TRPV4 Channels Contribute to Renal Myogenic Autoregulation in Neonatal Pigs

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Abstract

Myogenic response, a phenomenon in which resistance size arteries and arterioles swiftly constrict or dilate in response to an acute elevation or reduction, respectively in intravascular pressure is a key component of renal autoregulation mechanisms. Although it is well-established that the renal system is functionally immature in neonates, mechanisms that regulate neonatal renal blood flow (RBF) remain poorly understood. In this study, we investigated the hypothesis that members of the transient receptor potential vanilloid (TRPV) channels are molecular components of renal myogenic constriction in newborns. We show that unlike TRPV1-3, TRPV4 channels are predominantly expressed in neonatal pig preglomerular vascular smooth muscle cells (SMCs). Intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) elevation induced by osmotic cell swelling was attenuated by TRPV4, L-type Ca\(^{2+}\), and stretch-activated Ca\(^{2+}\) channel blockers, but not phospholipase A\(_2\) inhibitor. Blockade of TRPV4 channels reversed steady-state myogenic tone and inhibited pressure-induced membrane depolarization, [Ca\(^{2+}\)]\(_i\) elevation, and constriction in distal interlobular arteries. A step increase in arterial pressure induced efficient autoregulation of renal cortical perfusion and total RBF in anesthetized and mechanically ventilated neonatal pigs. Moreover, intrarenal arterial infusion of TRPV4 channel blockers HC 067047 and RN 1734 attenuated renal autoregulation in the pigs. These data suggest that renal myogenic autoregulation is functional in neonates. Our findings also indicate that TRPV4 channels are mechanosensors in neonatal pig preglomerular vascular SMCs, and contribute to renal myogenic autoregulation.
Healthy kidneys maintain constant renal blood flow (RBF) and glomerular filtration rate (GFR) despite physiological fluctuations in arterial pressure (10, 11, 40). This phenomenon known as the “renal autoregulation” serves to preserve renal function and protect the kidneys from glomerular injury (10, 11, 40). Two primary mechanisms have been demonstrated to mediate renal autoregulation: 1) the myogenic response and 2) tubuloglomerular feedback (TGF) mechanism. The renal myogenic response is based on the intrinsic ability of afferent arterioles to swiftly constrict or dilate in response to an acute elevation or reduction, respectively in renal perfusion pressure (10, 11, 40). Interlobular arteries have also been shown to contribute to renal myogenic autoregulation (27-29). TGF mechanism involves signal transduction events in which tubular flow rate-dependent changes in luminal sodium chloride concentration detected at the macula densa, adjust the GFR by altering preglomerular vascular tone (10, 11, 40).

Renal autoregulation mechanisms have largely been described in adults. However, the morphological and functional properties of the renal system differ between maturational stages (31, 65). The autoregulation range in healthy adults is between ~ 80 and 180 mmHg (11). The mean arterial pressure of term newborns is less than the lower limit of adult autoregulation range (44). Thus, renal autoregulation may occur at a lower perfusion pressure in neonates. Angiotensin II-induced increase in perfusion pressure during selective inhibition of its renal vascular effects elicited RBF autoregulation in ~ 4-5-week-old puppies (33). However, Buckley and colleagues suggested that renal autoregulation is insignificant at birth (8). This proposal was based on data showing that aortic compression decreased renal vascular resistance in 1-2-week- and 1-2-month-old pigs, but not in 1-4-day-old pigs (8). Moreover, step increases in kidney perfusion did not induce consistent autoregulation in piglets of all age groups (8). Hence, further studies are required to characterize neonatal renal myogenic autoregulation.

In myogenic arteries/arterioles, the mechanical stretch on the vascular wall exerted by elevated intravascular pressure induces smooth muscle cell (SMC) depolarization that activates voltage-dependent Ca^{2+} channels (VDCCs) (15, 52). VDCC activation results in extracellular Ca^{2+} influx, an elevation in intracellular Ca^{2+} ([Ca^{2+}]_{i}) concentration, and vasoconstriction (15, 52). The mechanism by which an elevation in intravascular pressure stimulates vascular SMC membrane depolarization involves activation of membrane-
resident mechanosensitive ion channels (15, 52). Proposed mechanosensitive ion channels in vascular SMCs include members of the transient receptor potential (TRP) channels (17). TRPC6, TRPM4, TRPP2, and TRPV2 channels have been shown to participate in cerebral or retinal artery/arteriole myogenic constriction (19, 41, 43, 70). However, the physiological roles of TRP channels in renal myogenic response remain unknown.

The TRPV subfamily of TRP channels is formed by 6 mammalian members (TRPV1-6). Unlike TRPV5 and TRPV6, TRPV1-4 channels are expressed in adult rat intralobar pulmonary arteries and aorta (74). Furthermore, TRPV1, TRPV4, TRPV5, and TRPV6 play unique functional roles in the kidneys. TRPV1 channels are expressed in renal tubules, pelvis, and nerves (34, 72). Activation of TRPV1 increased afferent renal nerve activity, GFR, and Na\(^+\) and water excretion in adult rats (37, 72, 76). Osmo- and mechanosensitive TRPV4 channels are expressed in adult mouse and rat nephrons, but restricted to ascending thin limb, thick ascending limb and distal convoluted and connecting tubules (14, 64). Both TRPV5 and TRPV6 are localized in adult mouse distal convoluted and connecting tubules and are involved in epithelial Ca\(^{2+}\) transport (30, 46, 67). Of note, TRPV channel expression and function in neuronal, inner ear, intestinal, renal, and endothelial cells are age-dependent (16, 36, 62, 66, 69).

