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Molecular Cardiology

Endothelial Cilia Are Fluid Shear Sensors That Regulate Calcium Signaling and Nitric Oxide Production Through Polycystin-1

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Background—When challenged with extracellular fluid shear stress, vascular endothelial cells are known to release nitric oxide, an important vasodilator. Here, we show that the ability of cultured endothelial cells to sense a low range of fluid shear depends on apical membrane organelles, called cilia, and that cilia are compartments required for proper localization and function of the mechanosensitive polycystin-1 molecule.

Methods and Results—Cells with the $Pkd1^{null/null}$ or $Tg737^{orpk/orpk}$ mutation encoded for polycystin-1 or polaris, respectively, are unable to transmit extracellular shear stress into intracellular calcium signaling and biochemical nitric oxide synthesis. Cytosolic calcium and nitric oxide recordings further show that fluid shear sensing is a cilia-specific mechanism because other mechanical or pharmacological stimulation does not abolish calcium and nitric oxide signaling in polycystin-1 and polaris mutant endothelial cells. Polycystin-1 localized in the basal body of $Tg737^{orpk/orpk}$ endothelial cells is insufficient for a fluid shear stress response. Furthermore, the optimal shear stress to which the cells respond best does not alter the apical cilia structure but modifies the responsiveness of cells to higher shear stresses through proteolytic modification of polycystin-1.

Conclusions—We demonstrate for the first time that polycystin-1 (required for cilia function) and polaris (required for cilia structure) are crucial mechanosensitive molecules in endothelial cells. We propose that a distinctive communication with the extracellular microenvironment depends on the proper localization and function of polycystin-1 in cilia. (***Circulation**. 2008;117:1161-1171.)*

Key Words: blood flow ■ blood pressure ■ endothelium ■ endothelium-derived factors ■ physiology ■ polycystic kidney diseases

Primary cilia are protruding structures found on the apical membranes of many cell types. The structure and maintenance of these cilia are regulated by intraflagella transport molecules, also known as intraflagella transport particles. Mutations in *Tg737*, an orthologous gene of *Chlamydomonas flagella IFT88*, results in cells with no cilia or shorter cilia.[1,2] Mice with mutations in the *Tg737* gene, encoding polaris, develop polycystic kidney disease (PKD).[3] Mutations in *Pkd1*, encoding polycystin-1, also result in PKD.[4] Polycystin-1 has been shown to localize in primary cilia and to mediate the sensitivity of kidney epithelial cells to fluid shear stress.[5,6] With its large extracellular domain and remarkable mechanical strength,[7] polycystin-1 has been proposed to be a mechanosensitive molecule.[8–10]

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Although enlarged cystic kidneys are the most obvious phenotype in PKD, extrarenal manifestations also are frequent. These include, for example, aneurysms, mitral valve prolapse, and other abnormalities in the vasculature, pancreas, and liver.[11] In particular, hypertension is observed in patients with PKD about a decade earlier than in the general population, even before the loss of renal function.[12,13] Although the mechanism underlying hypertension-related cardiovascular defects in PKD is not completely understood, the aggressive control of hypertension in PKD patient is clinically necessary and practically relevant.[12,13]

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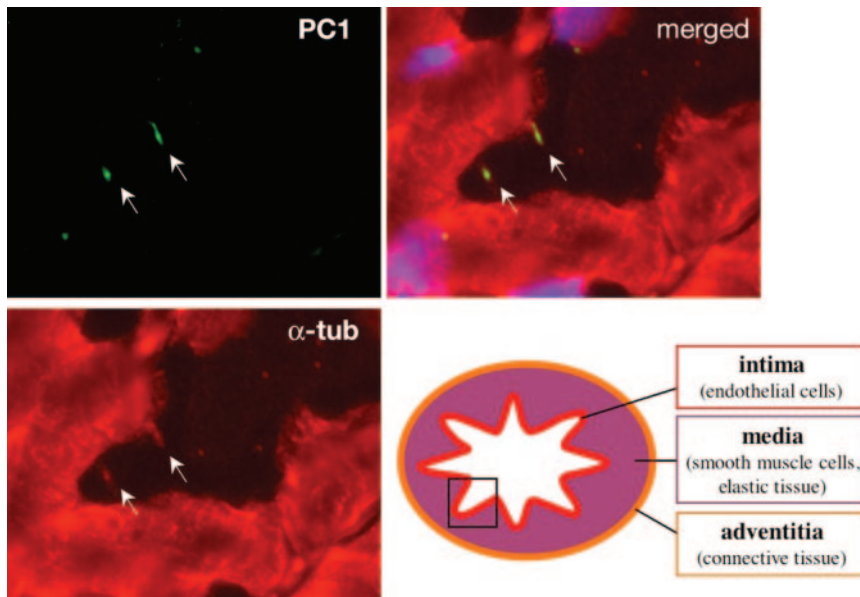
Figure 1. Polycystin-1 is localized in aortic endothelial cilia of embryonic mouse aorta. Isolated aorta from embryonic mouse was gently perfused with PBS to remove the remaining blood on the aorta wall and to help prevent the segment from collapsing. The aorta segment was cut to a thickness of 5 μm. The segment was stained for polycystin-1 (PC1; green) and the ciliary marker acetylated α-tubulin (α-tub; red). A merged image with nuclear marker (blue) also is shown. Arrows indicate specific staining for polycystin-1 and cilia. Images were taken at a magnification of ×90. The figure at the bottom right represents the region of an aorta segment where the micrograph of the section was taken, designated by a black box.

