Connexin45 is expressed in the juxtaglomerular apparatus and is involved in the regulation of renin secretion and blood pressure

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ABSTRACT

Connexin (Cx) proteins are known to play a role in cell to cell communication via intercellular gap junction channels or transiently open hemichannels. Previous studies have identified several connexin isoforms in the juxtaglomerular apparatus (JGA), but the vascular connexin isoform Cx45 has not yet been studied in this region. The present work aimed to identify in detail the localization of Cx45 in the JGA and to suggest a functional role for Cx45 in the kidney using conditions where Cx45 expression or function was altered. Using mice expressing LacZ coding DNA under the control of the Cx45 promoter, we observed β-galactosidase staining in cortical vasculature and glomeruli, with specific localization to the JGA region. Renal vascular localization of Cx45 was further confirmed using conditional Cx45-deficient (Cx45fl/fl:Nestin-Cre) mice which express EGFP instead of Cx45 only in cells that during development expressed the intermediate filament Nestin. EGFP fluorescence was found in the afferent and efferent arteriole smooth muscle cells, renin producing JG cells and in the extra- and intraglomerular mesangium. Cx45fl/fl:Nestin-Cre mice exhibited increased renin expression and activity, as well as higher systemic blood pressure. The propagation of mechanically-induced calcium waves was slower in cultured vascular smooth muscle cells (VSMCs) from Cx45fl/fl:Nestin-Cre mice as well as in control VSMC treated with a Cx45 gap mimetic peptide that inhibits Cx45 gap junctional communication. VSMCs allowed the cell-to-cell passage of the gap junction permeable dye Lucifer yellow and calcium wave propagation was not altered by addition of the ATP receptor blocker suramin, suggesting that Cx45 regulates calcium wave propagation via direct gap junction coupling. In conclusion, the localization of Cx45 to the JGA and functional data
from Cx45fl/fl:Nestin-Cre mice suggest that Cx45 is involved in the propagation of JGA vascular signals and in the regulation of renin release and blood pressure.
INTRODUCTION

The juxtaglomerular apparatus (JGA) is an important anatomical component of the renin-angiotensin system (RAS) and it plays a major role in regulating body fluid and electrolyte homeostasis and blood pressure. The JGA consists of a tubular component (the macula densa, MD), the extraglomerular mesangium, and a vascular component which includes the terminal part of the afferent arteriole containing the renin producing juxtaglomerular (JG) cells. Two major regulatory functions are performed by the JGA: the high distal tubular [NaCl]-induced afferent arteriolar vasoconstriction (tubuloglomerular feedback, TGF) and the low tubular [NaCl]-induced renin release.

Several connexin (Cx) isoforms have already been studied in the JGA. Cx37, Cx40, and Cx43 have all been identified in the endothelium of the proximal afferent arteriole (2, 17). Only Cx43 has been found within the efferent arteriole endothelium (42). JG cells express Cx37 and Cx40 and these Cxs are also found in the intra- and extraglomerular mesangial cells (42). Cx43 is also expressed within the glomerulus (35). These 3 isoforms, along with Cx45, are considered to be the predominant vascular connexins (39), yet Cx45 has not been studied within the renal vasculature and JGA.

Cx45 is expressed in several organ systems during embryogenesis and is essential for the proper development of the cardiac and vascular system (19, 20). In the adult animal, expression is reduced (1, 7) but is known to continue both in conductive cardiomyocytes (14) and distinct neuronal subpopulations in the adult brain (33). Although Cx45 has been previously found in the kidney, these studies focused on its expression in the developing kidney and in kidney derived cell lines (5, 32). In the mature kidney however, exact and detailed cellular localization of Cx45 was hampered by the
lack of specific detection methods. Recently, transgenic mouse techniques have been developed that provide not only a localization tool but also tissue or cell-specific deletion of connexins (19, 23). To aid in localization of Cx45 in all organ systems, a transgenic mouse in which one copy of the Cx45 coding DNA is replaced by the LacZ reporter coding DNA (Cx45±) was developed and studied (19). Additionally, since general deletion of Cx45 proves to be lethal during embryogenesis (19, 20), the Cre/loxP technique was recently used to generate a mouse with a Cx45 gene deletion that is restricted to cells expressing the protein nestin during development (23). This method has previously been used to achieve conditional expression of a variety of genes in the kidney (4, 15). By creating a mouse with the Cre recombinase gene under the control of kidney-specific promoters, gene deletions have been produced in renal structures including the vasculature and glomeruli, both of which express nestin during development (8).