Pig and human renal systems have similar anatomical features characterized by multipyramidal kidneys in contrast to unipyramidal kidneys of the mouse, rat, rabbit, dog, and sheep (22, 50). Pig and human also share similar renal maturational process (23, 45, 63). Hence, the newborn pig is an excellent large animal model of human neonatal renal physiology and pathophysiology. Here, we used pigs to investigate neonatal renal autoregulation. We tested the hypothesis that members of TRPV channels contribute to renal myogenic autoregulation mechanism in neonates.
Methods

Animals

All animal experimental procedures were approved and performed in accordance with the guidelines and regulations of the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center (UTHSC). Full-term male neonatal pigs (4-7 days old; Nichols Hog Farm, Olive Branch, MS) were used in this study.

Tissue preparation

Neonatal pigs were euthanized by intramuscular injection of ketamine/xylazine (100/10 mg/kg; i.m.) followed by exsanguination. The kidneys were immediately removed, decapsulated, hemisected, and placed in ice-cold (4°C) modified Krebs’ solution (MKS; 134 mM NaCl, 6 mM KCl, 2.0 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4). The kidneys were dissected to isolate interlobar, arcuate, and interlobular arteries and afferent arterioles using a Zeiss SteREO Discovery.V12 stereomicroscope (Carl Zeiss, Thornwood, NY).

Isolation of renal vascular smooth muscle and endothelial cells

Smooth muscle cells (SMCs) were isolated from renal arteries using a HEPES-buffered isolation solution containing (in mM) 134 NaCl, 6 KCl, 1 MgCl$_2$, 10 HEPES, and 10 glucose (pH 7.4). The arteries were incubated in isolation solution containing 1 mg/ml papain, 1 mg/ml dithioerythritol, and 1 mg/ml BSA for 16 min at 37 °C. Arteries were then incubated in isolation solution containing 0.5 mg/ml liberase blendzyme 1, 1 mg/ml BSA, and 100 nM CaCl$_2$ for 8 min at 37 °C. Digested vessels were subsequently washed in isolation solution and triturated using fire-polished glass Pasteur pipettes to yield single SMCs.

Renal vascular endothelial cells (ECs) were isolated using the Dynabeads CD31 EC isolation kit (Life Technologies, Grand Island, NY). To obtain a high yield of cells, ECs were isolated from interlobar arteries by gentle rubbing of the vessel luminal surface with small-tip cotton swabs. The detached endothelial linings were then incubated in CD31-coated magnetic beads for ~ 20 min with gentle rotation. A DynaMag magnet (Life
Technologies) was used to separate EC-bound Dynabeads. The beads were then washed 5 times in PBS containing 0.1% BSA. ECs were eluted from the magnetic beads using an RNA lysis buffer.

Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR)
For RT-PCR, total RNA was purified from intact arteries and SMCs using the RNAqueous-Micro Total RNA Isolation Kit (Life Technologies). For qRT-PCR, total RNA was purified from renal vascular SMCs and ECs using the Single Cell Real-Time RT-PCR Assay Kit (Signosis, Inc, Santa Clara, CA). cDNAs were synthesized from the arterial and SMC RNA samples using a cDNA Synthesis Kit (SuperScript VILO; Life Technologies). cDNAs were amplified by PCR using oligonucleotide primer pairs (Table 1). PCR reactions were performed in an Eppendorf Mastercycler (Eppendorf, Westbury, NY) with the following reaction conditions: an initial denaturation at 98°C for 2 min, followed by 40 cycles (denaturation at 98°C for 10 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s), with a final extension at 72°C for 10 min. Subsequently, PCR products were cleaned using the AxyPrep Mag PCR Clean-up Kit (Corning Life Sciences, Corning, NY) and resolved on a 2% agarose gel stained with SYBR Safe DNA gel stain (Life Technologies).

Renal vascular SMC and EC RNA samples were analyzed by qRT-PCR using specific primer pairs (Table 1) and an Applied Biosystems SYBR Green Master Mix kit (Life Technologies, Grand Island, NY). Reactions were performed in triplicate in an Applied Biosystems StepOnePlus Real-Time PCR System (Life Technologies). 18S ribosomal RNA was used as a housekeeping gene control.

Western Immunoblotting
Tissues were homogenized in ice-cold RIPA buffer using a Bead Mill Homogenizer (Omni International; Kennesaw, GA). Protein concentrations were determined using a Bio-Rad protein assay kit and SmartSpec 3000 Spectrophotometer (Bio-Rad, Hercules, CA). Protein lysates were then mixed with LDS sample buffer containing a reducing agent (Life Technologies) and boiled at 70 °C for 10 min. Proteins were separated by 4-20% ExpressPlus PAGE Gel (GenScript Corporation, Piscataway, NJ) using a Mini Trans Blot Cell (Bio-Rad) and transferred onto nitrocellulose membranes using a Pierce Fast Semi-Dry Blotter (Thermo Scientific). Nonspecific immunoreactive sites on the membranes were blocked with NAP blocking buffer (G-
Biosciences, St. Louis, MO) for ~1 h at room temperature. The membranes were then incubated with primary antibodies overnight at 4 °C. After several washes in Tris-buffered saline supplemented with 0.05% Tween 20 (TBS-T), the membranes were incubated in horseradish peroxidase-conjugated secondary antibodies for 45 min at room temperature and washed in TBS-T. Immunoreactive proteins were visualized using a chemiluminescent kit (G-Biosciences). Protein band intensities were analyzed by digital densitometry using Quantity One software (Bio-Rad).

Immunofluorescence

Renal vascular SMCs attached to collagen-coated coverslips were fixed in 4% formaldehyde for ~ 20 min and permeabilized with 0.2% Triton X-100 for ~ 15 min at room temperature. After 1 hour of incubation in PBS containing 5% BSA to block non-specific immunoreactive sites, cells were treated overnight at 4 °C with a TRPV4 antibody (1:50, each). Next day, cells were washed with PBS and incubated with Alexa 555-conjugated donkey anti-rabbit (1:500, each) for 1 h at room temperature. After several washes in PBS and mount, fluorescence images were acquired using a Zeiss laser-scanning confocal microscope.