The endothelial cells that line blood vessels and the chambers of the heart have the ability to sense external mechanical forces such as fluid shear stress. Although shear stress can activate many biochemical and molecular pathways in endothelial cells, the exact mechanism of mechanical sensing and mechanochemical conversion is not known. It is known, however, that with changes in external fluid shear stress, endothelial cells exhibit an increase in cytosolic calcium followed by release of nitric oxide (NO) that is critical for control of vascular contractility.[14] Furthermore, abnormalities in NO production or release have been associated with hypertension.[15] In the present study, we show that primary cilia are mechanosensory organelles in endothelial cells. The mechanosensory function of cilia further depends on the structural polaris molecule and the mechanosensing polycystin-1 protein. We propose that primary cilia may play important and distinct roles in sensing and transducing extracellular fluid shear stress into intracellular signaling and biochemical responses in vascular endothelial cells.

Methods

Embryonic Endothelial Cell Culture

Timed pregnancies were generated by intercrossing mice double heterozygous for the *Tg737[orpk]* or *Pkd1[null]* allele and the temperature-sensitive SV40 large T antigen (Charles River Laboratories, Wilmington, Mass). Because both *Pkd1[null/null]* and *Tg737[orpk/orpk]* mice are lethal at the embryonic stage,[3,16] aortas were isolated from 15.5-day embryos. Aortas from homozygous or wild-type embryos were dissociated with 1× trypsin/EDTA solution through a 1-cm³ 25G⅝ needle and plated in DMEM containing 2% fetal bovine serum, 0.75 μg/L interferon (IFN)-γ, 1.0 g/L insulin, 0.67 mg/L sodium selenite, 0.55 g/L transferrin, 0.2 g/L ethanolamine, 36 ng/mL hydrocortisone, 0.10 μmol/L 2,3,5-triido-L-thyronine, 100 U penicillin-G (base) combined with 0.30 mg/mL additional glutamine, 100 μg streptomycin sulfate, and 0.1 mmol/L citrate to maintain penicillin potency. All cell-culture supplements were obtained from Invitrogen, Inc (Carlsbad, Calif) except IFN-γ, hydrocortisone, and 2,3,5-triido-L-thyronine, which were obtained from Sigma (St Louis, Mo). Unless otherwise indicated, cells were grown at 37°C in the absence of IFN-γ for 4 to 5 days before experiments.

Fluorescence-Activated Cell Sorting

After trypsinization, 10^6 cells/mL were incubated with 10 mg/mL of the endothelial marker intracellular adhesion molecule-2 (ICAM-2; Santa Cruz Biotechnology, Santa Cruz, Calif). Fluorescein isothiocyanate (FITC)–conjugated ICAM-2 antibody was applied for 1 hour at room temperature at a dilution of 1:100 in PBS containing 1% fetal bovine serum to prevent any nonspecific binding of the antibody. After cells were washed 3 times to avoid nonspecific binding, they were analyzed with FACScan (Becton Dickinson, Franklin Lakes, NJ) at a wavelength of 525 nm (FITC, FL-1). Negative control cells were obtained exactly like cells from experimental groups except that FITC-conjugated anti-mouse antibody was used.

Western Blotting and Immunoprecipitation

Cells/tissues were lysed with 1× radioimmunoprecipitation assay buffer. Intracellular contents were collected by centrifugation at 100*g* for 10 minutes. Total cell lysate was analyzed by SDS-PAGE. In some experiments, cells were first challenged with different magnitudes of fluid shear stress (0, 1.1, or 7.2 dynes/cm²) for 10, 20, or 30 minutes. Cells were then rinsed vigorously with a small amount of lysis buffer[17] composed of 10 mmol/L EGTA, 5 mmol/L NaF, and a tablet of protease inhibitor mixture (Roche Applied Bioscience, Basel, Switzerland) in phosphate buffer, pH 7.2. Cell lysate was then subjected to immunoprecipitation study with anti–polycystin-1 (1:5 dilution). For Western blot, anti-SV40 (1:400 dilution; Santa Cruz Biotechnology), anti-endothelial NO synthase (eNOS; 1:200 dilution; Abcam, Cambridge, Mass), anti-actin (1:500 dilution; Sigma), or anti-polycystin-1 (1:50; P-15, Santa Cruz Biotechnology) antibodies were incubated with the blots and then with a peroxidase-conjugated secondary antibody (1:7500 dilution; Amersham Biosciences, Inc, Piscataway, NJ) for 1 hour each at room temperature.