The present work details the localization of Cx45 in the JGA of adult mouse kidney using genetic techniques. We then went on to identify its relevance in renal (patho)physiology. Our data show that Cx45 is expressed in the vascular component of JGA and is involved in the propagation of JGA vascular signals and in the regulation of renin release and blood pressure.
MATERIALS AND METHODS

Animals:

C57BL/6 mice were bred in house and were fed standard diets (Harlan Teklad, Madison WI) and provided drinking water ad libitum or water supplemented with 500mg/L captopril for one week. Cx45+/- and Cx45+/- mice, where the coding region of the Cx45 gene was replaced with the β-galactosidase (LacZ) reporter gene, were previously generated and described (17). Cx45fl/fl:Nestin-Cre mice were kindly provided by Dr. Marla Feller at the University of California in San Diego and had previously been described (23). The Cx45fl/fl:Nestin-Cre mice were developed from two mouse strains: Cx45fl/fl mice which were generated in C57BL/6 mice and backcrossed to C57BL/6 mice for at least 3 generations and Nestin-Cre mice which were generated in B6SJLF2 mice and backcrossed to C57BL/6 mice for at least 6 generations (The Jackson Laboratory, Bar Harbor, ME). By breeding these two mouse lines the background similarity to C57BL/6 was more than 90% percent (23). In these mice, the Cx45 coding DNA has been replaced by the EGFP reporter gene in cells which expressed nestin during development by way of the Cre/loxP site specific recombination system. Due to this recombination, EGFP gene transcription comes under the control of the Cx45 promoter. Therefore EGFP staining will only occur in cells that expressed nestin during development and that normally express Cx45. All animal protocols have been approved by the Institutional Animal Care and Use Committee at the University of Southern California.
\textit{\textbf{\textbeta-Gal staining:}}

Kidneys from Cx45\textsuperscript{+/+} and Cx45\textsuperscript{+/-} mice were frozen on dry ice, embedded in Tissue-Tec (Sakura, Zoeterwoude, The Netherlands), sectioned (10-20 \(\mu\m\)) on a cryostat (MICROM HM 500 OM) and transferred onto superfrost plus slides (Menzel, Braunschweig, Germany). Sections were fixed with 0.2\% glutaraldehyde in 0.1 M phosphate buffered saline (PBS), rinsed three times in LacZ washing buffer (0.1 M phosphate buffer, pH 7.4, 1.25 mM MgCl\textsubscript{2}, 5 mM EGTA, 0.2\% Nonidet P-40 and 0.01\% sodium doxycholate) and stained in LacZ substrate buffer (LacZ washing buffer supplemented with 0.4 mg/ml X-Gal [5-brom-4-chloro-3-indoly-\textbeta-D-glactopyranoside], 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide) overnight at 37\textdegree C. Sections were then washed in phosphate buffered saline (PBS), stained in 0.1\% eosin for 5 minutes, rinsed in water, and mounted in Entellan (Merck, Darmstadt, Germany).

\textit{\textbf{Antibodies:}}

The rabbit polyclonal renin antibodies for immunofluorescence studies were provided by Dr. Joël Menard (INSERM, Paris, France), while rabbit polyclonal renin antibodies for immunoblots were a kind gift from Dr. Tadashi Inagami (Vanderbilt University, Nashville, TN). Both renin antibodies were characterized in previous publications (6, 25). Rabbit polyclonal anti-Cx45 was kindly provided by Dr. Ulrike Janssen-Bienhold, University of Oldenburg, Germany, and was previously characterized (10).

\textit{Immunofluorescence labeling of Cx45fl/fl:Nestin-Cre mouse kidney tissue}
Kidneys were fixed in situ by perfusion with periodate-lysine-paraformaldehyde (PLP). Coronal kidney sections were incubated overnight in PLP at 4°C before overnight cryoprotection in 2.3 M sucrose. Tissue was embedded in O.C.T. embedding medium (Sakura, Torrance, CA) over dry ice. Thin sections were cut on a Leica CM cryostat (Leica Microsystems, Bannockburn, IL). Sections were fixed with 4% paraformaldehyde for 10 min, permeabilized for 10 min with 0.1% Triton-X in PBS, and subsequently incubated in a solution of 5% normal goat serum in PBS for 30 min to block non-specific binding. Additionally, some sections were also probed with antibodies against renin, at a dilution of 1:100 for 1 hour, followed by incubation with secondary Alex Fluor 594 goat-anti rabbit antibodies (Invitrogen, Carlsbad, CA) at a 1:500 dilution for 1 hour. Following a final wash step, all sections were mounted with Vectashield mounting media containing the nuclear stain DAPI (Vector Laboratories, Burlingame, CA) and examined with a Leica TCS SP2 confocal microscope.

**Measuring plasma renin activity**

Renin activity of mouse plasma was measured using a fluorescence resonance energy transfer (FRET)-based 5-FAM-conjugated renin substrate (Anaspec, San Jose CA) and a cuvette-based spectrofluorometer (Quantamaster-8, PTI, Birmingham, NJ). In the FRET-peptide’s native state, the fluorescence of 5-FAM is quenched by QXL-520. Upon cleavage of the substrate into two fragments by renin, 5-FAM will fluoresce. A similar method utilizing a FRET-based EDANS-conjugated renin substrate has been described before (16, 35). Briefly, 0.33 µM of the renin substrate in Krebs-Ringer (pH 7.4) was loaded into the cuvette and heated to 37°C. After taking a baseline reading, 30
µL of mouse plasma were mixed with the renin substrate in the 37°C chamber and the emitted fluorescence signal as an index of ANG I generation was measured at 520 nm in response to excitation at 490 nm for a period of 800 sec. The initial rate of the increase in 5-FAM fluorescence was then analyzed as a measure of renin activity using the FeliX32 software (PTI).