Diameter measurement in pressurized microvessels

Pressure-induced changes in interlobular artery luminal diameter were examined using the pressure myograph systems (Danish Myo Technology (DMT), Aarhus, Denmark and Living Systems Instrumentation, St. Albans, VT). Distal interlobular arteries were cannulated with a fabricated glass at each end in temperature-controlled chambers. The chambers were slowly and continuously perfused with MKS equilibrated with a 21 % O₂-5 % CO₂-74 % N₂ gas mixture and maintained at 37 °C. To alter intravascular pressure and prevent flow, a vessel “blind-sac” preparation was made by plugging the distal cannula. The proximal cannula was then connected to a software-controlled pressure interface (DMT) or pressure servo controller (Living Systems Instrumentation) for maintenance of steady-state intravascular pressure. Microvessels were visualized using charge-coupled device cameras attached to inverted microscopes. Changes in vessel luminal diameter were continuously acquired using the vessel dimension analysis software (DMT or IonOptix Corp, Milton, MA). Percentage
myogenic tone was calculated as \((1 - \text{active luminal diameter/\text{passive luminal diameter}}) \times 100\). Passive luminal diameter was determined in \(\text{Ca}^{2+}\) free, EGTA (2 mM)-containing MKS.

**Membrane potential measurement**

Renal vascular SMC membrane potential was measured by impaling borosilicate glass microelectrodes filled with 3 M KCl (tip resistances of 50-90 \(\text{m}\Omega\)) into the adventitial side of pressurized interlobular arteries. Membrane potential was recorded using an A-M Systems Intracellular Electrometer Model 3100 (A-M Systems; Carlsborg, WA) and digitized using a pClamp 10 software (Axon Instruments). A successful intracellular recording was based on the following criteria: 1) recording originates from 0 ± 2 mV baseline; 2) a fast, negative change in potential upon microelectrode impalement, 3) stable membrane potential for at least 1 min following microelectrode impalement, and 4) a fast, positive potential change upon microelectrode withdrawal from the impaled cell.

**Intracellular \(\text{Ca}^{2+}\) imaging**

Renal vascular SMCs on Cell-Tak (Corning Life Sciences, Corning, NY)-coated glass-bottom petri dishes were incubated with Fura-2-acetoxymethyl ester (Fura-2 AM; 10 \(\mu\text{M}\)), and 0.5% pluronic F-127 for ~ 1 h at room temperature in MKS. Cells were then washed for ~ 45 min to de-esterify Fura-2 AM molecules before imaging. [\(\text{Ca}^{2+}\)]\(_i\) concentrations were determined using a fluorescence photometry system (Ionoptix Corp., Milton, MA, USA). The fluorescence was collected simultaneously from cells located in the same field. Only one field was imaged per dish. To study pressure-induced changes in [\(\text{Ca}^{2+}\)], vessel luminal diameter and [\(\text{Ca}^{2+}\)]\(_i\) changes were simultaneously measured in pressurized interlobular arteries (56). Before cannulation, microvessels were incubated in MKS containing fura-2 AM/pluronic acid at room temperature. Vessels were allowed to de-esterify fura-2 AM before experimentation as described above.

Fura-2 AM fluorescence was recorded by exciting at wavelengths of 340 and 380 nm using a hyperswitch light source (Ionoptix). Background-subtracted Fura-2 AM ratios were collected at 510 nm using a MyoCam-S CCD digital camera (Ionoptix) and analyzed with IonWizard software (Ionoptix) using the following equation (26):

\[
[\text{Ca}^{2+}]_i = K_d \left[ \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)} \right] \delta.
\]

Where “R” is the 340/380 nm ratio, \(R_{\text{min}}\) and \(R_{\text{max}}\) are the
minimum and maximum Fura-2 ratios determined in Ca\textsuperscript{2+}-free + EGTA and Ca\textsuperscript{2+}-replete solutions, respectively.\n\[ \delta \] represents the ratio of the 380 nm excitation in Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+}-replete solution, while \( K_d \) is the apparent dissociation constant for Fura-2 (224 nM, (26)). \( R_{min} \), \( R_{max} \), and \( \delta \) were determined at the end of the experiments by perfusing the cells or pressurized arteries with 10 µM ionomycin and Ca\textsuperscript{2+}-free (plus 10 mM EGTA) or 10 mM Ca\textsuperscript{2+} solution.

**Renal blood flow autoregulation**

Neonatal pigs were acutely instrumented as we have previously described (55). Briefly, the pigs were anesthetized with ketamine/xylazine (20/2.2 mg/kg; IM) and maintained on \( \alpha \)-chloralose (50 mg/kg, intravenously). The animals were maintained at 37 °C, intubated via tracheostomy, and mechanically ventilated using a Bear Cub pediatric ventilator. Animals were continually monitored during experiments for anesthesia depth and re-dosed if necessary. Arterial blood gas, pH, and hematocrit (HCT) were measured periodically with a GEM Premier 3000 Blood Gas Analyzer (Instrumentation Laboratory, Bedford, MA). Ventilation was adjusted to maintain PCO\textsubscript{2}, PO\textsubscript{2}, and pH at physiological ~ 30 mmHg, > 85 mmHg, and 7.4, respectively. Urine was drained from the kidneys via a ureteral catheter. Mean arterial pressure (MAP) was recorded via a right femoral artery catheter connected to a physiological pressure transducer (ADI Instrument, Colorado Spring, CO). A femoral vein was catheterized for anesthetic and fluid administration. To administer pharmacological agents directly into the kidney, a catheter was inserted in the left femoral artery and advanced through the abdominal aorta until its tip was positioned at the junction of the aorta and left renal artery. The left kidneys were exposed retroperitoneally through flank incisions to permit access to the renal pedicles. RBF was measured with a flow probe (Transonic Systems Inc., Ithaca, NY) placed around the main renal artery and connected to a flowmeter (Transonic Systems). Renal cortical perfusion was measured by placing a Laser-Doppler probe (PF 407) attached to a holder (Perimed, Jarfalla, Sweden) on the kidney surface. Data were acquired and analyzed using a PowerLab data acquisition system and LabChart software (ADI Instrument).

