 phosphorylation and expression were decreased to the same extent in Wnk1<sup>+/−</sup> arteries, suggesting that L-WNK1 could regulate NKCC1 phosphorylation probably through the regulation of its total protein expression level. A decreased NKCC1 phosphorylation has also been observed in aortas from SPAK<sup>+/−</sup> mice. Therefore, hypococontractility phenotypes of SPAK and L-WNK1 heterozygous mice were both correlated to a significant decrease in NKCC1 phosphorylation. However, the level of expression of total NKCC1 varies between the 2 models. NKCC1 expression was increased in SPAK<sup>+/−</sup> mice and nonsignificantly decreased in Wnk1<sup>+/−</sup> mice. However, a knock-in mouse model in which SPAK cannot be activated by WNK kinases also showed reduced levels of NKCC1 phosphorylation and expression in the kidney, as observed in Wnk1<sup>+/−</sup> arteries. The possible regulation of NKCC1 protein expression by L-WNK1 thus appears to require further investigations. This hypothesis is actually further supported by the role of WNK1 in intracellular trafficking through its interaction with several proteins involved in endocytosis and exocytosis vesicles.

WNK1 notably phosphorylates synaptotagmin 2, modulating its plasma membrane binding and possibly affecting the release from or the insertion in the plasma membrane of several vesicle-associated proteins, such as ion cotransporters, which, if not integrated correctly in the membrane, could possibly be targeted for degradation. Interestingly, NKCC1 has also been shown to regulate arterial BP<sup>27,28</sup> and to mediate pathological conditions or following stress. Conditions but could participate in BP dysregulation in pathological conditions or following stress.

Perspectives

Our study establishes a novel and important role for L-WNK1 in regulating adult vascular function in vivo and provides evidence of a major vasoconstriction pathway linking α<sub>1</sub>-adrenergic receptors and transmural pressure to L-WNK1 and its downstream effectors SPAK and NKCC1. This study, therefore, provides important new insights to vascular physiology and the comprehension of the mechanisms involved in the control of BP during hypertension. Generation of L-WNK1 pharmacological inhibitors in the future would represent useful tools for the investigation of new antihypertensive drugs and for a better understanding of the action mechanism of α<sub>1</sub>-adrenergic antagonists used clinically.

Acknowledgments

We are grateful to Dr Chantal Boulanger, Prof Jo de Mey, and Prof Jean-Luc Elghozzi for their challenging discussions. We thank Julie Faugeroux, Rénette Saint-Fort, and Christelle Soukaseum for their everyday support.

Sources of Funding

This work was supported by INSERM, Agence Nationale pour la Recherche (ANR grant 05-MRAR-010-01), and the Leducq Transatlantic Network on Hypertension (Fondation Leducq Research Agreement 07 CVD 01/Jeuembaliare).

Disclosures

None.

References


WNK1 Regulates Vasoconstriction and Blood Pressure Response to $\alpha_1$-Adrenergic Stimulation in Mice

Sonia Bergaya, Sébastien Faure, Véronique Baudrie, Marc Rio, Brigitte Escoubet, Philippe Bonnin, Daniel Henrion, Gervaise Loirand, Jean-Michel Achard, Xavier Jeunemaitre and Juliette Hadchouel

_Hypertension_. 2011;58:439-445; originally published online July 18, 2011; doi: 10.1161/HYPERTENSIONAHA.111.172429

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2011 American Heart Association, Inc. All rights reserved.

Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://hyper.ahajournals.org/content/58/3/439

Data Supplement (unedited) at:

http://hyper.ahajournals.org/content/suppl/2011/07/14/HYPERTENSIONAHA.111.172429.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:

http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:

http://hyper.ahajournals.org//subscriptions/
WNK1 regulates Vasoconstriction and Blood Pressure Response to α1-adrenergic Stimulation in Mice

Short title: WNK1 Insufficiency Leads to Vascular Tone Defect

Sonia Bergaya, PhD1,2; Sébastien Faure, PhD3; Véronique Baudrie, BSc1,2,4; Marc Rio, BSc5; Brigitte Escoubet, MD, PhD6,7,8; Philippe Bonnin, MD, PhD7,9,10; Daniel Henrion, PharmD, PhD3; Gervaise Loirand, PhD5; Jean-Michel Achard, MD, PhD11; Xavier Jeunemaitre, MD, PhD1,2,4 and Juliette Hadchouel, VetMD, PhD1,2.