**Blood pressure measurement**

C57BL/6 mice and Cx45fl/fl:Nestin-Cre mice were anesthetized with a combination of Inactin (100 mg/kg body wt) and Ketamine (100 mg/kg body wt) intraperitoneally. To measure systemic blood pressure, a cannula was inserted into the left carotid artery and using an analog single-channel transducer signal conditioner and transducer, data was collected using data acquisition system QUAD-161 (World Precision Instruments, Sarasota, FL). Statistical significance was tested using an unpaired t-test and data are shown as mean ± SE.

**Isolation and culture of vascular smooth muscle cells from mouse kidneys**

Kidneys were collected from C57BL/6 and Cx45fl/fl:Nestin-Cre mice anesthetized with 100 mg/kg body weight Inactin. The terminal afferent arteriole was manually dissected on ice under a microscope from sagittal slices of kidney in DMEM culture medium containing 3% fetal bovine serum (FBS) (Invitrogen). The afferent arteriole was cut into short segments and transferred to tissue culture dishes containing circular glass coverslips. Explants gave rise to vascular smooth muscle cells (VSMC) approximately 2-3 days after attachment. Isolated VSMC were then grown to 90%
confluence on the glass coverslips in the following media: DMEM with 25 mM D-glucose with the addition of 3.7 g NaHCO₃, 20% FBS, and 1% Penicillin-Streptomycin. Cells were bathed in a modified Krebs-Ringer HCO₃ buffer during dye incubation and subsequent experiments. This buffer was composed of: 115 mM NaCl, 5 mM KCl, 25 mM NaHCO₃, 960 µM NaH₂PO₄, 240 µM Na₂HPO₄, 1.2 mM MgSO₄, 2 mM CaCl₂, 5.5 mM D-glucose, and 100 µM L-arginine. All solutions were adjusted to pH 7.4 using HCl and NaOH.

Immunoblotting of C57BL/6 and Cx45fl/fl:Nestin-Cre mouse and rat kidneys

Mice were anesthetized with 100mg/kg Inactin and kidneys were perfused with ice-cold PBS to remove blood. Tissue was then homogenized with a rotor-stator homogenizer in a buffer containing 20mM Tris-HCl, 1mM EGTA, pH 7.0, and a protease inhibitor cocktail (BD Bioscience, San Jose, CA). Samples were centrifuged at low speed to pellet cellular debris and supernatant was collected and assayed. Forty micrograms of protein were run per lane, separated on a 4-20% SDS-polyacrylamide gel (Biorad, Hercules CA), and then transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). After blocking the membrane in blocking buffer (Li-Cor, Lincoln, NE) blots were probed with rabbit polyclonal antibodies to Cx45 at a dilution of 1:1000 overnight. Reactivity of the primary antibodies was detected with IR680-labeled goat anti-rabbit antibodies (1:15,000 dilutions, Li-Cor). Blots were imaged using the Odyssey Infrared Imaging System (Li-Cor) and accompanying software. The blot was reprobed with a mouse monoclonal antibody to GAPDH (Ambion, Austin, TX) at a dilution of 1:4000 for 1 hour (Santa Cruz Biotechnology, Santa Cruz, CA) to test for protein loading.
and quality of transfer. Labeling was detected and imaged with an IR800-labeled goat anti-mouse antibody as described above (Li-Cor system).

**Immunoblotting of vascular smooth muscle cells:**

Afferent arteriole VSMCs isolated from C57BL/6 and Cx45fl/fl:Nestin-Cre mice were grown to confluence in plates as described above. Cells were removed from the plates by scraping and lysed using CellLytic-M lysis buffer (Sigma) according to manufacturer’s instructions. Protein concentration was assayed by a modified Bradford method (Quick Start Bradford protein assay, Biorad). Seven microgram samples were blotted and analyzed for Cx45 and GAPDH as described above.

**RT-PCR of vascular smooth muscle cells**

Total RNA was purified from confluent afferent arteriolar vascular smooth muscle cells derived from C57BL/6 and Cx45fl/fl:Nestin-Cre mouse kidneys using a Total RNA Mini kit in accordance with manufacturer’s instructions (Bio-Rad). RNA was then quantified using spectrophotometry and reverse-transcribed to single-strand cDNA using avian reverse transcriptase and random hexamers according to manufacturer’s instructions (Thermoscript RT-PCR systems, Invitrogen). Two microliters of cDNA were amplified using a master mix containing Taq polymerase (Invitrogen) and the following primers: Cx37 forward: 5’-GGC TGG ACC ATG GAG CCG GT-3’, Cx37 reverse: 5’-TTT CGG CCA CCC TGG GGA GC-3’; Cx40 forward: 5’-CCA GCT TTT AAT GCC GAG AG-3’, Cx40 reverse: 5’-CCA GCT TTT AAT GCC GAG AG-3’; Cx43 forward: 5’-TAC CAC GCC ACC ACT GGC-3’, Cx43 reverse: 5’-AAT CTC CAG GTC AGT-3’.
AGG-3’; β-actin sense: 5’-GGTGTGATGGGGAATGGGTC-3’, β-actin anti-sense: 5’-ATGGCGTGAGGGAGCATAGC-3’. All primer sequences were based on previous publications (36, 24).