To examine renal myogenic autoregulation, step decreases and increases in renal perfusion pressure were achieved by placing an inflatable vascular occluder cuff (4 mm In Vivo Metrics, Healdsburg, CA) around the aorta immediately upstream of the main renal arteries. The cuff was inflated with a BasixCOMPAK inflation
device (Merit Medical Systems Inc., South Jordan UT). MAP was increased by carefully tightening a ligature
each around the celiac and mesenteric arteries. MAP was then adjusted back to the baseline using the
occluder. Total RBF and renal cortical perfusion responses to a rapid change in perfusion pressure were
measured by reducing the MAP by ~20 mmHg followed by a ~20 mmHg increase for 60 s. After a recovery
time of ~10 min, the protocol was repeated at least three times. Autoregulatory index (AI) was calculated using
the Semple and DeWardener equation below (53):

\[
\text{AI} = \frac{RBF_2 - RBF_1}{RBF_1} \left( \frac{MAP_2 - MAP_1}{MAP_1} \right)
\]

Where \(RBF_1\) and \(MAP_1\) are the baseline values taken before the step increase in pressure, and \(RBF_2\) and
\(MAP_2\) are the values taken after the step increase in pressure. An AI of zero indicates perfect autoregulation,
an AI ≤ 0.2 signifies effective RBF/renal cortical perfusion autoregulation, whereas an AI near or above 1.0
indicates ineffective autoregulation (11, 53).

Antibodies and reagents
Rabbit polyclonal anti-TRPV4 (AP18990a; Western blot and blocking peptide: BP18990a), rabbit polyclonal
anti-TRPV4 (ab39260; immunofluorescence), and mouse monoclonal anti-beta actin (MA515739) primary
antibodies were purchased from Abgent Inc. (San Diego, CA), Abcam (Cambridge, MA), and Life
Technologies, respectively. HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased
from Abcam (Cambridge, MA). Unless otherwise specified, all reagents were purchased from Sigma-Aldrich
(St. Louis, MO). HC 067047 and RN 1734 were purchased from EMD Millipore (Billerica, MA). Fura-2 AM,
Pluronic F-127, ionomycin, GSMTX4, and liberase blendzyme 1 were obtained from Life Technologies,
AnaSpec (Fremont, CA), Cayman Chemical (Ann Arbor, MI), Smartox Biotechnology (Saint Martin d’Hères,
France), and Roche Life Science (Indianapolis, IN), respectively.
Statistical analysis

Statistical analysis was performed using the InStat statistics software (Graph Pad, Sacramento, CA). Data are presented as mean ± standard error of the mean. Student's t-test and Student-Newman-Keuls test were used for comparing paired or unpaired data and multiple data sets, respectively. Statistical significance implies a P-value < 0.05.
Results

TRPV4 channels are predominantly expressed in neonatal pig renal vascular SMCs

TRPV1-4 channels share structural and functional properties and are expressed in the vasculature (71, 74). Here, we determined whether TRPV1-4 channels are expressed in neonatal pig preglomerular vascular SMCs. Intact vessels contain both endothelial and smooth muscle cells, as shown by PCR amplification of both vWF and ACTA2, which are endothelial and smooth muscle cell markers, respectively (Figure 1A). Next, we determined TRPV1-4 expression in intact interlobular arteries. As shown in Figure 1A, TRPV1-4 amplicons were detected in cDNA samples from intact interlobular arteries. To study TRPV isoforms that are expressed specifically in renal vascular SMCs, isolated interlobular artery SMCs (~100) were individually selected under the microscope using a micropipette (Figure 1B). Only batches of SMCs that showed the absence of vWF were used to determine TRPV isoform expression (Figure 1C). Unlike intact vessels, only TRPV4 amplicons were detected in the cells (Figure 1C). To examine cellular localization of TRPV4 channels, isolated SMCs were immunostained with a TRPV4 channel antibody. Confocal microscopy indicated that unlike normal rabbit IgG (negative control; Figure 1D), TRPV4 channels are essentially localized to the plasma membrane of the cells (Figure 1D). TRPV4 channels are functionally expressed in vascular ECs (5). Here, we quantified TRPV4 channel expression in endothelial and smooth muscle cells isolated from neonatal pig renal interlobar arteries. Quantitative RT-PCR indicated that TRPV4 mRNA expression is >2-fold higher in neonatal pig renal interlobar artery SMCs when compared with ECs (Figure 1E,F). Together, these data suggest that TRPV4 channels are predominantly expressed in neonatal pig renal vascular SMCs.

TRPV4 channel protein expression levels in porcine kidney and renal preglomerular arteries are maturation-dependent

Western blotting using a rabbit polyclonal anti-TRPV4 antibody detected a prominent ~120 kDa immunoreactive band in neonatal pig renal cortex and medulla (Figure 2A). Similarly, protein lysates from interlobular arteries and afferent arterioles were positive for the ~120 kDa band (Figure 2B). A blocking peptide directed against the TRPV4 antibody completely blocked band detection, indicating that the ~120 kDa band corresponds to TRPV4 channels (Figure 2C). To investigate whether renal TRPV4 channel protein
expression is dependent on age, we obtained adult male (~6-month old) pig kidneys from a local slaughterhouse. Thereafter, we compared TRPV4 channel protein expression in neonatal versus adult pig kidneys and interlobular arteries. TRPV4 protein expression levels were ~54% and 74% higher in adult pig kidneys and interlobular arteries, respectively when compared with neonatal pigs (Figure 2D-G). These findings demonstrate age-dependent changes in the protein expression levels of renal TRPV4 channels in pigs.