Immunolocalization Analysis

Endothelial cells were grown to confluence and full differentiation. Cells were fixed with 4% paraformaldehyde in 2% sucrose solution for 10 minutes at room temperature. Cells were then incubated with anti-CD31 antibody (1:50 dilution; Sigma) for an hour followed by an FITC-labeled anti-mouse (1:500 dilution). For double labeling, an antibody to acetylated α-tubulin (1:10 000 dilution; Sigma) was premixed with a Texas Red–labeled anti-mouse (1:1000 dilution). The mixture was then added to the cells, and the cells were stained with 4′,6-diamidino-2-phenylindol (DAPI). To identify the expression patterns of polaris and polycystin-1, samples were incubated overnight at 4°C with anti–polycystin-1 antibody[8] (1:200 dilution)
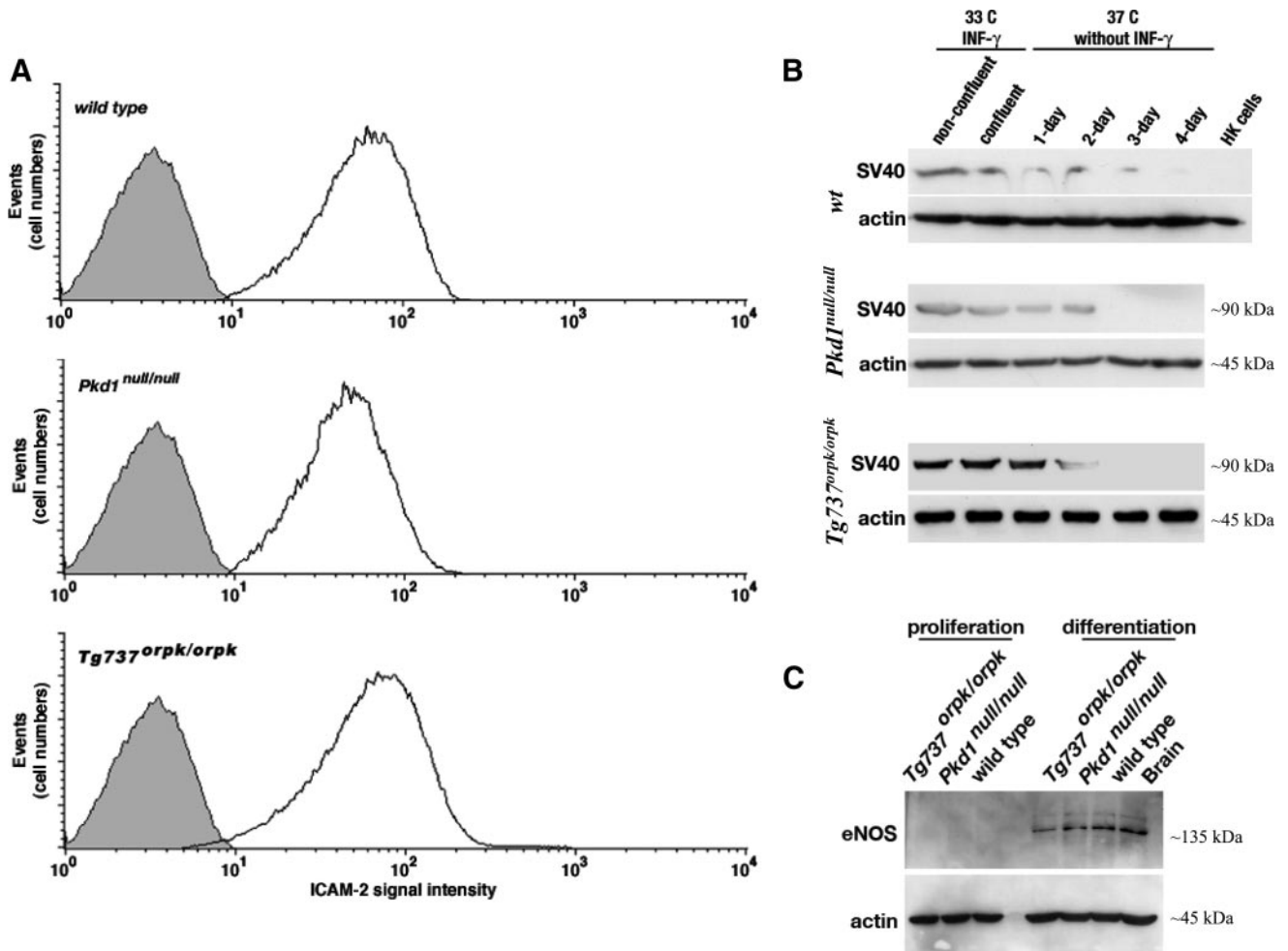
Figure 2. Endothelial cells express ICAM-2 and eNOS and are regulated by temperature and IFN-γ. A, Fluorescence cell-sorting study shows that cultured endothelial cells from mouse embryonic aortas are positive for the endothelial marker ICAM-2 (open curves). Cells treated with secondary mouse antibody were used as a negative control (filled curves). n=3. B, Endothelial cells under permissive conditions at 33°C in the presence of IFN-γ express SV40 large T antigen. When these cells are grown under nonpermissive conditions at 37°C in the absence of IFN-γ for 3 to 4 days, their expression of SV40 large T antigen can be suppressed. Human kidney (HK) cells were the negative control. Actin was used as the loading control. n=1. C, Western blot analysis confirms the expression of eNOS in differentiated cells. Cells undergoing proliferation do not show eNOS expression. Mouse embryonic brain tissue was used as a positive control. n=3.

and subsequently with an FITC-labeled anti-rabbit (1:500 dilution) antibody for 1 hour at room temperature. An antibody to polaris[18] (1:100 dilution) was premixed with a Texas Red–labeled anti-rabbit antibody (1:1000 dilution) and incubated with the samples for 1 hour at room temperature.