1INSERM, U970, Paris Cardiovascular Research Center PARCC, Paris, France. 2Université Paris Descartes, Paris, France. 3CNRS UMR 6214, INSERM U771, Angers University, Angers, France. 4Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges-Pompidou, Paris, France. 5INSERM UMR 915, CARDIEX PLATFORM, Nantes, France. 6INSERM U872, CEFI IFR02, Faculté de Médecine Xavier-Bichat. 7Université Paris Diderot, Faculté de Médecine, Paris, France. 8Assistance Publique-Hôpitaux de Paris, Hôpital Bichat, Paris France. 9INSERM U965, 10Assistance Publique-Hôpitaux de Paris, Hôpital Lariboisière, Paris, France. 11Division of Nephrology and Department of Physiology, Limoges University Hospital, Limoges, France.

Correspondence to Sonia Bergaya, PhD; INSERM U970, Paris Cardiovascular Research Center PARCC, 56 rue Leblanc, 75015 Paris, France. Phone: +33-1-53-98-80-74. Fax: +33-1-53-98-79-52. E-mail: sonia.bergaya@inserm.fr
Supplemental Methods

Mice
All animal studies have been approved and conducted in accordance with the relevant guidelines of the French Ministry of Agriculture (Authorisation Executive Order No. A 75-05-12) for scientific experimentation on animals, European Communities Council Directives and international ethical standards. Wnk1+/− mice were kindly provided and generated by Zambrowicz et al. on a mixed genetic background (129/SvEvBrd x C57BL/6J) by gene-trap mutation at the WNK1 locus by insertion of a β-galactosidase and neomycin resistance genes \(^1\). Our mice were obtained after back-crossing Wnk1+/− mice with C57BL/6N mice on 5 to 12 generations (N5 to N12). For all animal and expression studies, we solely used littermates adult males from wild-type (Wnk1+/+) and WNK1-haploinsufficient (Wnk1+/−) groups of mice. For each detailed protocol of physiological experiments described in the Online Supplement, we notified the precise number of backcrosses generation of the littermates male mice that we used and for which we verified that they were age- and weight-matched between groups for series of experiments.

Real time Quantitative Polymerase-Chain-Reaction (q-PCR)
Q-PCR were performed using a qPCR MasterMix Plus for SYBR Green I with Fluorescein (Eurogentec) either on kidney extracts isolated from Wnk1+/+ (n=6) and Wnk1+/− (n=6) littermates adult male mice or on thoracic aortas isolated from Wnk1+/+ (n=5) and Wnk1+/− (n=5) littermates adult male mice. Tissues were crushed in liquid nitrogen and RNA was extracted using subsequent steps of Trizol (Invitrogen), chloroform and ethanol 70%. We then followed the NucleoSpin RNA II protocol (from Macherey-Nagel) until the RNA elution. Q-PCR were then performed using a qPCR MasterMix Plus for SYBR Green I with Fluorescein (Eurogentec). HPRT was used as the reference gene for all the q-PCR performed on thoracic aortas (forward primer: 5’-CTCAACTTTAACTGGAAGATGTC-3’; reverse primer: 5’-TCCTTTTCACCAAGCAAG CT-3’) to express the level of RNA expression as a ratio gene of interest / HPRT. 18S was used as the reference gene for all the q-PCR performed on kidneys extracts (forward primer: 5’-CGCCGCTAGAGGTGAAATTC-3’; reverse primer: 5’-TCTTGGCAAATGCTTTCGC-3’) to express the level of RNA expression as a ratio gene of interest / 18S. The lecture of CT was performed using the CHROMO4 MJ Research (Biorad). Primers were designed in WNK1 exon 1 (forward primer: 5’-GACAGTCTACAAAGGTCTGGACAC-3’) and WNK1 exon 2 (reverse primer: 5’-GACCCTTTAACATTTACGCTCTTC-3’) to amplify L-WNK1 isoform, and primers were designed in WNK1 exon 4a (forward primer: 5’-TTGGTATTGAAATTTCTATTGCTG-3’) and WNK1 exon 5 (reverse primer: 5’-AGGAATTGCTACTTGTCAAAAATG-3’) to amplify KS-WNK1 isoform.

In vivo Pressure Response Study
Acute pressor responses to phenylephrine were measured in anesthetized mice. An arterial catheter was connected to a pressure transducer and a Gould RS 3400 polygraph, and a venous catheter was used for drug injection. We used only littermates adult male mice from Wnk1+/+ (n=5) and Wnk1+/− (n=5) groups of mice (number of backcrosses: N10 and N11) with comparable ages and weights (P>0.05 between groups). Acute pressor responses to phenylephrine as well as its respective ED50 (the log dose at half the maximal response) were measured and calculated as described earlier \(^2\), in mice anesthetized with pentobarbitalsodique (60mg/kg i.p.). Flexible plastic catheters were introduced into the abdominal aortic artery and in the jugular vein. The arterial catheter was connected to a pressure transducer and a Gould RS 3400 polygraph, and the venous catheter was connected to a syringe for drug injection. The BP signal was digitized using a 12-bit A/D converter and processed by an algorithm based on feature extraction to detect and measure the
characteristic of a BP cycle, ANAPRES 3.0 (Notocord Systems; Croissy/Seine, France). After at least 30 minutes - necessary to stabilize BP and heart rate - bolus injections of increasing doses of phenylephrine (0.01–700 μg/kg) and angiotensin II (0.0001–100 μg/kg) were administered at least 10-minutes intervals. Dose response curves were established for the peak of the BP responses.