**TRPV4 channels contribute to stretch-induced [Ca$^{2+}$] elevation and constriction in neonatal pig renal vascular SMCs**

Transmembrane hypoosmotic challenge causes vascular SMC swelling and stretch, leading to depolarization, [Ca$^{2+}$] elevation, and vasoconstriction (2, 9, 25). To test the hypothesis that cell swelling-induced neonatal renal vasoconstriction requires TRPV4 channels, we measured [Ca$^{2+}$] concentration in renal vascular SMCs and constriction in interlobular arteries exposed to a hypoosmotic solution. Phospholipase A$_2$ (PLA$_2$) contributes to hypotonic stress-induced activation of TRPV4 channels (68). Hence, we also examined whether PLA$_2$ mediate cell swelling-induced [Ca$^{2+}$] elevation and constriction in the vessels. A change in cell bath solution from 304 to 220 mOsm/L increased [Ca$^{2+}$] level by ~55 nM in interlobular artery SMCs. Hypoosmotic-induced [Ca$^{2+}$] elevation was attenuated by stretch-activated channel blocker tarantula toxin GsMTx4, selective TRPV4 channel blocker HC 067047 (20, 32), and L-type Ca$^{2+}$ channel blocker nimodipine (Figure 3A,B). By contrast, PLA$_2$ inhibitor OBAA did not alter hypoosmotic-induced [Ca$^{2+}$] elevation in the cells (Figure 3A,B). Similarly, hypoosmotic challenge reversibly stimulated neonatal pig renal artery constriction, an effect reduced by HC 067047, but not, OBAA (Figure 3C-F). These findings suggest that cell swelling-induced TRPV4 channel activation stimulates neonatal renal vasoconstriction independently of PLA$_2$.

**TRPV4 channels contribute to pressure-induced membrane depolarization, [Ca$^{2+}$] elevation, and constriction in neonatal pig renal preglomerular arteries**

To further examine the role of TRPV4 channels in the mechanisms that underlie neonatal renal artery myogenic constriction, we first measured membrane potentials in newborn pig distal interlobular arteries that
were pressurized to physiological renal arterial pressure (100 mmHg) following pre-incubation and continuous
superfusion with modified Krebs’ solution containing DMSO (control) or HC 067047. An elevation in
intravascular pressure from 20 to 100 mmHg depolarized the control arteries by ~ 37 mV (Figure 4A and B).
However, pressure-induced membrane depolarization was significantly inhibited by HC 067047 (Figure 4A and
B). Next, myogenic constriction and arterial wall $\text{[Ca}^{2+}\text{]}_i$ concentration were recorded in the arteries. An
increase in intravascular pressure from 20 to 100 mmHg elevated arterial wall $\text{[Ca}^{2+}\text{]}_i$ concentration from ~ 113
to 396 nM in control arteries (~ 282 nM change; Figure 4C and D). In contrast, arterial wall $\text{[Ca}^{2+}\text{]}_i$ concentration
was elevated from ~ 102 to 214 nM in HC 067047-treated arteries (~ 113 nM change; Figure 4C and D).
Correspondingly, pressure-induced constriction was reduced by ~ 73% in HC 067047-treated interlobular
arteries (Figure 4E and F). These findings demonstrate that TRPV4 channels are required for pressure-
induced membrane depolarization, $\text{[Ca}^{2+}\text{]}_i$ elevation, and constriction in neonatal pig renal preglomerular
arteries.

**TRPV4 channel blockade reversed steady-state myogenic tone in neonatal pig renal preglomerular
arteries**

Figure 4E and F indicate that blockade of TRPV4 channels before intravascular pressure elevation inhibits
myogenic constriction. Here, we examined the effect HC 067047 on steady-state myogenic tone. HC 067047
concentration-dependently overturned myogenic constriction in neonatal pig interlobular arteries pressurized to
100 mmHg (Figure 5A and B). These data support our hypothesis that TRPV4 channels contribute to neonatal
pig renal myogenic constriction.

**Blockade of TRPV4 channels does not alter voltage-gated $\text{Ca}^{2+}$ channel-mediated $\text{[Ca}^{2+}\text{]}_i$ elevation and
vasoconstriction in neonatal pig renal preglomerular arteries**

Nimodipine, an L-type $\text{Ca}^{2+}$ channel blocker essentially abolished stretch-induced $\text{[Ca}^{2+}\text{]}_i$ elevation in neonatal
pig renal vascular SMCs (Figure 3A,B). Nimodipine also reversed myogenic tone in neonatal pig renal
interlobular arteries by evoking vasodilation (Figure 6A,B). Removal of extracellular $\text{Ca}^{2+}$ in the presence of
nimodipine did not cause any further dilation of the vessels (Figure 6A,B), indicating that myogenic constriction
of neonatal pig renal preglomerular arteries occurs because of voltage-dependent L-type Ca\(^{2+}\) channel activation downstream of stretch-induced membrane depolarization. To confirm that inhibition of myogenic constriction by HC 067047 occurs independently of L-type Ca\(^{2+}\) channels, we studied its effect on depolarization-induced [Ca\(^{2+}\)]\(_e\) elevation and constriction. Depolarization by 75 mM K\(^+\) stimulated renal artery wall [Ca\(^{2+}\)]\(_i\) elevation and vasoconstriction that were blocked by nimodipine (Figure 7A-D). By contrast, HC 067047 did not alter depolarization-induced [Ca\(^{2+}\)]\(_i\) elevation and constriction in the arteries (Figure 7A-D). These findings demonstrate that HC 067047-induced inhibition of neonatal renal artery myogenic constriction is not mediated via L-type Ca\(^{2+}\) channels.