Calcium and NO Microfluorimetry

A nonfluorescent, CO_2-independent medium for imaging was formulated (pH 7.3) that contained (in mmol/L) $CaCl_2$ 1.26, $MgSO_4$ 0.81, KCl 5.4, KH_2PO_4 0.44, NaCl 137, Na_2HPO_4 0.34, D-glucose 5.6, L-glutamine 2.0, sodium pyruvate 1.0, HEPES 20, and 2% serum.[8] All chemicals were purchased from Sigma.

Cells were incubated for 30 minutes with the calcium-sensitive probe Fura 2AM (5 μmol/L) and/or NO-sensitive probe 4-amino-5-methyl-amino-2',7'-difluorofluorescein (DAF-FM; 20 μmol/L) at 37°C. These fluorescent probes were purchased from Invitrogen, Inc. Cells were then washed 3 times to remove excess probe. Cells were positioned under a Nikon TE2000 microscope equipped with a Coolsnap EZ cooled charge-coupled device monochrome digital camera using IPLab software for Macintosh (Scanalytics Inc, Billerica, Mass) or Metamorph/Metaflur software for PC (Molecular Devices Corp, Downington, Pa). Paired Fura images at excitation wavelengths of 340 and 380 nm were captured through an emission

filter of 510 nm.[19] DAF images at an excitation wavelength of 495 nm were captured through an emission filter of 515 nm.[20] In some experiments in which both calcium and NO signals were captured sequentially, a long-pass emission filter was used.

For fluid shear stress experiments, cells were placed in a perfusion chamber that was 0.0254 cm thick and 1.0 cm wide (GlycoTech, Gaithersburg, Md). Before a shear stress of 0.7 to 50 dynes/cm² was applied to the monolayer cells, they were equilibrated in fluid shear stress of 0, 1.1, or 7.2 dynes/cm² for 30 minutes. For experiments with mechanical cell pressing, a glass micromanipulator needle was first coated with BSA. Once the distance of the needle from a single targeted cell was determined, the needle was programmed to that position on the apical membrane of the cell with an automated and motorized controller (Eppendorf, Westbury, NY). In other experiments, 1 μmol/L acetylcholine (Sigma) was added to the cell population.

Statistical Analysis

All quantifiable experimental values are expressed as mean±SEM, and values of $P<0.05$ were considered statistically significant. All comparisons among means were performed using ANOVA with post hoc comparisons via Fisher's protected least-significant-difference test. All comparisons between 2 experimental groups were analyzed
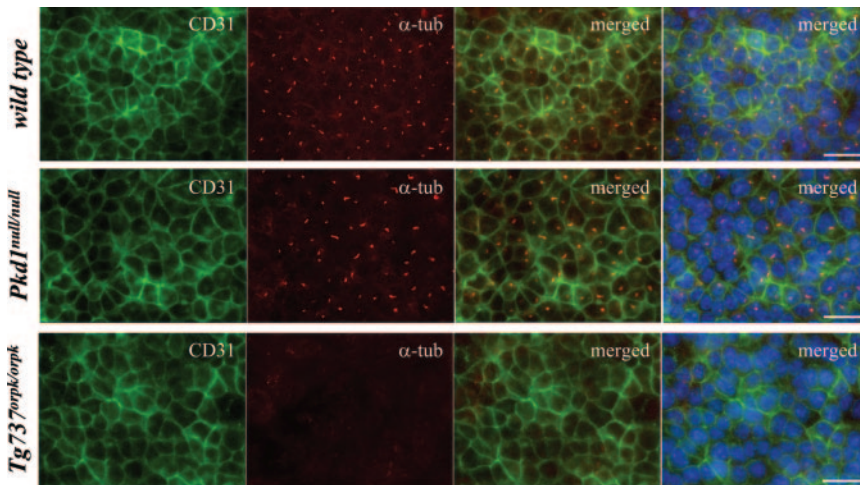
Figure 3. Endothelial cells express CD31 and have primary cilia. Cultured wild-type (top), *Pkd1^null/null^* (middle), and *Tg737^orpk/orpk^* (bottom) cells were stained for the endothelial marker CD31 (green), the ciliary marker acetylated α-tubulin (α-tub; red), and the nuclear marker DAPI (blue). Merged images with and without nuclear marker show that all cells were positive for CD31 and that each endothelial cell, except *Tg737^orpk/orpk^* cells, had 1 primary cilium. Bar=25 μm. n=2.

with the appropriate Student *t* tests. The numbers of experiments were determined sufficient if the statistical power analysis with a coefficient variant was ≤20%.

For immunofluorescence study, fluorescence images of the control and experimental groups were captured with the same exposure time and binning. Images were captured at several different planes of focus (z stack). The stack of immunofluorescence images was then analyzed 3-dimensionally with an up-to-date Metamorph version 7.0 software analysis program. In some cases, when the images were to be cropped, resized, or both, images from all control and experimental groups were treated exactly the same without altering their pixel calibration values.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Primary Cilia in Embryonic Endothelial Aorta

Primary cilia have been observed in cultured human umbilical vein endothelial cells.[21] More recently, the presence of endothelial cilia in the endocardium of the developing chicken was reported.[22] In this study, we show that cilia also are present in aortic endothelia of the embryonic E15.5 mouse (Figure 1). Primary cilia were identified with the ciliary marker acetylated α-tubulin. The presence of polycystin-1 in cilia also was observed. To further study the cellular and molecular functions of the cilia and polycystin-1, we isolated and characterized endothelial cells from *Pkd1^null/null^* and *Tg737^orpk/orpk^* mouse models.

