Notch3 Is Essential for Regulation of the Renal Vascular Tone

Nada Boulos, Frank Helle, Jean-Claude Dussaule, Sandrine Placier, Paul Milliez, Sonja Djudjaj, Dominique Guerrot, Anne Joutel, Pierre Ronco, Jean-Jacques Boffa, Christos Chatziantoniou

Abstract—The Notch3 receptor participates in the development and maturation of vessels. Mutations of Notch3 in humans are associated with defective regulation of cerebral blood flow. To investigate the role of Notch3 in the regulation of renal hemodynamics, we used mice lacking expression of the Notch3 gene (Notch3−/− mice). Bolus injections of norepinephrine and angiotensin II increased renal vascular resistance and decreased renal blood flow in a dose-dependent manner in wild-type mice. In sharp contrast, renal vascular resistance of Notch3−/− mice varied little after boluses of norepinephrine and angiotensin II. Inversely, bradykinin and prostacyclin relaxed renal vasculature in wild-type mice. Both vasodilators had a negligible effect on renal vascular resistance of Notch3−/− mice. Afferent arterioles freshly isolated from Notch3−/− mice displayed decreased thickness of vascular wall compared with wild-type mice and showed a deficient contractile response to angiotensin II. To examine the physiopathological consequences of the above-described deficiency, hypertension was induced by continuous infusion of angiotensin II. Angiotensin II gradually increased blood pressure in both strains, but this increase was lesser in the Notch3−/− mice. Despite this blunted systemic effect, Notch3−/− mice displayed high mortality rates (65%) attributed to heart failure. In the kidney, the surviving Notch3−/− mice showed focal structural alterations characteristic of nephroangiosclerosis. These data show that Notch3 is necessary for the adaptive response of the renal vasculature to vasoactive systems. A deficiency in the expression of Notch3 could have important physiopathological consequences in the adaptation of the cardiac and renal function to chronic increase of blood pressure. (Hypertension. 2011;57:1176-1182.) • Online Data Supplement

Key Words: Notch3 • renal hemodynamics • renal vascular response • angiotensin

Notch3 is a mammalian heterodimeric transmembrane receptor that, under normal conditions, is almost exclusively expressed by vascular smooth muscle cells (VSMCs)1. It belongs to the NOTCH gene family, which encodes 4 members of highly conserved receptors involved in cell fate control and particularly in vascular development in mammals. Notch3 implications in cell–cell interactions, regulation of cell growth, differentiation, and apoptosis have been clearly established.2,3 Notch3 mutations cause CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), a hereditary autosomal dominant disease characterized by progressive degeneration of smooth muscle cells of small brain vessels leading to dementia and death.4-6 A murine model carrying the CADASIL mutation displayed attenuated myogenic responses and reduced caliber of brain arteries as well as impaired cerebrovascular autoregulation and functional hyperemia.7

Little is known about the regulation of blood pressure and renal function in CADASIL patients and an eventual interaction between Notch3 function and renal disease. An association between renal arteriopathy and mesangial IgA deposits with focal segmental proliferation was reported in a patient with CADASIL, suggesting a possible renal involvement in this systemic disease.8 Recently, a case of a CADASIL patient with mild hypertension was reported. This patient displayed an important decline in his renal function and histological lesions of nephroangiosclerosis that were unusually severe for the level of the recent, mild hypertension.9

Targeted deletion of the expression of Notch3 gene (Notch3−/−) does not affect viability or general growth in mice.10 However, small brain and tail arterioles of Notch3−/− mice show age-related structural defects, affecting the shape and the cytoskeleton of smooth muscle cells. These defects are probably attributable to impaired postnatal mechanism(s) of cell differentiation and maturation.11 In contrast, large conduit arteries and even some small diameter vessels such as mesenteric arteries do not display these defects.12

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In the present study, we investigated the role of Notch3 in renal hemodynamics. We examined renal vascular responses to vasoconstrictor or vasodilator agents in Notch3/H11002/H11002 mice, and we compared these responses with those of wild-type littermates. We found that the renal vascular reactivity of the Notch3/H11002/H11002 mice is significantly impaired. This deficient response is associated with a structural deformation of the vascular wall of the afferent arterioles. In addition, Notch3/H11002/H11002 mice display a severe cardiac and renal phenotype during hypertension. We believe that the lack of expression or mutations in Notch3 can severely impair the adaptation of renal hemodynamics to the abrupt or chronic alterations of blood pressure.

Materials and Methods
This section is available as an online data supplement at http://hyper.ahajournals.org.