**TRPV4 channels contribute to renal autoregulation in neonatal pigs**

To investigate whether the myogenic mechanism observed in vitro can be recapitulated in vivo, we examined autoregulation of total RBF and renal cortical perfusion in anesthetized and mechanically ventilated neonatal pigs. As described in the methods, MAP was increased by carefully tightening a ligature each around the celiac and superior mesenteric arteries. This procedure increased steady-state MAP from 86.9 ± 3.0 to 114.1 ± 2.3 mmHg (n = 15; P<0.05). Following MAP elevation, step decreases and increases in MAP were made using a vascular occluder. We observed whether an increase in arterial pressure induces an efficient autoregulation of neonatal pig RBF and renal cortical perfusion. As shown in Figure 8A, an increase in MAP by 20 mmHg resulted in autoregulation of total RBF and renal cortical perfusion within 10 s. The RBF and renal cortical perfusion autoregulation indexes following 60 s of increased MAP were ~ 0.1 and 0, respectively, indicating efficient autoregulation (11). Intrarenal arterial infusion of L-type Ca\(^{2+}\) channel blocker nicardipine for ~ 30 min reduced basal MAP by 23.5 ± 7.4 mmHg (n=4; P<0.05) and abolished renal autoregulation in the pigs (Figure 8B,C). These data suggest that renal autoregulation is efficient in neonatal pigs. Our data also suggest that L-type Ca\(^{2+}\) channel-mediated myogenic constriction induces neonatal renal autoregulation.

Next, we investigated whether TRPV4 channels contribute to myogenic autoregulation in the pigs. Intrarenal infusion of TRPV4 channel blockers HC 067047 and RN 1734 for ~ 30 min did not alter basal MAP [change in MAP (mmHg): control: -4.3 ± 0.64; n=4 vs. HC 067047: -7.3 ± 1.05; n=5 vs. RN 1734: -5.5 ± 2.11; n=4; P>0.05]. However, HC 067047 and RN 1734 inhibited RBF and renal cortical perfusion autoregulation in
the pigs (Figure 9A-C). The effects of HC 067047 and RN 1734 on RBF and renal cortical perfusion autoregulation indexes following 10 s of MAP elevation are presented in Figure 9D. Our data indicate that TRPV4 channel blockade attenuates myogenic renal autoregulation in neonatal pigs.
Discussion

TRPV channels are highly expressed in the kidney, but their physiological functions in renal vascular bed are poorly understood. Similarly, mechanisms that control immature kidney perfusion are unclear. In this study, we show that unlike TRPV1-3, TRPV4 channels are expressed in neonatal pig preglomerular vascular SMCs. TRPV4 expression is higher in renal vascular SMCs than in ECs. A TRPV4 channel blocker attenuated cell swelling-induced \( [\text{Ca}^{2+}]_e \) elevation in renal vascular SMCs and vasoconstriction. Blockade of TRPV4 channels also reversed steady-state myogenic tone and inhibited pressure-induced membrane depolarization, \( [\text{Ca}^{2+}]_e \) elevation, and constriction in distal interlobular arteries. Moreover, intrarenal arterial infusion of TRPV4 channel blockers inhibited renal autoregulation in the pigs. These findings identify for the first time the physiological function of vascular SMC TRPV4 channels in neonatal renal microcirculation.

Data here show that TRPV1-3 are expressed in intact preglomerular arteries, but not in SMCs that were uncontaminated with ECs. Hence, renal vascular TRPV1-3 may be restricted to ECs in neonatal pigs. We demonstrate that TRPV4 is predominantly expressed in neonatal pig renal vascular SMCs, suggesting that they may participate in SMC-dependent neonatal renal vasoregulation. In addition, localization of TRPV4 channels in the plasma membrane of freshly isolated neonatal renal vascular SMCs is consistent with a mechanotransduction function.

Vasoregulation by TRPV4 channels appears to depend on vascular bed type, vessel size, or the predominant location of the channels within the vasculature. Cerebral and mesenteric artery vasodilation induced by local \( [\text{Ca}^{2+}]_e \) signals, including \( \text{Ca}^{2+} \) sparks and sparklets have been associated with SMC and EC TRPV4 channels (18, 57, 58). Low intraluminal pressure stimulated rat cremaster arteriolar vasodilation via activation EC \( \text{Ca}^{2+} \) signaling mediated by myoendothelial junction TRPV4 channels (3). In pial arterioles, TRPV4 is expressed in ECs (35). By contrast, the channels are colocalized with astrocyte markers in parenchymal arteriole adventitia and mediate flow/pressure-induced myogenic constriction (35). Both flow- and arachidonic acid-induced dilation of human coronary arterioles are dependent on EC TRPV4 channels (7, 75). However, intravital microscopy of the lung showed that TRPV4 ablation attenuates hypoxia-induced constriction of mouse pulmonary arterioles (24). In adult mice, TRPV4 agonist GSK1016790A relaxed large renal arteries precontracted with phenylephrine and vasa recta precontracted with norepinephrine (12).
Whether pharmacological modulation or pressure-induced activation of TRPV4 channels directly regulates the
diameter of resistance size renal vessels was previously unexplored. Our qRT-PCR data suggest that TRPV4
channels are expressed in neonatal pig renal vascular ECs, albeit at a much lower level than in SMCs. The
physiological function of renal vascular EC TRPV4 in neonates is unclear and requires further investigation.

Both in vivo and in vitro studies have demonstrated that TRPV4 is a sensor of osmotic/mechanical stress, but conflicting evidence exists (14, 39, 49). Stretch-induced \([Ca^{2+}]\) elevation was reduced in TRPV4-deficient urothelial cells (42). TRPV4 channels were hypotonic-gated, but not responsive to membrane stretch when transfected in HEK-293 cells (59). Genetic ablation of TRPV4 abolished cell-swelling-induced \([Ca^{2+}]\), in renal tubules (60). Hypotonic stress has also been shown to induce \([Ca^{2+}]\), elevation and whole-cell currents in Chinese hamster ovary (CHO) cells overexpressing TRPV4 (38). By contrast, another study reported that cell inflation, but not hypotonic stress significantly activated TRPV4 currents in CHO cells transiently expressing TRPV4 (61). Consistent with a previous report on pulmonary artery SMCs (73), we show in this study that hypoosmotic challenge triggered TRPV4-dependent \([Ca^{2+}]\) elevation in neonatal pig renal vascular SMCs. Conceivably, the functional role of TRPV4 as osmo-mechanosensitive channels is dependent on cell type.