Measurement of calcium wave propagation in vascular smooth muscle cells

Coverslips containing the VSMC monolayer were mounted to a chamber of the Leica TCS SP2 confocal microscope system and imaged in the absence of any dyes to establish any EGFP fluorescence (excitation at 488 nm, emission at 520 ± 20nm). VSMCs were then loaded for 20 minutes with the ratiometric calcium dyes Fluo-4 AM (excitation at 488 nm, emission at 520 ± 20nm) and Fura red AM (excitation at 488 nm, emission at >600nm) (Invitrogen) at a final concentration of 1 µM and 5 µM respectively. A transmitted light detector and differential interference contrast imaging (DIC) was used to visualize the position of the pipette prior to and during mechanical stimulation. All experiments were performed using the same instrument settings and data acquisition and analysis were done using the Leica LCS imaging software (LCS 2.61.1537). Calcium wave velocity was calculated using the formula: velocity = distance/time, where distance was defined as the length between the point of mechanical stimulation and the center of a cell with increased [Ca^{2+}],. For each experimental group, n=6. Statistical significance was calculated by a one-way ANOVA analysis followed by Dunnett’s post-hoc comparison with data shown as mean + SE.

Mechanical stimulation of vascular smooth muscle cells
A single VSMC of the monolayer was stimulated with a glass micropipette (Drummond Scientific Company, Broomall, PA) pulled to 2-3 µm diameter using a micropipette puller (PP-830, Narishige, Japan). A micromanipulator (ROE-200, Sutter Instruments, Novato, CA) was used to position and lower the micropipette to contact the monolayer.

Pharmacological treatment of vascular smooth muscle cells

In cell calcium wave experiments, the gap junction uncoupling agent 18α-glycyrrhetinic acid (18α-GA, 25 µM) (Sigma-Aldrich, St. Louis, MO) was used as a non-specific gap junction inhibitor. To specifically block Cx45 in the same experiments, a Cx45 gap mimetic peptide of sequence QVHPFYVCSRLPCPHK (amino acids 202-217) was synthesized (USC/Norris Cancer Center DNA Core Facility, Los Angeles, CA) based on the work of Li and Simard (21). Cell monolayers were incubated with the gap mimetic peptide at a concentration of 500 µM for 3 hours at 37°C, as previously described. The non-selective purinergic receptor antagonist suramin was applied to cell monolayers at a concentration of 50 µM for 10 minutes at 37°C.

Dye spreading assay

Coverslips containing a confluent VSMC monolayer were mounted to a chamber of the Leica confocal microscope system and bathed with 1mL modified Krebs-Ringer HCO₃ buffer. Hoechst 33342 (10µM, Invitrogen) was added to the bath prior to the experiment to identify nuclei. A single cell within the VSMC monolayer was then injected with a micropipette loaded with Lucifer Yellow (700 µM, Invitrogen) and the
dye was allowed to diffuse to adjacent cells for five minutes. Images were recorded every 15 seconds. Both Hoechst 33342 (emission between 400-450nm) and Lucifer Yellow (emission > 550nm) were excited using two-photon excitation at 800nm by a MaiTai laser (Spectra-Physics, Mountain View, CA).
RESULTS

*LacZ localization in the renal cortex of Cx45+/− mice*

Kidneys from Cx45+/− mice, where one copy of the Cx45 gene is replaced by the LacZ reporter gene were sectioned and stained with X-Gal, in order to determine Cx45 renal expression. Expression of a transgenic reporter gene was used for localization instead of immunohistochemistry, since specific antibodies were not available. LacZ staining was found in the renal cortex, specifically in blood vessels and glomeruli (Fig 1A). While these results confirm previously published data on the gross renal localization of Cx45 (19), Cx45 expression within these structures was not investigated in earlier studies. Using higher power magnification, we were able to further elucidate the localization of Cx45 in the JGA. Both afferent and efferent arterioles were positive for LacZ, as well as the extra- and intraglomerular mesangium (Fig 1B and 1C). No LacZ labeling was detected in sections stained in parallel from Cx45+/+ mice, which served as a negative control for endogenous galactosidase activity. (Fig 1D). These results indicate that Cx45 is expressed throughout the vascular components of JGA.

*EGFP localization in the JGA of Cx45fl/fl:Nestin-Cre mice*

To provide a further line of evidence that Cx45 is expressed in the JGA, the expression of EGFP in kidney sections of Cx45fl/fl:Nestin-Cre mice was analyzed (Fig 2). In these mice, the coding region of Cx45 was flanked by loxP sites. Additionally, these mice expressed cre recombinase in cells expressing the intermediate filament protein nestin during development. In the nestin-positive cells, cre recombinase excised the floxed Cx45 coding region, leaving the EGFP reporter gene under the control of the
Cx45 promoter. Therefore EGFP expression indicates Cx45 expression only in cells that express nestin during development and express Cx45 in the adult.

At the JGA, strong EGFP signals were observed in vascular smooth muscle cells of both the afferent and efferent arterioles (Fig 2A-B), and presumably in extraglomerular and intraglomerular mesangial cells (Fig 2C). To verify JGA localization, a consecutive section was co-labeled with renin antibodies (Fig 2B). The renin labeling was consistent with the anticipated JGA location in the afferent arteriole. As predicted, the EGFP signal overlapped with the renin labeling confirming the expression of Cx45 in renin producing JG cells. EGFP was not detected in any endothelial cells.