Results

The Notch3 Receptor Is Expressed in Renal Resistance Vessels
These data are shown in the online supplement (supplemental Figure 1).

Basal Characteristics of Systemic and Renal Hemodynamics of Notch3−/− Mice
There was no strain difference in the basal systemic and renal hemodynamic parameters (see online supplement).

The Renal Vasculature of Notch3−/− Mice Does Not Respond Properly to Norepinephrine
Bolus administration of norepinephrine induced a very rapid, transient response in wild-type animals. In the example shown in Figure 1A; a bolus of 10 ng of norepinephrine rapidly increased mean arterial pressure (MAP) by 33%; then, MAP gradually returned to baseline (Figure 1A). This increase was accompanied by a 45% transient fall of renal blood flow (RBF; Figure 1B) and a 2.5-fold increase of renal vascular resistance (RVR). Norepinephrine increased MAP similarly in Notch3−/− mice (Figure 1A), but contrary to what happened in wild-type mice, RBF increased by 35% (Figure 1B), and RVR (Figure 1C) exhibited a negligible change.

Figure 1D through 1F summarizes the maximum responses of wild-type and Notch3−/− animals to 2, 4, and 10 ng of norepinephrine (n=12 and 8 for Notch3−/− and wild-type mice, respectively). MAP increased in a similar dose-dependent manner in both strains (Figure 1D). RBF decreased in a dose–response-dependent fashion in wild-type mice but increased in Notch3−/− mice (Figure 1E).
dose dependently in wild-type mice but remained around the baseline values in Notch3−/− mice (Figure 1F).

**Notch3−/− Mice Display a Blunted Renal Vascular Response to Angiotensin II**

To check whether this abnormal vascular response was specific to norepinephrine or generalized to other vasoconstrictors, additional experiments were performed with angiotensin II (Figure 2A through 2C). In wild-type mice, angiotensin II induced a dose-dependent increase in MAP and RVR, concomitant to a dose-dependent decrease of RBF. In Notch3−/− mice, the systemic response to angiotensin II did not statistically differ at 0.5 ng (NS from baseline), and it slightly decreased at 1 or 2 ng; it remained significantly lower compared with wild-type response (Figure 2B; *P<0.01*). RVR varied little with the 3 doses (Figure 2C; *P>0.05* versus wild type). Interestingly, for a similar systemic pressor effect between wild-type and Notch3−/− mice (0.5 for wild-type compared with 2 ng for Notch3−/−), the renal vascular response was 2-fold lower in Notch3−/− mice.

To investigate whether the abnormal vascular response to vasoconstrictors was attributable to a deficient signaling of the Notch3 pathway, wild-type animals were treated with the γ-secretase inhibitor DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-buty1 ester) and then injected with norepinephrine or angiotensin II. Cleavage of Notch3 by γ-secretase is the rate-limiting step of Notch3 activation, and administration of DAPT has been used as a pharmacological tool to block the Notch signaling pathway in vivo.13,14

The renal vascular response was similar before and after inhibition of γ-secretase (RBF decrease after 4 ng of norepinephrine: ~17.6±2.1 versus ~19.2±2.2% before and after DAPT administration, respectively; *n=5*); similarly, DAPT treatment did not change the effect of 10 ng of norepinephrine or of 1 ng of angiotensin II (data not shown).

**Notch3−/− Mice Show a Defective Transient Response During Mechanical Clipping**

To investigate whether the deficient renal vascular response can also be observed after acute mechanical increase of blood pressure, experiments were performed by mechanically clipping the abdominal aorta just below the left renal artery. RBF increased transiently after clipping and returned to baseline relatively rapidly in wild-type mice (Figure 2D). RBF also increased rapidly in Notch3−/− mice, but this increase was not transient because RBF did not return to baseline values as long as the aorta remained clipped (Figure 2D and 2E).

**Notch3−/− Mice Exhibit a Deficient Renal Vascular Response to Vasodilators**

To examine whether this deficient renal vascular response applied to vasodilators as well, we administered bradykinin, an agent stimulating the cGMP pathway, or the prostacyclin...
Afferent Arterioles Isolated From Notch3−/− Mice Do Not Contract Normally to Angiotensin II

In this study, we provide evidence indicating the importance of Notch3 in the regulation of renal hemodynamics. First, we show that Notch3 is expressed in the vascular wall of renal resistance vessels under control conditions. Second, we demonstrate that mice lacking the expression of Notch3 display compromised renal vascular reactivity. The renal vasculature of these animals did not contract properly to acute administration of norepinephrine or angiotensin II in vivo. A blunted vascular relaxation was observed with vasodilators, indicating a deficient adaptation of the renal resistance vessels in abrupt changes of systemic blood pressure. Ex vivo studies confirmed that afferent arterioles of Notch3−/− mice display deficient contractility and suggested an abnormal structure of the vascular wall as a possible cause of this deficiency. Finally, we show that this deficiency has important physiological implications because Notch3−/− mice have an altered blood pressure increase and develop a severe cardiac and renal phenotype when hypertension is induced.