**Ex vivo Vascular Reactivity Experiments**

Thoracic aortas were dissected and cut segments were mounted in a Multi Wire Myograph system (Model 610 M – Danish Myo Technology - Denmark). Mesenteric arteries were dissected and cannulated at both ends in a video-monitored perfusion system (Living System Instrumentation, Burlington, VT, USA). Blinded experiments were performed for all. For each individual series of experiments, we used only littermates adult male mice from Wnk1+/+(n=6 to 9) and Wnk1+/−(n=6 to 10) groups of mice (number of backcrosses: N5 to N7), with comparable ages and weights (all through the series, mice were aged from 15 to 22 weeks and had weights between 25g to 32g; P>0.05 between groups within each single serie). For experiments performed on thoracic aortas, mice were anesthetized with a mixture of Ketamin/Xylazin as described above, and a midsternal thoracotomy was performed. Thoracic aortas were carefully exposed and rapidly dissected. For desendothelialization experiments, the endothelium was mechanically removed by a gentle rubbing of the intimal surface with a sewing thread. Arterial segments were cut and then mounted in a Multi Wire Myograph system (Model 610 M – Danish Myo Technology - Denmark) and data were recorded with the use of an ADI PowerLab Data Acquisition System. Arterial segments were suspended by 2 tungsten wires and bathed in a 5 ml organ chamber containing a physiological saline solution (PSS) (in mmol/L: NaCl 118.99, KCl 4.69, MgSO4·7H2O 1.17, KH2PO4 1.18, CaCl2·2H2O 2.5, NaHCO3 25, EDTA disodium salt 0.03, Glucose 5.5). The solution was continuously warmed at 37°C and gassed with a mixture of 95%O2/5%CO2. Segments were put under tension and were equilibrated for 45 mins until they reached a stable plateau at their resting tension of 9.8 milliNewton (mN). Before every single experiment, vessels were stimulated with a potassium chloride enriched PSS solution (KCl concentration: 60 mmol/L) and the integrity of the endothelium was tested by contracting the vessel with 1 μmol/L of phenylephrine (Phe) and by dilating it with 1 μmol/L of acetylcholine (Ach). Only arteries which were able to dilate more than 60% of the delta contraction imposed by Phe were kept for the experiments. On the opposite, specifically for the desendothelialization experiments, only arteries which were able to dilate less than 10% of the delta contraction imposed by Phe were kept for the denuded-arteries experiments. Concentration-response curves were performed for phenylephrine (from 10⁻⁹ mol/L to 10⁻⁴ mol/L), for norepinephrine (from 10⁻⁹ mol/L to 3×10⁻⁷ mol/L) and for angiotensin II (from 10⁻¹⁰ mol/L to 10⁻⁶ mol/L). Phenylephrine concentration-response curves were also reproduced in presence of L-NAME (N⁴-nitro-L-arginine methyl ester) inhibitor (100 μmol/L) which was incubated 30 mins prior to Phe concentration-response curves. Acetylcholine concentration-response curves (from 10⁻⁹ mol/L to 10⁻⁴ mol/L) were also performed following a pre-constriction with Phe which corresponds to ~60% of the maximal contraction obtained with the initial KCl contraction. Also, we examined contractions caused by potassium chloride - enriched isotonic PSS containing a final KCl concentration at 30 mmol/L, 60 mmol/L and 125 mmol/L in isotonic conditions *i.e.* with the same osmolarity as found in the PSS solution. The corresponding arterial tension (in mN) was recorded in real time. Data are expressed as increases in force (MilliNewton, mN) and as percentage of relaxation of phenylephrine-induced pre-contraction.