Effects of Cx45 on renin expression and blood pressure

Since Cx45 was expressed in the JGA, it may play a role in renin expression and blood pressure regulation. Samples of whole kidney homogenate from Cx45fl/fl:Nestin-Cre mice (n=4) and C57BL/6 mice (control) (n=5) were run on SDS-PAGE gels, transferred and blotted for renin (Fig 3A). Densitometry analysis (Fig. 3B) revealed a significant up-regulation (approximately 50%) of renin expression in Cx45fl/fl:Nestin-Cre mice when compared to C57BL/6 mice (p<0.05).

Using spectrofluorometry, plasma renin activity was analyzed in C57BL/6 and Cx45fl/fl:Nestin-Cre mice (Fig. 3C). Plasma samples were mixed with a fluorogenic renin substrate and the emitted FRET-signal (representative of the generation of AngI) was detected. Renin activity (AngI generation) was plotted as a function of time and the initial activity was quantified. Plasma renin activity was significantly higher by approximately 70% in Cx45fl/fl:Nestin-Cre mice compared to control mice (control: 121
± 10; Cx45fl/fl:Nestin-Cre: 204 ± 24, n=5, p<0.05). This increase in plasma renin activity was comparable to the increase observed in captopril-treated mice (194 ± 14, p<0.05 vs. control, p>0.05 vs. Cx45fl/fl:Nestin-Cre).

The blood pressure of control and Cx45fl/fl:Nestin-Cre was measured by pressure transducer catheterization. C57BL/6 mice had an average mean arterial blood pressure (MAP) of 90 ± 2 mmHg (n=3), while Cx45fl/fl:Nestin-Cre had an average MAP of 116 ± 5 mmHg (n=3) (Fig. 3D). The 28% increase in MAP in Cx45fl/fl:Nestin-Cre mice was found to be significant (p<0.05)

Characterization of vascular smooth muscle cells from C57BL/6 and Cx45fl/fl:Nestin-Cre mice

VSMCs derived from Cx45fl/fl:Nestin-Cre mice were positive for EGFP (Fig 4A), indicating that the Cx45 coding region was excised in these cells. Under the same microscope and laser settings, no EGFP signal was observed in VSMCs cultured from C57BL/6 (control VSMC) mice (Fig 4B).

In order to quantify the excision of the Cx45 coding region from Cx45fl/fl:Nestin-Cre VSMCs, protein extracts from C57BL/6 (control) and Cx45fl/fl:Nestin-Cre VSMCs were run on SDS-PAGE gels, transferred to membranes and probed with Cx45 antibodies (Fig 4C). Whole kidney homogenate from C57BL/6 and Cx45fl/fl:Nestin-Cre mice were also analyzed by immunoblot to provide a means to compare Cx45 expression in a cell culture with total renal Cx45 expression. A single band approximately 50 kDa in size appeared on all blots probed with Cx45 antibodies. Cx45 expression was approximately 50% lower in both VSMCs and kidneys from the Cx45fl/fl:Nestin-Cre mouse compared
to control VSMCs and kidneys based on densitometry (not shown), indicating effective Cx45 excision in a significant cell population of Cx45fl/fl:Nestin-Cre VSMCs and kidneys. Both blots were also probed with GAPDH as a confirmation of equal protein loading (Fig. 4C).

Since VSMCs are subject to phenotypic changes in culture, we sought to identify any differences in Cx expression that may have arisen during the culture process. Total RNA was purified from confluent C57BL/6 (control) and Cx45fl/fl:Nestin-Cre VSMC and amplified by RT-PCR with primers for Cxs 37, 40 and 43. Bands at the predicted band size (344bp, 323bp and 391bp) were detected for both control and Cx45fl/fl:Nestin-Cre VSMC samples (Fig 4D). Amplification with a β-actin primer pair, which served as a positive control for the experiment, also produced bands of the expected size (400bp) for both cell culture types.

Effects of Cx45 on calcium wave propagation in vascular smooth muscle cells

When control and Cx45fl/fl:Nestin-Cre VSMCs were loaded with Fluo-4/Fura red and one VSMC in the center of the microscope field was mechanically stimulated with a glass micropipette, an increase in \([Ca^{2+}]_i\) in the stimulated cell was observed (Fig 5A). This increase in \([Ca^{2+}]_i\) propagated to adjacent cells (Fig 5A).

The propagation speed (µm/s) of the calcium wave was analyzed in control, Cx45fl/fl:Nestin-Cre, and pharmacologically treated control VSMCs (n=6 for all experimental groups) (Fig 5B). In control VSMCs, the propagation speed was 17 ± 5 µm/s. The speed of propagation in Cx45fl/fl:Nestin-Cre VSMCs was significantly lower (7 ± 1 µm/s, p<0.05). The non-specific gap junction inhibitor 18α-GA failed to
significantly lower the speed of calcium wave propagation in control VSMCs (12.4 ± 2.0 µm/s, p>0.05), however control VSMCs treated with a Cx45-specific gap mimetic peptide (GAP) did exhibit a significant reduction in calcium wave propagation speed (5.7 ± 1.6 µm/s, p<0.05).