VSMC plasticity is an important ingredient of the functional and structural adaptations of the vascular wall in acute or chronic changes of blood pressure. Although several studies have investigated the myogenic mechanisms of renal autoregulation, little is known regarding genetic determination of renal vascular reactivity in vivo. Notch3 has recently been reported as an important receptor controlling cerebral autoregulation.11,12 Our results clearly show that the kidney, another organ in which autoregulation is very important, is profoundly affected by the lack of Notch3. The RVR of Notch3−/− mice responded very little to acute changes of blood pressure, whether these changes elicited contraction or relaxation centrally or by peripheral vasoconstrictors.

The isolation of afferent arterioles allowed the measurement and comparison of lumen diameter and vessel wall thickness. The lumen diameter was similar in both strains (Figure 3B). However, vascular wall thickness was significantly lower in afferent arterioles of Notch3−/− mice (Figure 3C), indicating structural abnormalities in the development and maturation of the vascular wall. Examination of semithin sections confirmed abnormalities in the structure of renal vessels (supplemental Figure III). Contrary to the strain difference seen with the renal afferent arterioles, skin arterioles from Notch3−/− mice responded to angiotensin II in a similar way compared with wild-type mice (supplemental Figure IV).

Notch3−/− Mice Show Increased Mortality After Chronic Infusion of Angiotensin II

Next, we examined whether the above-observed abnormalities of vascular reactivity can affect the adaptation of mice to chronic increase of blood pressure. To this end, angiotensin II was infused in Notch3−/− and wild-type mice (n=16 per strain) for 28 days. In both strains, angiotensin II induced a gradual increase of systolic blood pressure. Interestingly, this increase was lesser in the Notch3−/− mice (Figure 3A). Despite this blunted systemic effect, 10 of 16 Notch3−/− mice developed peripheral edema and died between days 5 and 15 of chronic angiotensin II infusion (Figure 4B). Autopsy excluded encephalic hemorrhage and end-stage renal disease as causes of this relatively rapid mortality. Measurements of cardiac parameters showed cardiac hypertrophy and increased left ventricular diastolic diameter in Notch3−/− mice compared with wild-type mice (Figure 4C). At the end of the protocol (28 days), the kidneys of the surviving Notch3−/− mice showed important focal structural alterations (Figure 4D) contrasting with the quasi-normal aspect of wild-type kidneys (glomerular damage index: 1.2±0.2 versus 0.2±0.2; P<0.01).

Discussion

In this study, we provide evidence indicating the importance of Notch3 in the regulation of renal hemodynamics. First, we show that Notch3 is expressed in the vascular wall of renal resistance vessels under control conditions. Second, we demonstrate that mice lacking the expression of Notch3 display compromised renal vascular reactivity. The renal vasculature of these animals did not contract properly to acute administration of norepinephrine or angiotensin II in vivo. A blunted vascular relaxation was observed with vasodilators, indicating a deficient adaptation of the renal resistance vessels in abrupt changes of systemic blood pressure. Ex vivo studies confirmed that afferent arterioles of Notch3−/− mice display deficient contractility and suggested an abnormal structure of the vascular wall as a possible cause of this deficiency. Finally, we show that this deficiency has important physiological implications because Notch3−/− mice have an altered blood pressure increase and develop a severe cardiac and renal phenotype when hypertension is induced.

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relaxation. The experiments with mechanical clipping indicate that this defect is because of a deficient myogenic response and is not dependent exclusively on hormone responses. A similar deficient response was also observed ex vivo in the caudal artery but not in skin microvessels, mesenteric arteries of third order, nor in large conduit vessels. These data indicate that Notch3 is involved in the regulation of contractility in specific vascular beds, apparently in those in which autoregulation is a major function. It will be interesting to investigate the relative abundance of expression or signaling activation of Notch3 during the development of the brain and renal vasculature.