In separate experiments, mice were sacrificed by CO₂ inhalation and mesenteric arteries were dissected and a segment of second-order mesenteric arteries (~4mm long) was cannulated at both ends in a video-monitored perfusion system (Living System Instrumentation, Burlington,
VT, USA) as described in Pressure-Diameters Curves Methods Section. Pressure was controlled by a servo-perfusion system and flow was generated by a peristaltic pump. Before every single experiment, vessels were stimulated with a potassium chloride enriched PSS solution (KCl concentration: 80 mmol/L) and the integrity of the endothelium was tested by contracting the vessel with 1 µmol/L of Phe and by dilating it with 1 µmol/L of Ach. Only arteries which were able to dilate more than 60% of the delta contraction imposed by Phe were kept for the experiments. Concentration-response curves were performed for phenylephrine from $10^{-9}$ mol/L to $10^{-5}$ mol/L and for angiotensin II (from $10^{-12}$ mol/L to $10^{-6}$ mol/L). Phe concentration-response curves were also reproduced in presence of L-NAME (100 µmol/L) which was incubated 30 mins prior to Phe concentration-response curves. Acetylcholine concentration-response curves (from $10^{-9}$ mol/L to $10^{-5}$ mol/L) were also performed following a pre-constriction with phenylephrine and serotonin in order to obtain a similar level of pre-constriction (PC) in both groups of mice (PC = 102.4±11.9 µm and PC = 103.2±15.0 µm, respectively in Wnk1+/+ and Wnk1+/− mice, P=0.97). Flow-induced dilation was studied by increasing flow rate by steps from 3 to 100 µl/min. We first pre-constricted mesenteric arteries with phenylephrine in order to obtain a similar level of pre-constriction in both groups of mice before imposing the step-increases in flow (PC = 79.7±15.3 µm and PC = 88.8±15.2 µm, respectively in Wnk1+/+ and Wnk1+/− mice, P=0.68), with intraluminal pressure set at 75 mmHg. Flow-induced relaxation was expressed as percentage of relaxation of phenylephrine-induced pre-contraction.

Myogenic response was determined by increasing intraluminal pressure by steps from 10mmHg to 125mmHg without intraluminal flow and was calculated as follows: Arterial diameters were first measured for each pressure-step in normal physiological salt solution and were considered as diameters under active tone or "active diameter". At the end of each experiment, arterial segments were superfused with a Ca²⁺-free physiological salt solution containing EGTA (2mmol/L), sodium nitroprusside (10µmol/L) and papaverine (10µmol/L), and pressure-steps were repeated to determine the "passive diameter" of the arteries. Myogenic response was then expressed as the difference between passive and active diameters (in delta microns) for each step of pressure.

**Pressure-Diameter Curves**

Carotid and mesenteric arteries were dissected and mounted ex vivo in an arteriograph which is a video-monitored perfusion system (Living System Instrumentation, Burlington, VT, USA). Blinded experiments were performed for both types of vessels. Mice were anesthetized with an intraperitoneal injection of a mixture of Ketamin (diluted 1/10th in PBS) / Xylazin (diluted 1/10th in PBS) (2/3rd + 1/3rd respectively) (for carotid arteries) or sacrificed by CO₂ inhalation for mesenteric arteries. Carotid arteries as well as a segment of second-order mesenteric arteries (~ 4mm long) from Wnk1+/+(n=5 to 8) and Wnk1+/−(n=5 to 7) littermates adult male mice (number of backcrosses: N5 and N7, respectively for carotids and mesenteric arteries) were carefully dissected and mounted ex vivo in an arteriograph as described earlier. Arteries were cannulated at both ends and bathed in a 5 mL organ bath thermostated at 37°C containing physiological salt solution (PSS) of the following composition (in mmol/L): for carotid arteries: 118.3 NaCl, 5.5 glucose, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, and 5 HEPES, pH 7.4; and for mesenteric arteries: 135 NaCl, 15 NaHCO₃, 4.6 KCl, 1.5 CaCl₂, 1.2 MgSO₄, 11 glucose, and 5 HEPES, pH 7.4. For carotid arteries, solutions were gassed with a mixture of 95%O₂/5%CO₂. For mesenteric arteries, the pO₂ was maintained at a value of 160 mmHg and the pCO₂ at a value of 37 mmHg. Carotid arteries were first equilibrated for 45mins and then submitted to different step of pressures (from 20mmHg to 160mmHg) for which we measured carotid arteries outer diameters, referred as "active diameters". The same experiments were repeated in a PSS containing 0 calcium, EGTA (2 mmol/L) and sodium nitroprusside (SNP) (100 µmol/L) in order to obtain "passive diameters".
representing the maximal state of dilatation of the arteries for each level of pressure. Similarly, arterial segments of mesenteric arteries were also submitted to step-increases in pressure (from 10mmHg to 125mmHg) for which we measured their active diameters in normal PSS. At the end of each experiment, arterial segments were superfused with a Ca$$^{2+}$$-free physiological salt solution containing EGTA (2mmol/L), sodium nitroprusside (10 µmol/L) and papaverine (10 µmol/L), and pressure steps were repeated to determine the “passive diameter” of the arteries. Results are given in microns for artery diameters. Furthermore, cross-sectional compliance (CSC) (µm$$^2$$/mmHg) as well as cross-sectional distensibility (CSD) (µm/mmHg) were calculated respectively as CSC=ΔS/ΔP and CSD=(1/S) x ΔS/ΔP (S represents the surface and P the pressure) as described earlier.$^{4}$