Since these experiments pointed to a role for Cx45 in calcium wave propagation we sought to establish whether Cx45 regulates calcium wave propagation indirectly by an extracellular agent such as ATP or directly via intercellular coupling. Generally, calcium wave speeds above 100 µm/s are thought to be due to direct coupling between cells (28, 31), while slower calcium wave speeds (as we observed) are associated with the release of ATP via Cx hemichannels (9, 36). To test whether ATP played a role in the calcium wave propagation, control VSMCs were treated with the non-selective purinergic receptor antagonist suramin (Fig 5B). This treatment did not significantly reduce calcium propagation speed when compared to control (10.3 ± 2.4 µm/s, p>0.05). To determine whether VSMCs were directly coupled, a dye spreading assay was conducted. A single VSMC was loaded by microinjection with Lucifer Yellow (Fig 5C). Lucifer Yellow, which does not cross cell membranes but does permeate gap junctions, spread to adjacent VSMCs within 60 seconds of injection, indicating that cultured VSMCs were coupled by gap junctions.
DISCUSSION

Here we report that Cx45 is found in the renal cortex of the adult mouse kidney, specifically in the vasculature and glomeruli. In the JGA, Cx45 was expressed in the mesangium and the smooth muscle cells of the afferent and efferent arterioles, and in renin producing JG cells. Both renal renin expression and plasma renin activity were markedly increased in Cx45fl/fl:Nestin-Cre mice, which are considered deficient in their JGA expression of Cx45. VSMCs cultured from afferent arterioles of Cx45fl/fl:Nestin-Cre mice had reduced calcium wave propagation speed when compared to VSMCs cultured from C57BL/6 mice. This decrease in speed was replicated in control VSMCs treated with a Cx45-specific gap mimetic peptide. The non-specific gap junction blocker 18α-GA did not significantly reduce propagation speed.

Due to the lack of specific antibodies against Cx45, two transgenic mice with reporter gene constructs were studied to determine the intrarenal localization of Cx45. Using mice expressing the LacZ gene under the control of the Cx45 promoter, Cx45 was detected in glomeruli and vascular structures of the renal cortex, which affirms previous findings in these mice (19) (Fig. 1A). In addition, we observed β-gal staining under higher magnification and identified positive structures as the afferent and efferent arterioles, glomeruli, and mesangial cells (Figs. 1B-C). We utilized a second method to substantiate these findings. Cx45fl/fl:Nestin-Cre mice have a deletion of the Cx45 coding region in cells expressing nestin during embryogenesis. While nestin is typically thought of as a neuronal marker, it is also expressed during development by the metanephric mesenchyme, the progenitor to all renal cell types, except collecting duct epithelial cells (8). Therefore Cx45 is functionally knocked-out in JGA cells and its expression is
replaced by EGFP expression. By detecting EGFP signal in Cx45fl/fl:Nestin-Cre mice we observed a continuous Cx45 labeling in vascular smooth muscle cells of the afferent and efferent arteriole and the adjacent extraglomerular mesangial cells (Figs. 2A-C). This localization data suggests the possibility of fast and direct coupling between the afferent and efferent arterioles and the mesangium. There is indeed evidence for simultaneous propagation of the TGF calcium wave into these areas (26). The colocalization of EGFP with renin in the afferent arteriole (Fig. 2B) suggests that this Cx isoform may also play a role in renin synthesis and release.

The importance of Cx45 to the vasculature was already evident from previous studies. Cx45-deficient mice are characterized by abnormal development of the vasculature and this genetic modification proves to be lethal (19). Functional Cx45 gap junctions have also been discovered in the vascular smooth muscle cells of cerebral arteries, where it has been suggested that they play a role in regulating blood flow in the nervous system (21, 22). However, the appearance of Cx45 in the renal vasculature does not allow us to presume a functional role for the protein.

Therefore, to ascertain the physiological relevance of Cx45 in the kidney, in-vivo and in-vitro experiments were performed with Cx45fl/fl:Nestin-Cre mice. These mice had increased renin expression and activity and increased mean arterial pressure (Fig. 3). These changes in blood pressure and the renin-angiotensin system (RAS), as well as the localization, mirror those observed in another Cx transgenic mouse, the Cx40 deficient mouse (Cx40−/−) (18, 38). Cx40−/− mice have renin-dependent hypertension. Wagner et al (38) concluded that this hypertension is due to a failure to properly engage the AngII and
intrarenal blood pressure negative feedback loops on renin. Clearly, both mouse model studies indicated that Cxs exert an effect on renal blood pressure regulation.

There are also differences between our study and those performed in the Cx40\textsuperscript{-/-} model. While Cx45fl/fl:Nestin-Cre mice had a significant elevation in blood pressure, they were not hypertensive. This limited blood pressure increase may be due to a restriction of the gene knockout to nestin-expressing cells, as opposed to the systemic knockout in the Cx40\textsuperscript{-/-} mouse model. As with Cx40\textsuperscript{-/-}, the elevated blood pressure we observed does appear to be RAS-dependent in nature, given the increase in renin levels and activity. However, the precise feedback mechanism through which renin is dysregulated in Cx45fl/fl:Nestin-Cre mice requires further study. Blood pressure regulation and renin secretion can also be regulated by the renal sympathetic nerve (12). Therefore, we cannot exclude the possibility that the observed elevations in blood pressure and renin were due to the effects of a conditional knockout in other Cx45- and nestin-expressing cells, including those of the nervous system (33, 39, 43). However, the effects of Cx45 loss in isolated VSMC primary cultures on calcium waves does suggest that Cx45 plays a role in JGA function at the local level and independent of sympathetic innervation. In addition to in-vivo and in-vitro analysis of Cx45fl/fl:Nestin-Cre mice, we also attempted to block Cx45 function pharmacologically. Surprisingly, the non-specific gap junction inhibitor 18\alpha-GA did not significantly reduce VSMC calcium wave propagation in control VSMC. Recently published data in mouse embryonic stem cells suggests that 18\alpha-GA may not inhibit Cx45 (40). In these cells (which express Cx31, 43, and 45), inhibition of gap junction intracellular communication by 18\alpha-GA required the expression of Cx43. It has been established that Cx45 and Cx43 can interact together to
form functional gap junctions (11, 40), but only Cx43 mRNA, and not protein, has been found in the smooth muscle cells of the renal vasculature (2, 42). Therefore, it seems unlikely that Cx43 and Cx45 form heteromeric channels in VSMCs and the lack of these channels could explain the inability of 18α-GA to reduce calcium wave propagation we observed.