Differences appeared between norepinephrine and angiotensin II responses. Norepinephrine induced a similar blood pressure increase in the 2 strains, and the renal vasculature of Notch3−/− mice responded passively by increasing RBF. In contrast, there was a strain difference in systemic pressure response to angiotensin II, which was blunted in Notch3−/− mice. Despite this blunted systemic response, there was a greater renal vasoconstrictor response to angiotensin II compared with norepinephrine in Notch3−/− mice. One hypothesis is that constriction of efferent arterioles in vivo could be involved in the angiotensin II response independently of Notch3 expression. Another possibility is that the effect of angiotensin II to increase tubuloglomerular feedback sensitivity could cause decreased RBF in vivo. Alternatively, it is possible that these vasoconstrictor systems affect differentially renal and total resistance depending on the signaling pathway(s) affected, as has been previously shown for the renal vasculature of the rat.

Defects in VSMC contractility in resistance arteries could affect systemic blood pressure and the function or structure of organs in which autoregulation is a major mechanism. We, as others before us, did not observe a significant pathological cardiovascular or renal phenotype of Notch3−/− mice under control conditions. Thus, either other factors compensate throughout the development or Notch3 is not necessary for the maintenance of a normal phenotype in nonstressed animals. The importance of Notch3 was unmasked when hypertension was induced in Notch3−/− mice. First, we observed that the angiotensin II–induced pressor response continued to be blunted even in a chronic experimental setting. Despite this blunted systemic effect, 65% of Notch3−/− mice died before the second week of chronic angiotensin II infusion (Figure 4B). This period of time is relatively short to consider cardiac fibrosis as the cause of the early mortality, and autopsy excluded encephalic hemorrhage or end-stage renal disease as alternative causes. In addition, mortality was preceded by the development of peripheral edema, and Notch3−/− mice hearts were dilated despite lower blood pressure increase (Figure 4A and 4C). These data suggest congestive heart failure as the most probable cause of the rapid mortality of Notch3−/− mice. Future studies are necessary to identify the underlying cellular mechanism(s) by

![Figure 4. Systolic blood pressure increase (A), survival rate (B), heart/body weight, and left ventricle diameter (C) after angiotensin II infusion for 28 days in wild-type and Notch3−/− mice. Values are means ± SE; n = 16; *P < 0.05 vs wild-type mice. D, Representative examples of renal cortical morphology in wild-type or Notch3−/− mice surviving after 28 days of angiotensin II infusion. Note the important structural alterations (dilated glomerular capillaries, renal fibrosis, tubular dilation) in the renal cortex of Notch3−/− mice. WT indicates wild type; KO, knockout; Ang II, angiotensin II; CT, control.](http://hyper.ahajournals.org/doi/10.1161/HYPERTENSIONAHA.110.189040)
which lack of Notch3 produces heart failure. Notch3−/− mice survivors developed lesions characteristic of hypertensive chronic kidney disease (Figure 4D), despite the fact that their background is C57/B6j, a background known to be resistant to the development of renal fibrosis in hypertension-induced renal disease.

An important question arising from our results is the mechanisms whereby Notch3 deletion affects the renal vascular response. Because Notch3 is particularly involved in the maturation and differentiation of VSMCs, the deficient response may be attributable to a structural defect occurred during the development of the vascular wall of resistance vessels. In this regard, it was previously reported that although classical histological examination and staining with smooth muscle cell–specific markers did not show a difference between Notch3−/− and wild-type vessels, high-resolution microscopy and ultrastructural analysis revealed that VSMCs of Notch3−/− mice had important alterations in size and shape.11 Notch3−/− VSMCs were thinner, and during adulthood, this defective size resulted in thinner vascular wall and a less festooned elastica lamina in cerebral microvessels. Subsequent studies showed that although the expression of several proteins participating in the regulation of myogenic tone was similar in the Notch3−/− mice and wild-type mice, the myosin light chain phosphorylation was significantly blunted in response to mechanic stress in the Notch3−/− mice. This defect was attributed to a deficient activation of the RhoA/Rho kinase pathway.12 Other investigations raised the possibility that deletion of Notch3 gene is associated with a decreased expression of several genes controlling the contractile machinery. Transcriptomic analysis indicated decreased expressions of Cacna1g and Cacna2d2, genes encoding for the alpha 1G subunit and the alpha-2-delta-2 mutant subunit 1 of the voltage-operated T-type calcium channel, respectively.16 Again, these abnormalities were observed in the small cerebral vessels, whereas VSMCs from large conduit vessels of Notch3−/− were similar to normal.