Characterization of Endothelial Cells

Aortic endothelial cells were isolated from wild-type, *Pkd1^null/null^*, and *Tg737^orpk/orpk^* embryonic mice. The purity of these endothelial cells was verified by confirming their expression of ICAM-2, a well-established endothelial marker involved in inflammation[23] and angiogenesis[24] (Figure 2A). These endothelial cells were immortalized with the SV40 gene, the promoter of which is regulated by temperature and IFN-γ. We confirmed that cells grown under permissive conditions in the presence of 0.75 μg/L IFN-γ at 33°C express SV40 large T antigen regardless of the status of their confluence (Figure 2B). When switched to nonpermissive conditions in the absence of IFN-γ at 37°C, the endothelial cells completely shut down the SV40 gene. Cells under nonpermissive

conditions were grown to differentiated state, and their expression of eNOS, a general marker for endothelial cells, was confirmed. Hyperproliferative cells under permissive conditions did not express eNOS (Figure 2C).

To further characterize the endothelial cells, we performed an immunofluorescence study by using CD31, another well-accepted endothelial marker. CD31, also known as platelet/endothelial cell adhesion molecule-1, is an important cell adhesion molecule involved in endothelial cell-cell contact, cell migration, angiogenesis, wound healing, and other processes.[25] The study demonstrates that all endothelial cells were stained positive for CD31 (Figure 3). When the cells were characterized for the presence of cilia with acetylated α-tubulin as a ciliary marker, each wild-type and *Pkd1^null/null^* cell was shown to possess a single primary cilium (Figure 3). Note that some of the cilia were not visible because of a different focus level at different cell heights (z distance) relative to the apical membrane of neighboring cells. On the other hand, no cilia or short stubby cilia were observed in *Tg737^orpk/orpk^* cells.

Mechanosensory Functions of Polaris and Polycystin-1

We next performed immunofluorescence microscopic analysis on wild-type, *Pkd1*, and *Tg737* cells to confirm the cellular expression and localization of polaris and polycystin-1 (Figure 4A). Immunofluorescence study of wild-type endothelial cells confirmed that each cell possesses a single primary cilium on its apical surface and expresses both polycystin-1 and polaris in the cilia. Ciliary polycystin-1 is absent from the primary cilia of *Pkd1^null/null^* cells, although polaris is present. In *Tg737^orpk/orpk^* cells, no polaris is detected, whereas polycystin-1 appears to be concentrated in the basal body.