A previously developed Cx45 gap mimetic peptide was also used to inhibit of Cx45 (21). The Cx45 peptide was designed to be homologous to earlier identified Cx43 and Cx40 blocking peptides. By patch-clamping smooth muscle cell pairs, it was demonstrated that the Cx45 peptide altered conductance in a manner that was consistent with channel blockade (21). In applying the Cx45 peptide to control VSMC, we were able to reduce calcium wave propagation to levels observed with Cx45fl/fl:Nestin-Cre VSMC. However, our study marks the first attempt to use this mimetic peptide in a cell culture model and the results therefore should be interpreted with caution.

One possible mechanism through which Cx45 could affect renin regulation and blood pressure is calcium signaling. Basolateral ATP released from the macula densa (3) initiates a propagating calcium wave in the JGA and beyond (26) during TGF that controls renal blood flow and glomerular filtration rate (29). The increase in $[\text{Ca}^{2+}]_i$ accomplishes two mechanisms: inhibition of renin release from JG cells (30) and contraction of VSMCs in the afferent arterioles (41). Gap junctions are known to be instrumental in calcium wave propagation in several cells types (9, 13, 34) and it has been previously demonstrated that the calcium wave of TGF can be abolished by gap junction blockers (26).
Our observation that calcium wave propagation in VSMCs is dependent on the expression and function of Cx45 (Fig 5B) suggests that Cx45 may play a role in the propagation of TGF calcium wave (26). Calcium propagation involving Cxs occurs by two mechanisms: either by intercellular gap junction communication or via the release of an extracellular mediator such as ATP. Calcium wave propagation speeds above 100 µm/s have previously been found in intact preglomerular smooth muscle cells (28). In the present study however, the VSMC calcium propagation speeds measured were at least 5-fold slower suggesting that the calcium wave in cultured VSMCs does not rely solely on fast and direct gap junctional coupling, but instead involves an extracellular mediator, such as ATP. A recently published paper from Toma et al (36) examined the role of Cx40 on calcium wave propagation in a glomerular endothelial cell (GENC) culture. Calcium wave propagation speeds similar to those we presently observed in VSMCs were recorded in GENCs and the authors concluded that Cx40’s control of calcium wave propagation was mediated by ATP (36). It is well established that extracellular ATP can cause cell-to-cell calcium signaling via Cxs (9).

However, when we tested this slow wave hypothesis in cultured VSMCs, our data pointed towards gap junction intercellular communication as the mechanism behind the calcium wave. The purinergic receptor antagonist suramin failed to significantly reduce calcium wave propagation speeds and a dye spreading assay using lucifer yellow provided evidence of direct VSMC coupling. Our findings are supported by several other studies that have reported intercellular coupling in both smooth muscle cultures and preparations (27, 31). The reason for the slow calcium wave propagation in VSMCs in spite of the presence of direct gap junctional coupling is unknown, but the discrepancy
could be possibly explained by the different techniques (cultured VSMCs versus intact vessels) used. Future investigation of the TGF calcium wave in intact preglomerular vessels from control and Cx45fl/fl:Nestin-Cre mice could further support the physiological significance of Cx45 in the propagation of calcium waves in the JGA.

In comparing our findings to the recent work on Cx40 in GENCs (36), Cxs appear to be a critical factor for calcium signaling in the JGA, but they seem to use at least two different mechanism to achieve calcium wave propagation: direct gap junctional coupling (Cx45) and cell-to-cell signaling via extracellular ATP (Cx40). The different characteristics of the two cell types (endothelial cells vs. smooth muscle cells) or the functional differences that exist between the Cx40 and 45 isoforms may explain why both indirect and direct methods of calcium wave propagation occur in the JGA.
PERSPECTIVES AND SIGNIFICANCE

In conclusion, in this study we reported the localization of Cx45 to the renal cortical vasculature, glomeruli, and the JGA region. In the JGA, the afferent and efferent arterioles, and intra- and extraglomerular mesangial cells were all Cx45-positive. Renin expression, plasma renin activity, and blood pressure were all increased significantly in Cx45fl/fl:Nestin-Cre, which have reduced JGA Cx45 expression. The speed of calcium wave propagation in VSMC cultured from Cx45fl/fl:Nestin-Cre mice was significantly lower than in control VSMCs. Treatment of control VSMCs with a Cx45-specific gap mimetic peptide also reduced calcium wave propagation. Blockade of purinergic receptors failed to reduce calcium wave propagation, while a dye spreading assay provided evidence of cell-to-cell coupling between VSMCs. The localization of Cx45, its effects on renin and calcium wave propagation all suggest a role for Cx45 in TGF, renin regulation and systemic blood pressure maintenance. While, the precise mechanism through which Cx45 controls these regulatory systems remains to be determined, a model that utilizes intercellular gap junction communication is likely involved.
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REFERENCES