**Plasma and Urinary Electrolyte Concentrations Measurements**

Littermates \(Wnk1^{+/+}\) (n=6) and \(Wnk1^{-/-}\) (n=6) male mice (N5) were housed in metabolic cages and fed a standard diet (SAFE A03; 0.29% Na$$^+$$ - 0.41% Cl$$^-$$) with free access to tap water. Creatinine, plasma and urine electrolytes were determined using an Olympus AU400 analyser. After a 2-day adaptation period, urines were collected over a 24h period. Blood samples from the same groups of mice were collected, following tail-nick, in lithium-heparin capillary coated tubes (SARSTEDT, Germany). At the end of the experimental period, animals were sacrificed with ketamine and xylazine (0.1 and 0.01 mg/g of body weight, respectively).

**Telemetry Measurements**

Blood pressure and heart rate were measured in conscious, unrestrained mice using a radiotelemetry system (PA-C10 and Dataquest software, Data Sciences International). They were performed in \(Wnk1^{+/+}\) (n=7) and \(Wnk1^{-/-}\) (n=6) littermates adult male (number of backcrosses: N8 and N9) mice which display comparable ages (between 20 and 27 weeks; \(P>0.05\)). Blood pressure and heart rate were measured in conscious, unrestrained mice using a radiotelemetry system (PA-C10 and Dataquest software, Data Sciences International) as described previously.$^{5}$ After a 1 week recovery period from surgery, recordings were obtained every 15 minutes for 60 seconds and were analyzed for 12h day-time (7-19h) and 12h night-time (19-7h) periods, respectively, to evaluate circadian variation in hemodynamics.

**Echocardiography**

Echocardiography was performed in \(Wnk1^{+/+}\) (n=6) and \(Wnk1^{-/-}\) (n=6) littermates adult male mice (N5) as previously described$^{6}$ with a Toshiba Powervision 6000, SSA 370A device equipped with an 8-14 MHz linear transducer under isoflurane anesthesia (0.75-1.0% in oxygen) with spontaneous ventilation. The body temperature was maintained with a heating pad. Data were transferred on-line to a computer for off-line analysis (Ultrasound Image Workstation-300A, Toshiba). The left ventricle (LV) was imaged in parasternal long axis view to obtain measurements of the left atrium (LA) and LV (LV end diastolic diameter: LVEDD, shortening fraction: SF, ejection fraction: EF) in time-motion mode. Pulse-wave (PW) Doppler tissue images were obtained from the posterior wall for the measurement of maximal systolic wall velocities (Spw). The apical view was used for PW Doppler measurements of LV mitral inflow (E wave) and LV aortic outflow (ejection time: ET), time-motion color Doppler mode (E wave propagation velocity: EpV), and for tissue Doppler measurement of mitral annulus velocities (systolic wave: Sa, and diastolic wave: Ea). E/Ea was computed as an estimate of LV filling pressure$^{7}$.

**Western-blotting**

Immunoblots were performed using a rabbit anti-L-WNK1 (1:500 - Alpha Diagnostic), a mouse monoclonal anti-GAPDH (1.1000 - Chemicon) and a goat anti-ACTIN (1.1000 – Santa Cruz biotechnolgy) antibodies. The other antibodies were produced in sheep by Dundee University (Scotland): anti-NKCC1 (1-260) (2.5µg/ml), anti-NKCC1 Phospho...
Thr207+Thr212+Thr230 (2μg/ml) pre-incubated with the NKCC1 non-phospho peptide (10μg/ml), anti-SPAK (0.5μg/ml), anti-SPAK Phospho Ser373 (5μg/ml).

Protein lysates were obtained from abdominal aortas of littermates adult males (n=4 to 16 mice in each group). Aortas were carefully isolated and immediately crushed in lysis buffer containing Tris-HCl pH7.4 (50 mmol/L), NaCl (150 mmol/L), EDTA (1 mmol/L), SDS (0.1%), NP-40 (1%), proteases inhibitors cocktail tablets (cOmplete mini - Roche), phosphatases inhibitors cocktail tablets (PhoSTOP - Roche), activated sodium orthovanadate (1 mmol/L) and Dithiothreitol (DTT-1 mmol/L), followed by sonication, incubation at +4°C on a rotator and a centrifugation step at 11200g for 10mins at +4°C. Each single aorta was lysed in 50 μl of lysis buffer. Proteins were then denaturated at 95°C for 5 mins in a laemli buffer containing DTT. For western-blotting experiments (Figure 7), 25μg of proteins of each sample (n=4 Wnk1+/+ and n=4 Wnk1−/for each gel) were then loaded on a 10% polyacrylamide gel. Western-blotting were performed after migration of the gel (30 mins at 90 Volts then 1hour at 140 Volts), followed by its transfer on to a nitrocellulose membrane in 10% ethanol (2 hours at 250 mA at +4°C).