Our results with the injections of vasoactive agents are more consistent with the structural abnormality hypothesis. First, the thickness of the vascular wall of afferent arterioles was significantly thinner in Notch3−/− (Figure 3C). As was the case with the cerebral vessels, this difference was unnoticed with a classical histological approach, whereas it became detectable when arterioles were isolated from the rest of the kidney. In addition, semithin sections revealed structural irregularities in the vascular wall of renal microvessels similar to the abnormalities observed previously in brain vessels.11 Our results with the γ-secretase, which did not alter the renal vascular reactivity to angiotensin II or norepinephrine in wild-type mice, do not support the de novo signaling hypothesis. Further, the renal vascular response to boluses of vasoactive agents is a transient phenomenon occurring within seconds, whereas activation of Notch3 pathway requires far more time.

Notch3 signaling can regulate the plasticity and the adaptation of VSMCs during pulmonary hypertension in humans.13 Expression of Notch3 in small pulmonary artery VSMCs was correlated to the severity of the disease. Prolonged hypoxic conditions activated the Notch3–Hex5 signaling pathway, which, in turn, induced proliferation of pulmonary VSMCs in mice. In contrast, pulmonary vessels of Notch3−/− mice did not change phenotype in response to hypoxia, and thus, Notch3−/− mice were protected against the development of pulmonary hypertension. A partial protection was also observed when wild-type mice were treated with the γ-secretase inhibitor DAPT. Our results with angiotensin II–induced hypertension show that the disease phenotype was attributable to a deficient myogenic response (blunted increase of blood pressure, damaged glomeruli). At least a part of this deficient vascular reactivity can be attributed to the structural abnormalities of the Notch3−/− resistance vessels. This finding does not exclude a de novo activation of Notch3 signaling to promote vascular remodeling during chronic pressure increase. According to this hypothesis, lack of Notch3 signaling impairs the ability of vessels to adapt against pressure load, which, in turn, leads to organ failure. It is possible that Notch3 plays a dual role: the vessel response to acute changes of blood pressure depends on the Notch3–induced regulation of contractile genes during development, whereas the adaptation of vessels to chronic changes of blood pressure can also depend on a de novo activation of Notch3 signaling. Development of a blocker specific for the Notch3 pathway or the creation of time- and tissue-conditional transgenic mice will provide valuable tools to test these hypotheses.

RBF autoregulation is an important physiological mechanism in renal adaptation to blood pressure variations.17 Benign nephroangiiosclerosis is the major renal disease related to chronic hypertension. Its precise pathophysiology is incompletely understood, but transmission of systemic hypertension to renal vessels and glomerular capillaries is believed to initiate the progression of this pathological process. To date, the only available data linking Notch3 to vascular dysfunction in humans comes from CADASIL patients. The vascular phenotype of these patients is characterized by nonspecific neointimal fibrosis and hyalinosis of the arterial wall reducing vascular lumen. Progressive VSMC degeneration and loss of normal anchorage to extracellular matrix and adjacent cells were found in small arteries in CADASIL patients.4,18 In addition, in vivo alterations in skin microvascular reactivity were also detected in CADASIL patients.19 Recently, a CADASIL patient was reported to have renal failure, glomerular enlargement, and severe fibrous endarteritis in the absence of long-term systemic hypertension or other cardiovascular risk factors. The isolated nephroangiiosclerosis lesions and the presence of granular osmiophilic material suggested a specific Notch3 mutation–associated renal disease through autoregulation impairment.5 CADASIL patients have mis-sense mutations and do not lack completely the expression of Notch3. Thus, our findings cannot be extrapolated to CADASIL patients. However, our data clearly show that Notch3 deletion impairs renal vasoreactivity and induces a severe pathological phenotype during hypertension. It will be interesting to investigate a possible pathophysiological relationship between Notch3 deletion (or malfunction) and the development of heart or renal failure in selected patient cohorts.
Clinical Perspectives
In this study, we provide evidence that Notch3 is a very important regulator of the renal myogenic response and of the renal vascular tone. Genetic deletion of the Notch3 receptor profoundly alters the acute renal vascular response to vasoactive agents. This deficiency also has important implications under chronic increase of blood pressure because Notch3−/− mice show a severe cardiac and renal phenotype. It is important to perform future clinical investigations to understand how alterations in the expression or function of Notch3 contribute to the development of cardiac and renal complications during hypertension.

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Disclosures
None.

References
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MATERIALS AND METHODS

Animal Handling and Care

All animals were handled in strict accordance with good animal practice as defined by the relevant national animal welfare bodies of France, and all animal work was approved by the appropriate committee of Inserm and the University Pierre et Marie Curie, Paris. These rules are in accordance to the NIH guidelines for the care and use of laboratory animals.