To test our hypothesis that cilia are mechanosensitive organelles, we challenged the cells with various magnitudes of fluid shear stress (Figure 4B). When cells were equilibrated for 30 minutes under static condition (0 dynes/cm^2), we found that wild-type mouse embryonic endothelial cells responded best to optimal fluid shear stress of 7.2 dynes/cm^2 by increasing their cytosolic calcium. When cells were equilibrated for 30 minutes under a low shear stress (1.1
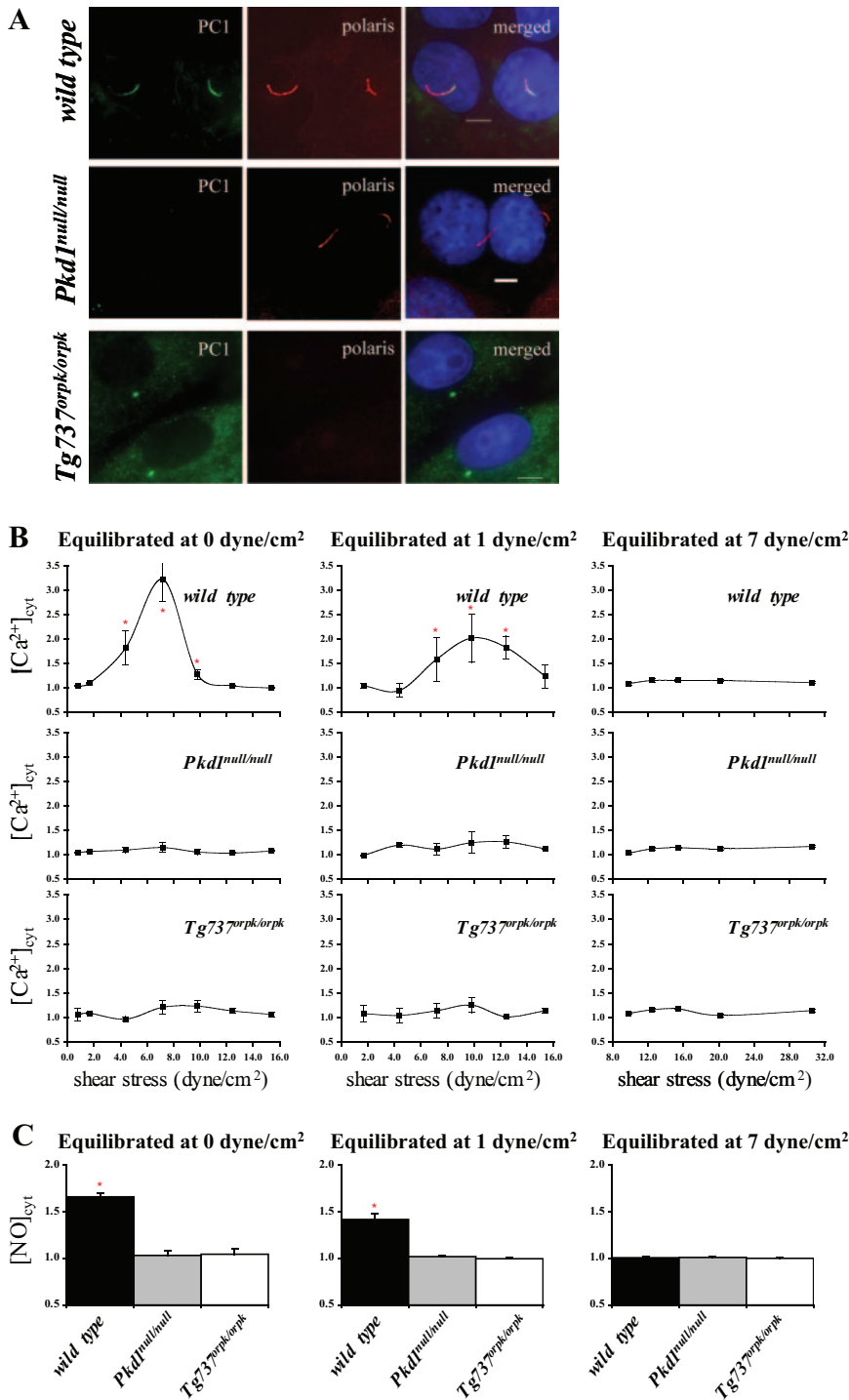
Figure 4. Endothelial cilia respond to a specific range of fluid shear stresses. A, Immunostaining studies show colocalization of polycystin-1 (PC1; green) and polaris (red) counterstained with nuclear marker DAPI (blue). Note that polycystin-1 appears to be concentrated in the basal body of the *Tg737* mutant cells. Bar=5 μm. n=4. B, All cells were first exposed to a fluid shear stress of 0, 1.1, or 7.2 dynes/cm^2 for 30 minutes, followed by the corresponding step increase in shear stress as depicted in the graphs. Cytosolic calcium ($[Ca^{2+}]_{cyt}$) was measured as a readout in response to fluid shear stress. Although wild-type cells respond to a range of shear stress, neither mutant cell shows any apparent response to different magnitudes of shear stress. n=3 to 6 for each time point in a given shear stress condition. C, Cumulative measurements of cytosolic NO biosynthesis ($[NO]_{cyt}$) at the predicted optimal ranges of shear stress. n=3 to 6 for each time point in a given shear stress condition.

dynes/cm^2) before a step increase in various magnitudes of shear stress, we found that wild-type cells responded best at fluid shear stress of 9.8 dynes/cm^2 followed by 12.4 and 7.2 dynes/cm^2. Although the magnitude of cytosolic calcium increase was greater in wild-type cells equilibrated at static compared with 1.1 dynes/cm^2, neither *Pkd1*$^{null/null}$ nor *Tg737*$^{orpk/orpk}$ cells exhibited this calcium response to shear stress. When cells were equilibrated for 30 minutes under a higher shear stress (7.2 dynes/cm^2), wild-type and mutant cells did not show a cytosolic calcium increase in response to a step increase in various magnitudes of fluid shear stress. A further increase in the magnitude of shear stress to 50 dynes/cm^2 did not provoke a calcium response in any of the cells because they tended to detach from the matrix at higher shear (data not shown).

Fluid Shear Stress Induces Polycystin-1 Cleavage

Endothelial cells exposed to fluid shear stress for hours have been shown to lose their cilia from the apical membrane.[21] It also has been reported that polycystin-1 can undergo proteolytic cleavage, and this cleavage has been implicated to be associated with the mechanical fluid stimulus.[26,27] To exam-
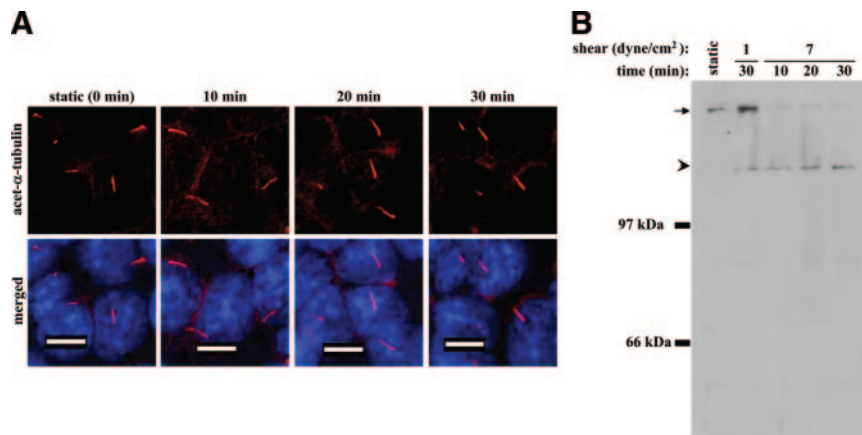
Figure 5. Optimal fluid shear preserves the presence of cilia and induces apparent proteolytic cleavage of polycystin-1. A, Endothelial cells equilibrated at an optimal shear stress of 7.2 dynes/cm^2 for various time points maintain their apical primary cilia. The presence of cilia in every cell is confirmed with acetylated α-tubulin (acet-α-tubulin). Bar=10 μm. n=2 for each time point. B, Cells were equilibrated at 0, 1.1, or 7.2 dynes/cm^2 for 10, 20, or 30 minutes. Only a single full-length polycystin-1 band is observed in static control cells (arrow), whereas fluid shear stress induces a second lower–molecular-weight polycystin-1 band (arrowhead). Note that the band intensity of full-length molecular-weight polycystin-1 in cells equilibrated at 7.2 dynes/cm^2 is much weaker than that of static control or 1.1 dynes/cm^2. n=2.