The membrane was then blocked with 5% milk in TBST-1X or TTBS-1X (2 hours at room temperature). The upper panel of the membrane was blotted overnight either with anti-L-WNK1, anti-NKCC1 or anti- Phospho NKCC1 primary antibodies. The lower parts of the membrane were blotted overnight either with anti-SPAK or anti- Phospho SPAK, and either with anti-GAPDH or anti-ACTIN primary antibodies. Secondary anti-rabbit (1:500), anti-sheep (1:1000), anti-mouse (1:1000) and anti-goat (1:1000) IgG horseradish peroxidase-linked whole antibodies (from Donkey) antibodies (GE Healthcare) were used. Detection of the proteins was performed using the Amersham/ECL plus western blotting detection system (GE Healthcare) and a luminescent Image Analyzer (LAS-4000 mini).

**Drugs and Reagents**

Pentobarbital sodium (CEVA Santé animale, France); Ketamine (Imalgene 1000, MERIAL France); Xylazine (ROMPUN 2%, BAYER, Germany); Acetylcholine chloride, L-Phenylephrine hydrochloride, (+)-Norepinephrine (+)-bitartrate salt, Angiotensin II and Nω-nitro-L-Arginine methyl ester hydrochloride (L-NAME) were purchased from SIGMA (France).

**Statistical Analysis**

All data were expressed as mean ± SEM. A student’s t-test for unpaired series was used to analyse q-PCR data, Western-blotting quantification, plasma and urinary electrolyte concentrations, vascular and cardiac echo Doppler parameters, ED50 of phenylephrine- an angiotensin II- induced BP responses, KCl contractions as well systolic, diastolic and mean arterial BP between Wnk1+/+ and Wnk1−/− mice (P≤0.05 was considered to be statistically significant). To analyse the circadian rhythms of the systolic and diastolic BP between day and night periods as well as the effect of L-NAME on Phe concentration-response curves within each group of mice, we performed a Student’s t-test for paired series (P≤0.05 was considered to be statistically different). Finally, we performed an ANOVA for repeated measures to analyse pressure-diameter curves, CSC curves, CSD curves, phenylephrine (+/-L-NAME), norepinephrine, angiotensin II and acetylcholine dose- or concentration- response curves as well as for flow-induced dilatations and myogenic response measurements (P≤0.05 was considered to be statistically significant). NS means that P>0.05 and * means that P≤0.05 between two groups or two conditions and ** means that P≤0.01 between two groups or two conditions.
Supplemental References


### Supplemental Tables and Figures

**Table S1. Plasma electrolyte concentrations and urinary electrolyte excretion**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wnk1^{+/+}</th>
<th>Wnk1^{+/-}</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Age (weeks)</td>
<td>17.1 ± 0.5</td>
<td>18.5 ± 0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>25.9 ± 0.4</td>
<td>25.5 ± 0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Food intake (g)</td>
<td>3.7 ± 0.5</td>
<td>3.5 ± 0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Water intake (g)</td>
<td>3.0 ± 0.4</td>
<td>3.3 ± 0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Urine volume (mL)</td>
<td>0.96 ± 0.11</td>
<td>1.05 ± 0.11</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**Plasma**

<table>
<thead>
<tr>
<th></th>
<th>Wnk1^{+/+}</th>
<th>Wnk1^{+/-}</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na^+ (mmol/L)</td>
<td>145.3 ± 1.8</td>
<td>148.0 ± 0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>K^+ (mmol/L)</td>
<td>3.8 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Cl^- (mmol/L)</td>
<td>109.0 ± 1.4</td>
<td>109.5 ± 0.3</td>
<td>0.7</td>
</tr>
</tbody>
</table>

**Urine**

<table>
<thead>
<tr>
<th></th>
<th>Wnk1^{+/+}</th>
<th>Wnk1^{+/-}</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na^+ (nmol/min)</td>
<td>158.6 ± 19.8</td>
<td>185.7 ± 16.4</td>
<td>0.3</td>
</tr>
<tr>
<td>K^+ (nmol/min)</td>
<td>203.4 ± 23.4</td>
<td>223.8 ± 22.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Cl^- (nmol/min)</td>
<td>189.0 ± 19.8</td>
<td>199.2 ± 14.0</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Urinary ionograms correspond to the third day of urinary collections during a 3-day metabolic cage experiment.
Table S2. Echocardiography exploration