Animal Model

Experiments were performed on mice lacking expression of Notch3 gene (Notch3 -/-) on a C57Bl6/J background. The generation of Notch3-/- mice is described elsewhere (1). In all experiments aged-matched wild type (WT) littermates were used as controls. Animals were bred in the local animal facility with free access to water and food.

Renal Hemodynamics

Experiments were performed on male 5-6 month-old Notch3-/- and WT mice weighing 29 ± 1 and 31 ± 1 g, respectively. Animal surgery and renal blood flow (RBF) measurements were performed according to previously established methodology (2, 3). Briefly, after anesthesia by pentobarbital sodium (50–60 mg/kg body weight ip, Nembutal, Abbott, Chicago, IL), animals were placed on a servo-controlled table kept at 37°C and the trachea was cannulated to facilitate respiration. The left femoral artery was catheterized for measurement of arterial pressure, and a femoral venous catheter was used for infusion of volume replacement. Bovine serum albumin (4.75 g/dl of saline solution) was infused initially at 50 µl/min to replace surgical losses, and then at 10 µl/min for maintenance.

Arterial pressure was measured via a pressure transducer (Statham P23 DB); RBF was measured by a flowmeter (0.5v probe, Transonic systems TS420, Ithaca, NY). RBF values were controlled for zero offset determined at the end of an experiment after cardiac arrest. Data were recorded, stored and analyzed using DataTranslation analog-to-digital converter and the IOX software (EMKA Technologies, Paris France). RBF was measured during intravenous bolus injections of angiotensin II (0.5, 1 and 2 ng), norepinephrine (2, 4 and 10 ng), bradykinin (50 and 100 ng) and iloprost, a stable analogue of prostacyclin (50 and 100 ng). In separate experiments, RBF was measured before and during mechanically clipping of the abdominal aorta just below the left renal artery. Experiments were performed in a total of 70 Notch3-/- and 63 WT mice. The exact number of animals per experiment per group is given in the corresponding part of results.

Angiotensin II-induced hypertension

Angiotensin II (Sigma Chemical, MO, USA) was infused subcutaneously (1 µg/kg/min) using osmotic mini-pumps (Model 1004, Alzet, CA, EU) for 4 weeks. In previous studies (4) we have established that this infusion rate of angiotensin II was gradually increasing blood pressure from day 3.

Measurement of Systolic Arterial Pressure

Systolic arterial pressure (mmHg) was measured in angiotensin II treated animals with a tail-cuff sphygmomanometer adapted to the mouse, using the automated system CODA 6 (Kent Scientific) following the instructions of the manufacturer. To avoid variations in blood pressure due to day cycle, all measurements were carried out between 9 and 11A.M. Animals were accustomed for several days before measurements. Only animals that did not display signals of stress and that showed stable and reproducible values of blood pressure for at least
three consecutive days were considered for blood pressure measurements. Ten measurements from each mouse were taken at two minutes intervals then a mean value was determined.

**Morphologic evaluation**

Sections of kidneys were examined on a blinded basis for the level of glomerular ischemia, glomerular sclerosis and peri-glomerular and peri-vascular infiltration using a 0-4+ injury scale as previously described (4). At least 200 glomeruli were scored to estimate the sclerotic index of an animal.

**Echocardiography**

Transthoracic echocardiography was performed on anesthetized angiotensin II treated mice using an echocardiograph (General Electric Vivid 7, Paris France) equipped with a 8-14 MHz linear transducer. This allows the non-invasive measure of cardiac dimensions (end-systolic and end-diastolic wall thicknesses, end-systolic and end-diastolic LV diameters) and functional parameters (LV fractional shortening, percent of wall shortening). LV outflow velocity is measured by pulsed wave Doppler, which allows calculating left ventricular end-diastolic pressure (LVEDP), stroke volume and cardiac output, as previously described (5).

**Ex vivo Contractility of Afferent Arterioles**

Afferent arterioles were isolated using the agarose-infusion method originally described by Loutzenhiser (6) and later on adapted and modified in our laboratory (7). Bright-field images were acquired using a 20x objective on an Olympus DP20 camera (1600x1200 pixels) at baseline and every 30 seconds for 6 minutes after addition of the agonist. The inner outline of the vessel-wall was traced using Olympus DP-Soft 5.0 software. For contractility studies, one or two arterioles from each animal were used in each experiment, 16 and 10 arterioles were used in each experimental group. For measurements of afferent arteriole lumen and vessel wall thickness, between 3 and 4 arterioles were used from each animal, for a total of 30 and 25 arterioles for Notch3-/- and wild type mice, respectively.