Renal vascular SMC \([Ca^{2+}]\), elevation induced by hypoosmotic solution was inhibited by stretch-activated and L-type \(Ca^{2+}\) channel blockers, indicating that cell swelling stimulates stretch-activated channels and subsequent membrane depolarization in the cells. Cell swelling activates PLA\(_2\) in several cell types, including tumor cells, fibroblasts, and pancreatic islets (4, 47, 48). Swelling-induced channel activation in TRPV4-transfected HEK-293 cells has also been shown to be dependent on PLA\(_2\) (68). We show here that at the concentration that did not alter high \(K^+\)-induced \([Ca^{2+}]\), elevation and constriction, HC-067047 attenuated \([Ca^{2+}]\), elevation and vasoconstriction induced by hypoosmotic challenge. These data suggest that cell swelling activates TRPV4 channels in neonatal pig renal vascular SMCs. The lack of effect of a PLA\(_2\) inhibitor on hypoosmotic-induced \([Ca^{2+}]\), elevation and vasoconstriction indicates that osmotic cell swelling-induced TRPV4 activation in neonatal pig renal vascular SMCs occurs independently of PLA\(_2\). Together, our data suggest that neonatal pig renal vascular SMC TRPV4 channels are mechanosensitive, and contribute to renal myogenic constriction. The identities and functions of mechanosensitive ion channels that underlie pressure-induced depolarization and successive \([Ca^{2+}]\), elevation and constriction in renal vascular SMCs are
understudied. So far, only epithelial Na⁺ channels (ENaC) have been shown to contribute to mechanotransduction in renal vascular SMCs. Knockdown of the β-subunits of ENaC (βENaC) attenuated stretch-induced currents in adult mouse renal vascular SMCs (13). Afferent arteriole myogenic constriction and renal autoregulation were also reduced in mice that lacked βENaC (21). Both ENAC and TRPV4 proteins interact with actin cytoskeleton (51). Whether ENAC and TRPV4 form a mechanosensitive signaling complex in renal vascular SMCs is unclear and requires additional studies.

Although isolated tissue experiments in this study utilized resistance arteries, our data indicate that TRPV4 channels are also expressed in afferent arterioles. Hence, the effect of TRPV4 channel blockers on neonatal pig renal autoregulation may reflect the contribution of both interlobular arteries and afferent arterioles. Attenuation of neonatal pig renal autoregulation by TRPV4 channel blockers is consistent with the data on myogenic constriction of isolated vessels and strongly support our hypothesis that mechanotransduction by TRPV4 channels in renal vascular SMCs contributes to neonatal pig renal myogenic autoregulation. Osmotic and mechanical stress trigger ATP release (6). Studies have also shown that hypotonicity- and stretch-induced ATP release by adult rat thick ascending limb and adult mouse urothelial cells, respectively, are dependent on TRPV4 channels (42, 54). Given that ATP release in the macula densa contributes to TGF-mediated autoregulation, it is possible that TRPV4 is also involved in TGF mechanisms. However, TRPV4 is absent in adult rat macula densa (64). Whether the absence of TRPV4 in the macula densa is consistent across species, and age is unknown. TGF mechanism may also be immature in neonates (1). Thus, our current findings provide no insight into the functional role of TRPV4 channels in neonatal pig TGF mechanism.

TRPV4 protein expression levels and agonist-induced [Ca²⁺] elevation in primary mesenteric artery ECs were higher in young (3 months old) when compared with aged (22 months old) rats, suggesting age-dependent regulation of the expression and function of vascular TRPV4 channels (16). Data here indicate that TRPV4 protein expression levels in pig kidneys and renal arteries are significantly higher in adults when compared with neonates. Perhaps, TRPV4 channels are also involved in renal myogenic autoregulation mechanism in adults. Hence, the functional significance of renal maturation-dependent expression of the channels requires further investigation.
In summary, we have uncovered a new physiological role for renal TRPV4 channels. We show that neonatal pig renal vascular SMC TRPV4 channels are activated by an elevation in intravascular pressure, and this reaction results in membrane depolarization, extracellular Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels, and vasoconstriction. We also demonstrate that TRPV4-dependent myogenic constriction contributes to renal autoregulation in neonatal pigs.
Acknowledgments

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Disclosure

None.


Figure Legends

Figure 1: **TRPV4 channels are predominantly expressed in neonatal pig renal vascular SMCs.** A, an agarose gel image showing the expression of vWF (EC marker), ACTA2 (SMC marker), and TRPV1-4 channels in neonatal pig interlobular arteries. **B,** Images showing selection of a neonatal pig renal vascular SMC using a patch pipette. **C,** an agarose gel image indicating that individually selected SMCs expressed only ACTA2 and TRPV4 channels. **D,** confocal microscopy images of neonatal pig renal vascular SMCs immunostained with normal rabbit IgG (negative control) and TRPV4 antibodies. **E,** an agarose gel image, and **F,** bar graphs summarizing mean data (n=3) for qRT-PCR experiments that compared TRPV4 mRNA expression in neonatal pig renal interlobar artery SMCs versus ECs. *P<0.05 vs. ECs. Scale bar = 10 µM.

Figure 2: **TRPV4 channel protein expression levels in porcine kidney and renal preglomerular vessels.** **A** and **B,** Western blot images showing the expression of TRPV4 channels in neonatal pig renal cortex, medulla, and preglomerular vessels. **C,** A blocking peptide directed against TRPV4 antibody abolished TRPV4 immunoreactive band detection. **D** and **E,** Western blot images and bar graphs (n=3 each) illustrating TRPV4 protein expression levels in neonatal and adult pig kidneys and **F** and **G,** interlobular arteries. *P<0.05 vs. neonates.