ine why our endothelial cells equilibrated at a fluid shear stress of 7.2 dynes/cm^2 became insensitive to further shear stimuli, we investigated both cilia structure and polycystin-1 in response to various fluid shear stresses. Endothelial cells challenged with shear stresses of 1.1 dynes/cm^2 (data not shown) or 7.2 dyne/cm^2 (Figure 5A) for 10, 20, and 30 minutes did not lose their cilia. This suggests that the mechanosensory ciliary compartments are still intact but that the cells have lost their sensory functions, probably because of the alteration in sensory polycystin-1 after the fluid shear stress. To investigate this possibility, we performed immunoblotting experiments on polycystin-1 at different shear stresses and time points (Figure 5B). In contrast to static control cells, we observed secondary truncated bands of polycystin-1 in cells exposed to either 1.1 or 7.2 dynes/cm^2 at various time points. We suspect that our endothelial cells pre-equilibrated at 7.2 dynes/cm^2 for 30 minutes had almost lost the entire functional full-length polycystin-1.

Cilia Play Distinct Roles in Fluid Shear Sensing

To validate ciliary roles in fluid sensing, we challenged the cells from static condition to a step increase in an optimal fluid shear stress of 7.2 dynes/cm^2 to induce increases in cytosolic calcium and NO. We performed simultaneous measurements of cytosolic calcium and NO levels (Figure 6A). Peaks of cytosolic calcium followed by NO production were seen in wild-type cells challenged with a shear stress of 7.2 dynes/cm^2, but no changes in cytosolic calcium or NO were observed in mutant cells. Changes in cytosolic calcium were plotted in line graphs from randomly picked cells (Figure 6B). Similarly, individual cells were randomly selected, and the amounts of cytosolic production of NO by the cells were averaged and plotted (Figure 6C). Peaks of NO observed in wild-type cells are the only statistically significant responses. These data indicate that both polycystin-1 and polaris are required for proper calcium signaling and NO production by endothelial cells in response to fluid shear stress.

To confirm the specificity of cilia function, we mechanically activated a single cell by gently touching its apical membrane with a micromanipulator and recording simultaneous measurements of cytosolic calcium and NO levels (Figure 7A). Calcium propagation as evidenced by the spreading of the calcium signal from a single touched cell to neighboring cells was observed in wild-type and mutant cells. Surprisingly, we did not observe signal propagation for NO. Only cells that had been touched and were immediately adjacent to the touched cell showed an increase in cytosolic NO. Ten cells within a population were randomly selected from the touched cell to the cells farther away from this cell, and their calcium profiles were plotted (Figure 7B). The NO response of these 10 cells also was plotted (Figure 7C). The cell that was directly touched showed the highest increase in cytosolic calcium. Similarly, the touched cell had the highest NO production. To better visualize the data, cell populations were grouped on the basis of their distance away from the touched cell, and both their calcium and NO profiles were plotted (Figure 7D). No significant change in NO profile was observed within groups. In contrast, the farther the cells were from the point of force application, the smaller the changes were in cytosolic calcium. More important, no obvious differences were present in the calcium and NO patterns of wild-type and mutant cells, indicating that polycystin-1 and polaris are not involved in this mechanotransduction response involving direct physical distortion of the apical membrane.