FIGURE LEGENDS

Figure 1: Localization of Cx45 transcription in Cx45^{+/−} mouse kidney sections by X-Gal staining. A: β-gal was detected in the renal cortex, predominately in vasculature (arrow) and glomeruli (arrowhead). B and C: Within the juxtaglomerular apparatus (JGA), β-gal staining was observed in the afferent (AA) and efferent arterioles (EA) as well as the extra- and intraglomerular mesangial cells (arrows). D: No β-gal staining was detected in sections from Cx45^{+/+} mouse kidneys; G: glomerulus. Bar = 50µm.

Figure 2: Detection and localization of EGFP in Cx45fl/fl:Nestin-Cre mouse kidney sections. EGFP expression is controlled by the Cx45 promoter only in cells that expressed nestin during development. A: EGFP (green) was detected in the afferent and efferent arterioles (AA, EA) and in the extraglomerular mesangial cells. G: glomerulus; Arrow: macula densa. B: Sections were labeled for renin (red) in a consecutive serial section. EGFP was co-localized to the renin positive region of the AA. C: EGFP was also found in the extraglomerular mesangium (arrow). Bars = 20µm.

Figure 3: Renal renin expression, plasma renin activity, and systemic blood pressure in C57BL/6 and Cx45fl/fl:Nestin-Cre mice. A: C57BL/6 (control, n=5) and Cx45fl/fl:Nestin-Cre (n=4) kidney homogenate samples were blotted with renin antibodies. B: Densitometric analysis of immunoblot indicated that the expression of renin in Cx45fl/fl:Nestin-Cre mice was significantly higher than that of C57BL/6 mice (*p<0.05). C: Plasma renin activity was measured in real-time using a FRET-based fluorogenic renin substrate. Renin activity was significantly higher in Cx45fl/fl:Nestin-
Cre mice compared to C57BL/6 mice and this increase was comparable to the increase observed in captopril-treated mice (*p<0.05, n=5 per group). D: Mean arterial blood pressure was significantly increased by approximately 30% in Cx45fl/fl:Nestin-Cre mice (*p<0.05, n=3 per group). Values are mean+SE.

Figure 4: Characterization of vascular smooth muscle cell (VSMC) primary cultures from C57BL/6 and Cx45fl/fl:Nestin-Cre mice. A: EGFP expression (green) was detected in VSMC derived from Cx45fl/fl:Nestin-Cre mice. B: Under the same microscope and laser settings VSMCs derived from C57BL/6 mice (control VSMC) were negative for the EGFP signal. Differential interference-contrast (DIC) background was added and merged with fluorescence in A and B. 40x magnification. C: Immunoblots with protein samples from C57BL/6 VSMCs and kidney (control) and Cx45fl/fl:Nestin-Cre VSMCs and kidney probed for Cx45 and the loading control GAPDH. Cx45 expression was detected in all four samples, but expression was reduced in Cx45fl/fl:Nestin-Cre VSMCs and kidney extracts. D: Cxs 37, 40, and 43 mRNA was detected in C57BL/6 (control) and Cx45fl/fl:Nestin-Cre VSMCs by RT-PCR. mRNA from all three Cxs at their expected bands sizes (344bp, 323bp, and 394bp respectively) was observed. β-actin (400bp) served as a positive control.

Figure 5: Calcium wave propagation in vascular smooth muscle cells (VSMC) from C57BL/6 and Cx45fl/fl:Nestin-Cre mice. A: Representative pseudocolor Fluo-4/Fura red ratio images from control VSMC shown at different time points (0-8 s) after the stimulation of a single cell (indicated by X). B: Speed of calcium wave propagation in control VSMC, Cx45fl/fl:Nestin-Cre VSMC, and control VSMC treated with 18α-
glycyrrhetinic acid (18α-GA), Cx45 gap mimetic peptide (GAP), or suramin. Calcium wave propagation was significantly slower in Cx45fl/fl:Nestin-Cre and GAP-treated VSMC (n=6, *p<0.05). Bar= 20µm. C: The gap junction permeable dye Lucifer Yellow was loaded by microinjection into a single VSMC (indicated by X) and spread to adjacent cells within 60 seconds. Nuclei were labeled blue with Hoechst 33342. Bar= 10µm.
Figure 1
Figure 2
Figure 3

A

Cx45fl/fl:Nestin-Cre Control

50kDa

B

Normalized band intensity

control Cx45fl/fl: Nestin-Cre

C

Plasma renin activity (U)

control Cx45fl/fl: Nestin-Cre captopril

D

Blood pressure (mmHg)

control Cx45fl/fl: Nestin-Cre
Figure 5

A

![Image of Ca²⁺ concentration over time](image)

B

![Graph showing speed comparison](image)

C

![Images showing cellular activity over time](image)