**Ex vivo Contractility of Pre-capillary Abdomen Skin Arterioles**

Tissue from abdomen skin was used to isolate agar-filled pre-capillary arterioles suitable for diameter measurements. The protocol used for isolating these vessels was the same as for renal vessels described above. Arterioles were selected using 3 criteria: presence of capillaries indicating 1st order branch, agar-filled portions with easily identifiable vessel-wall lumen outline, and lumen diameter between 5 and 15 µm. Eight and 7 arterioles were isolated from Notch3-/- and WT mice, respectively.

**Immunohistochemistry**

Immunohistochemistry for Notch3 was performed by staining acetone-fixed cryosections of kidney tissue or on 3 µm paraffin sections of methyl-Carnoy’s-fixed kidneys (in the latter case, tissue was deparaffinized with xylene and graded ethanol baths). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 minutes, and blocked with phosphate-buffered saline with 1% bovine serum albumin for 20 min. The tissue was then incubated with anti-Notch3 (R&D systems) or anti-Notch3 M20 (Santa Cruz). Secondary antibody was peroxidase-linked and applied by Histofine. Antibody binding was revealed with AEC substrate (Diagnostic Biosystems), followed by hematoxylin counterstaining (Vector Laboratories). In additional experiments, semi-thin sections of the renal cortex coloured with toluidine blue were examined for microvessel structure according to previously described protocol (8).
**Total RNA extraction and quantitative Real time PCR**

Total RNA was extracted from renal cortex using TRIzol reagent (Invitrogen) and methyl trichloride according to the manufacturer’s instructions. RNA quality was checked by control of optical density at 260 and 280 nm, and by electrophoresis. Contaminating genomic DNA was removed by RNase-free DNase (Quiagen) for 15 min at room temperature. cDNA was synthesized from 1µg of purified RNA using oligodt and superscript II RT (Quiagen) for 1h30 at 37°C and 10 min at 70°C. Real-time PCR amplification was performed with ABI PRISM 5700 Sequence Detection System using SYBR Green PCR Master Mix (Quiagen) as described previously (9). Primers are listed in the Table S1. All samples were assayed in triplicate, and the average value of the triplicate was used for quantification. Final results are expressed as the ratio of a given gene /gene reference (GAPDH and/or beta actin) cDNA.

**Statistical Analysis**

Statistical analyses for the in vivo studies were performed using one-way ANOVA followed by Fisher's protected least significance difference test in the Statview software package. Diameter values, vessel wall thickness and media/lumen ratio were compared using one-way ANOVA with a Student-Newman-Keuls post hoc test using SigmaStat 3.1 software. Results with $P < 0.05$ were considered statistically significant. All values are means ± SE.
RESULTS

Basal characteristics of systemic and renal hemodynamics of Notch3-/- mice

As previously reported, Notch3-/- mice do not show an obvious developmental defect, at least up to the age of 6 months (1). At this age, body and kidney weights of Notch3-/- mice are similar to wild type controls (29 ± 1 vs 31 ± 1 g, and 212 ± 6 vs 214 ± 9 mg, for body and kidney weight, respectively). In addition, baseline hemodynamic parameters were similar between the two strains (mean arterial pressure: 77 ± 2 vs 78 ± 2 mm Hg; renal blood flow: 1.5 ± 0.1 vs 1.4 ± 0.1 ml/min; renal vascular resistance: 54 ± 3 vs 56 ± 3 mmHg.min/ml).

The Notch3 receptor is expressed in renal resistance vessels

Afferent arterioles freshly isolated from control, wild type animals expressed significant levels of Notch3 mRNA (0.096 ± 0.009 relative ratio vs GAPDH and beta-actin), whereas vessels from Notch3-/- mice were devoid of Notch3 expression. To verify that these vessels were indeed afferent arterioles, we checked whether renin is also expressed. Subsequent immunohistochemistry studies, using two different anti-bodies and fixation protocols, indicated that Notch3 is localized in renal resistance vessels (Fig S1). Additional experiments showed that several members of the Notch3 superfamily such as the ligands Jagged 1 and 2 and the effectors Hes1 and Hey2 are also significantly expressed in the renal cortex of wild type animals under control conditions (data not shown).

Notch3-/- mice show a deficient renal vascular response to vasodilators

Bradykinin administration decreased MAP similarly in the two strains (Fig S2-A, n=7 and 6 for Notch3-/- and wild type mice, respectively). RBF increased in a dose-dependent manner in the wild type animals, whereas it changed little (5% increase) in Notch3-/- mice (Fig S3-B, p<0.01 vs wild type).