Figure 3: **TRPV4 channels contribute to stretch-induced [Ca^{2+}]_i elevation and constriction in neonatal pig renal vascular SMCs.** **A,** traces and **B,** bar graphs showing changes in [Ca^{2+}]_i induced by transmembrane hypoosmotic challenge (n=5) in control and SMCs pretreated with GsMTx4 (300 nM; n=4), nimodipine (1 µM; n=4), HC 067047 (1 µM; n=5), or OBAA (1 µM; n=4). **C-F,** traces and bar graphs demonstrating that hypoosmotic challenge-induced constriction of neonatal pig renal interlobular arteries is inhibited by HC 067047 (1 µM; n=7 each), but not OBAA (1 µM; n=5 each). Cells/vessels were pretreated with inhibitors/blockers 10-15 minutes before hypoosmotic challenge. *P<0.05 vs. control (hypoosmotic).
Figure 4: TRPV4 channels contribute to pressure-induced membrane depolarization, $[\text{Ca}^{2+}]_i$ elevation and constriction in neonatal pig renal preglomerular arteries. A and B, traces and bar graphs showing SMC membrane potentials in neonatal pig renal interlobular arteries that were pressurized to 100 mmHg in the presence of DMSO (control; n=4) or HC 067047 (1 µM; n=7). C and D, traces and bar graphs illustrating pressure-induced changes in $[\text{Ca}^{2+}]_i$ concentration in neonatal pig renal interlobular arteries in the presence of DMSO (control; n=4) or HC 067047 (1 µM; n=5). E and F, traces and bar graphs showing myogenic tone in neonatal pig renal interlobular arteries that were pressurized to 100 mmHg in the presence of DMSO (control; n=12) or HC 067047 (1 µM; n=12). Vessels were pretreated and continuously superfused with DMSO or HC 067047. *P<0.05 vs. control.

Figure 5: TRPV4 channel blockade reversed steady-state myogenic tone in pressurized neonatal pig renal preglomerular arteries. A, traces and B, bar graphs (n=8 each) summarizing the concentration-response effect of HC 067047 (1 µM) on steady-state myogenic tone in neonatal pig interlobular arteries pressurized to 100 mmHg. *P<0.05 vs. control.

Figure 6: Myogenic constriction of neonatal pig renal interlobular arteries is dependent on L-type voltage-dependent $\text{Ca}^{2+}$ channels. A, a trace showing that nimodipine (1 µM) abolished myogenic tone in neonatal pig renal interlobular arteries by blocking L-type $\text{Ca}^{2+}$ channels and that the removal of extracellular $\text{Ca}^{2+}$ in the presence of nimodipine did not cause any further dilation of the vessels. B, bar graphs summarizing mean data (n=3) for luminal diameter of neonatal pig interlobular arteries in the presence of nimodipine and nimodipine + $\text{Ca}^{2+}$ free/EGTA bath medium.

Figure 7: Blockade of TRPV4 channels does not alter L-type $\text{Ca}^{2+}$ channel-mediated $[\text{Ca}^{2+}]_i$ elevation and vasoconstriction in neonatal pig renal preglomerular arteries. A and C, traces and B and D, bar graphs demonstrating that depolarization by 75 mM $\text{K}^+$ stimulated renal artery wall $[\text{Ca}^{2+}]_i$, elevation (n=5) and vasoconstriction (n=7) that were abolished by nimodipine ($[\text{Ca}^{2+}]_i$: n=4; vasoconstriction: n=5), but not HC 067047 ($[\text{Ca}^{2+}]_i$: n=4; vasoconstriction: n=5). *P<0.05 vs. 75 mM $\text{K}^+$. 
Figure 8: **Renal autoregulation is efficient in neonatal pigs, and dependent on L-type Ca\(^{2+}\) channels.**

A, traces illustrating that an increase in MAP by 20 mmHg induces autoregulation of RBF and renal cortical perfusion in neonatal pigs. 

B, traces showing that intrarenal arterial infusion of nicardipine (1 µg/kg/min; 30 min) abolishes RBF and renal cortical perfusion autoregulation in neonatal pigs. 

C, bar graphs summarizing mean (n=4 each) RBF and renal cortical perfusion autoregulation indexes in the absence and presence of nicardipine. MAP: mean arterial pressure; RBF: total renal blood flow; RCoP: renal cortical perfusion; *P<0.05 vs. control.

Figure 9: **TRPV4 channels contribute to myogenic renal autoregulation in neonatal pigs.**

A-C, Graphs showing steady-state MAP, RBF, and renal cortical perfusion (RCoP) without (n=4) and with intrarenal arterial infusion of HC 067047 (20 µg/kg/min; 30 min; n=5) and RN 1734 (20 µg/kg/min; 30 min; n=4). 

D, bar graphs illustrating RBF and RCoP autoregulation indexes in the absence (n=4) and presence of HC 067047 (n=5) and RN-1734 (n=4). *P<0.05 vs. control.
Table 1:
Oligonucleotide primer sequences

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TRPV4
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TRPV4 NM_001130729.1 105
Forward 5' - CAGGCTACTACCAGCCTCTG - 3'
Reverse 5' - GGACCCCATAGAAGAGTG - 3'

* Partial cds

# Predicted
Figure 1

(A) Intact renal interlobular arteries

(B) Interlobular artery SMCs

(D) Rabbit IgG

(E) TRPV4

(F) Relative TRPV4 mRNA abundance

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

(A) 

3 \mu m

3 min

0 Ca^{2+}

Nimodipine

(B) 

Nimodipine
Nimodipine + 0 Ca^{2+}

Luminal diameter (\mu m)

60
50
40
30
20
10
0
Figure 7
Figure 8

(A) Control

(B) Nicardipine

(C) Control

Nicardipine

* * *
Figure 9