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wnk1^{+/+}</th>
<th>Wnk1^{+/−}</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Age (days)</td>
<td>102.8 ± 3.4</td>
<td>112.7 ± 3.7</td>
<td>0.10</td>
</tr>
<tr>
<td>BW (g)</td>
<td>25.9 ± 0.2</td>
<td>25.9 ± 0.5</td>
<td>0.88</td>
</tr>
<tr>
<td>Heart rates (bpm)</td>
<td>503.9 ± 10.2</td>
<td>491.2 ± 11.1</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Cardiac remodeling

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wnk1^{+/+}</th>
<th>Wnk1^{+/−}</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LA (mm)</td>
<td>2.4 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>0.83</td>
</tr>
<tr>
<td>LV EDD/BW (mm/g)</td>
<td>0.153 ± 0.004</td>
<td>0.15 ± 0.01</td>
<td>0.62</td>
</tr>
<tr>
<td>LV mass/BW (mg/g)</td>
<td>4.0 ± 0.3</td>
<td>3.7 ± 0.3</td>
<td>0.46</td>
</tr>
</tbody>
</table>

LV systolic function

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wnk1^{+/+}</th>
<th>Wnk1^{+/−}</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>EF (%)</td>
<td>88 ± 1</td>
<td>81 ± 2 *</td>
<td>0.03</td>
</tr>
<tr>
<td>Vcfc (circ/s)</td>
<td>3.5 ± 0.1</td>
<td>3.1 ± 0.2</td>
<td>0.06</td>
</tr>
<tr>
<td>Sa (mm/s)</td>
<td>26.1 ± 0.2</td>
<td>28.9 ± 0.1</td>
<td>0.33</td>
</tr>
<tr>
<td>Spw (mm/s)</td>
<td>26.8 ± 0.2</td>
<td>24.6 ± 0.2</td>
<td>0.42</td>
</tr>
</tbody>
</table>

LV diastolic function

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wnk1^{+/+}</th>
<th>Wnk1^{+/−}</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IVRT (ms)</td>
<td>17.3 ± 1.2</td>
<td>17.7 ± 0.9</td>
<td>0.84</td>
</tr>
<tr>
<td>Ea (mm/s)</td>
<td>46.7 ± 0.3</td>
<td>48.2 ± 0.1</td>
<td>0.66</td>
</tr>
<tr>
<td>Epw (mm/s)</td>
<td>38.0 ± 0.1</td>
<td>33.9 ± 0.3</td>
<td>0.25</td>
</tr>
<tr>
<td>E/Ea</td>
<td>19.5 ± 0.8</td>
<td>18.4 ± 1.2</td>
<td>0.49</td>
</tr>
</tbody>
</table>

BW indicates body weight; LA: left atrium; LV EDD: left ventricle end diastolic diameter; EF: ejection fraction; Vcfc: velocity shortening of circumferencial fibers; Sa, Spw: maximal systolic velocity of the mitral annulus and posterior wall; IVRT: isovolumic relaxation time; Ea and Epw: maximal diastolic velocity of the mitral annulus and the posterior wall; E: maximal velocity of the LV inflow. * P≤0.05 versus Wnk1^{+/+}. 
S1. L-WNK1 and KS-WNK1 expression. A, L-WNK1 mRNA level of expression measured by q-PCR was significantly reduced by half in Wnk1<sup>−/−</sup> thoracic aortas and kidney tissue when compared to Wnk1<sup>+/+</sup> respective tissues (n=5 and n=6 in each group, respectively for thoracic aortas and kidneys extracts) (**P<0.01). The kidney-specific isoform (KS-WNK1) mRNA level of expression in kidney was comparable between both groups of mice (n=6 in each group) (NS, P>0.05). B, Immunoblots performed on aorta evidence a significant decrease of L-WNK1 protein expression in Wnk1<sup>−/−</sup> mice when compared to Wnk1<sup>+/+</sup> mice (*P=0.017; n=4 per group).
**Figure S2.**