Similar observations were obtained when injecting iloprost. The effect on MAP didn’t differ statistically between the two strains (Fig S2-C, n=8 and 7 for Notch3-/- and wild type mice, respectively). RBF increased significantly in wild type mice, but changed very little and remained closed to the baseline in the Notch3-/- mice (Fig S2-D, ns compared to baseline in Notch3-/- mice, p<0.01 vs wild type).

Renal resistance vessels of Notch3-/- mice display structural abnormalities

Toluidine blue staining of semithin sections show that the vascular wall of renal microvessels of Notch3-/- exhibited structural abnormalities such as presence of vacuoles, heterogenous thickness and a less festooned elastica lamina (Fig S3). These abnormalities were similar to those previously observed in brain microvessels (8).

Pre-capillary arterioles isolated from the skin of Notch3-/- mice contract normally to Ang II

To test whether the deficient contractility to Ang II was a generalized defect applying in other resistance vessels as well, pre-capillary arterioles were isolated from the abdomen skin using the agarose-perfusion method (Fig. S4-A, arrows). Baseline diameters were similar between the two strains (around 10 μm). Angiotensin II induced a dose-dependent contraction in skin microvessels reaching 35-40% of baseline at the concentration of 10^-7 M in the wild type animals (Fig S4 B-C). Contrary to the strain-difference seen with the renal afferent
arterioles, skin arterioles from Notch3-/- mice responded to angiotensin II in a similar way compared to WT (Fig S4 B-C).
SUPPLEMENTAL REFERENCES


Table S1. List of the primers used for the Real Time-PCR of the different genes as mentioned in the results section.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
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<tbody>
<tr>
<td>Notch3 sens</td>
<td>AGCTGGGTCTCCTGAGGTGAT</td>
</tr>
<tr>
<td>Notch3 antisens</td>
<td>AGACAGAGCCGGTTGCAAT</td>
</tr>
<tr>
<td>Hes1 sens</td>
<td>ACACCGGACAACAAACAAAGAC</td>
</tr>
<tr>
<td>Hes1 antisens</td>
<td>CGCCTCTTCTCCATGATAGG</td>
</tr>
<tr>
<td>Hey2 sens</td>
<td>GTGGGGGAGCGAGAAACAATTA</td>
</tr>
<tr>
<td>Hey2 antisens</td>
<td>GTTGTCGGTGAATTGGACCT</td>
</tr>
<tr>
<td>Jag1 sens</td>
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</tr>
<tr>
<td>Jag1 antisens</td>
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<tr>
<td>Renin sens</td>
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<tr>
<td>Renin antisens</td>
<td>GGGGCAGCTCGGTGACCTCT</td>
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<tr>
<td>AT1a sens</td>
<td>ACTCACAGCAACCCCTCCAAAG</td>
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<td>beta-actin sens</td>
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<tr>
<td>beta-actin antisens</td>
<td>ACGGATGTCCAACGTCACACT</td>
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Figure S1: Representative example of Notch3 expression in the renal cortex of wild type mice (A: formalin fixation; B: frozen sections; C: negative control; AA: afferent arteriole). Note that Notch3 is mainly expressed in renal vessels. Bar = 20 mm.
Figure S2: Maximum changes in arterial pressure (A & C), and renal blood flow (B & D) by 50 and 100 ng of bradykinin (A & B) or iloprost (C & D), in Notch3-/- and wild type littermates. Values are means ± SEM for 8 Notch3-/- and 7 wild type mice, respectively. ** P<0.01 vs WT.
Figure S3: Semithin sections colored with toluidine blue of renal cortex from WT (A) and Notch3-/- (B & C) mice. Note that the vascular wall of renal microvessels of Notch3-/- exhibited structural abnormalities such as presence of vacuoles (arrows), heterogeneous thickness and a less festooned elastica lamina (arrowheads). These abnormalities were not uniformly distributed as some microvessel sections appeared to have normal structure (vessel on the left corner of B).
Figure S4. Preparation of freshly isolated arterioles from abdomen skin of mice. Only agar-filled portions with discernible vessel wall (arrow heads) were used for diameter measurements (A). Angiotensin II-induced dose-dependent vasocontraction expressed as absolute (B) or % of baseline (C) changes of the diameter of skin arterioles of Notch3-/- and wild type littermates. Values are means ± SEM of 8 and 7 afferent arterioles per group, respectively.