### A

**S2. Basal Blood Pressure measurements by telemetry.** A. Graphs represent the daily rhythm of the systolic and diastolic BP recorded over a 24-hours period (n=7 *Wnk1*+/+ mice and n=6 *Wnk1*+/- mice). The curves pattern evidence the circadian rhythm of BP in both groups of mice throughout day and night periods. Within each group of mice, the means of the systolic and diastolic BP are significantly different between the 12-hours day period (7h to 19h) and the 12-hours night period (19h to 7h) (**P<0.01** day period versus night period). B, Graphs show no significant difference of the systolic and diastolic BP between *Wnk1*+/+ and *Wnk1*+/- mice, during both day and night periods (NS, P>0.05).
S3. Endothelial function evaluation. Graphs represent endothelium-dependent relaxations elicited ex vivo by isolated arteries. A and B, Aortic rings (mounted in a wire-myograph) and second-order mesenteric arteries (mounted in an arteriograph) from Wnk1+/+ (n=9 and n=7, respectively) and Wnk1+/- (n=9 and n=6, respectively) mice were submitted to increased concentrations of acetylcholine after being pre-constricted with phenylephrine. Graphs represent the percentage of relaxation of phenylephrine pre-contraction in function of acetylcholine concentration (in mol/L). C, Second-order mesenteric arteries from Wnk1+/+ (n=7) and Wnk1+/- (n=6) mice were mounted in an arteriograph where they were pressurized at 75 mmHg and pre-constricted with phenylephrine before being submitted to stepwise increases in intraluminal flow. Graph represents the percentage of relaxation of phenylephrine-induced pre-contraction in function of flow rates (µl/min). Both graphs evidence no statistical difference between Wnk1+/+ and Wnk1+/- mice (NS, P>0.05).
Figure S4.

S4. Norepinephrine- and Angiotensin II- mediated contractions. Norepinephrine concentration-response curves (A) were performed on thoracic aortic rings (n=8 and n=7 respectively for Wnk1+/+ and Wnk1+/− mice) while angiotensin II concentration-response curves were performed on abdominal aortic rings (B) (n=8 and n=5 respectively for Wnk1+/+ and Wnk1+/− mice) and on second-order mesenteric arteries (C) (n=6 Wnk1+/− and n=7 Wnk1+/+ mice). Ex vivo contractions induced by increases in norepinephrine concentration were significantly reduced in Wnk1+/− mice when compared to Wnk1+/+ mice (* means that P ≤ 0.05 between the two groups). Ex vivo angiotensin II-induced contractions were comparable between both groups of mice, in both types of arteries (NS, P>0.05). D. In vivo angiotensin II pressor responses were comparable between Wnk1+/+ mice (n=5) and Wnk1+/− mice (n=4) (NS, P>0.05).
Figure S5.

S5. KCl-induced contractions. A, Bars represent contractions (expressed in delta milliNewton) of thoracic aortic rings from Wnk1<sup>+/+</sup> (n=7) and Wnk1<sup>−/−</sup> (n=10) mice mounted ex vivo in a myograph in response to potassium chloride-enriched isotonic physiological solution containing a final concentration of KCl at 30 mmol/L, 60 mmol/L and 125 mmol/L. B, Bars represent contractions (expressed in delta microns) of second-order mesenteric arteries from Wnk1<sup>+/+</sup> (n=7) and Wnk1<sup>−/−</sup> (n=6) mice mounted ex vivo in an arteriograph in response to potassium chloride-enriched isotonic physiological solution containing a final concentration of KCl at 80 mmol/L. There was no statistical difference in KCl contractions between both groups of mice in both types of arteries (NS, P>0.05).
Figure S6.

**S6. Phenylephrine- and KCl- induced contractions in desendothelialized arteries.** Concentration-response curves were performed *ex vivo* in a wire-myograph on denuded thoracic aortic segments in which the endothelium was removed. A, Graphs represent contractions (in delta milliNewton) elicited in response to phenylephrine (in mol/L) in Wnk1+/+ (n=6) and Wnk1+/− (n=10) denuded aortic rings. Phenylephrine contractions were significantly reduced in Wnk1+/− mice (*P*≤0.05 versus Wnk1+/+). B, Bars represent contractions (expressed in delta milliNewton) in response to potassium chloride-enriched isotonic physiological solution containing a final concentration of KCl at 30mmol/L, 60mmol/L and 125mmol/L. KCl contractions at 60mmol/L and 125mmol/L were significantly reduced in Wnk1+/− mice (n=10) (NS, *P*>0.05 and *P*≤0.05 versus Wnk1+/+ (n=6)).
Figure S7.

S7. Basal vascular resistances. Carotid arteries (n=5 in each group) (A) as well as mesenteric arteries (n=8 Wnk1+/+ and n=7 Wnk1+/−) (B) were mounted ex vivo in an arteriograph and submitted to different levels of intraluminal pressure. We measured their passive outer diameters (in microns) for each step-increase in pressure (mmHg) in conditions where they exhibit their maximal basal level of dilatation. We also calculated from these pressure-passive diameter curves their arterial cross-sectional compliance (CSC) as well as their arterial cross-sectional distensibility (CSD) for each corresponding delta pressure. Graphs represent diameter-pressure, compliance-pressure and distensibility-pressure curves (from left to right). For each measured or calculated parameter, there was no statistical difference between both groups of mice (P>0.05 for each graph).