To further confirm that polycystin-1 and polaris are specific for the sensation of fluid shear stress through primary cilia, we challenged the cells with a pharmacological agonist, acetylcholine. Acetylcholine is known to induce an increase in cytosolic calcium through the muscarinic receptor, which in turn activates eNOS and results in NO release.[28] Treatment with 1 μmol/L acetylcholine increased both cytosolic calcium and NO in wild-type and mutant cells (Figure 8). Immediate increases in calcium and NO were observed in wild-type and mutant cells, and their levels remained high in all cell types, indicating that polycystin-1 and polaris mediate fluid shear
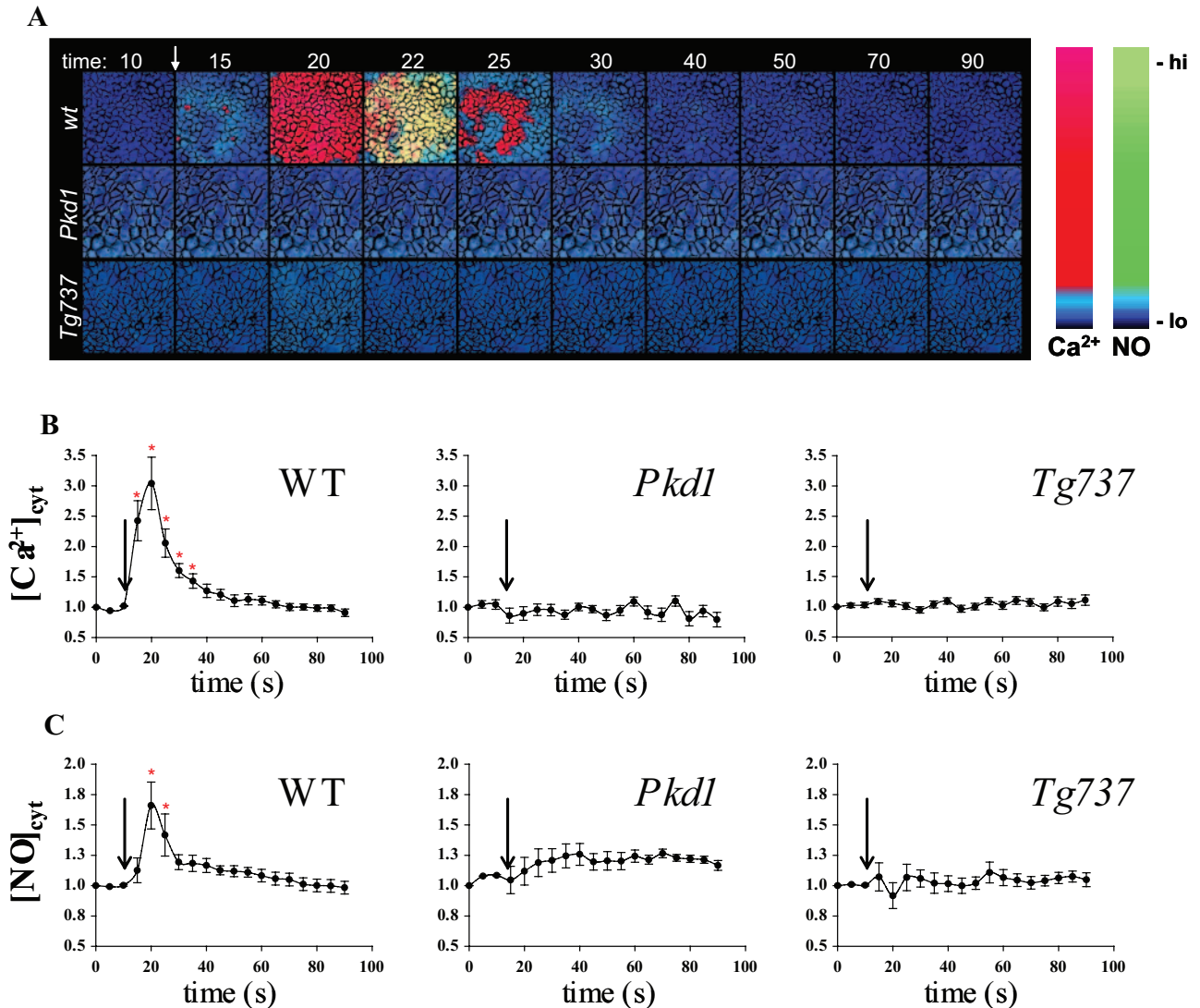
A

B

C

Figure 6. Fluid shear–induced calcium and transient NO require polaris and polycystin-1. A, Response of individual wild-type (wt), *Pkd1*[null/null] (Pkd1), and *Tg737*[orpk/orpk] (Tg737) cells from static condition to fluid shear stress of 7.2 dynes/cm^2 is pseudocolored. The calcium (red) and NO (green) are superimposed. The color bar indicates the calcium (Ca^{2+}) or NO level relative to the corresponding baseline level. Black and blue represent a low (lo) level; red and green denote a higher (hi) level of calcium and NO, respectively. B, The average cytosolic calcium ([Ca^{2+}]$_{cyt}$) response to shear stress. C, The average cytosolic NO ([NO]$_{cyt}$) response. Fluid shear stress was applied after the baseline values of calcium and NO were obtained for at least 14 seconds as indicated by arrows. All the units of time are seconds. n=5. *Statistically significant responses from the baseline values.

stress–dependent changes in calcium and NO via a distinct pathway.

Discussion

Our study provides strong evidence that the primary cilium, specifically its constituent polycystin-1 and polaris proteins, mediates a mechanism by which endothelial cells sense and respond to fluid shear stress. Using endothelial cells isolated from *Pkd1* and *Tg737* knockout mice, we showed that primary cilia are crucial organelles for fluid shear sensing in endothelial cells. We believe that primary cilia act as antennas to sense and transduce extracellular fluid shear into changes in biochemical signaling inside vascular endothelial cells. The absence of primary cilia in *Tg737*[orpk/orpk] endothelial cells clearly abrogated the increases in cytosolic calcium and NO normally elicited by application of physiological levels of

fluid shear stress. This indicates that the presence of polycystin-1 in the cells by itself is not sufficient to maintain the ability of the cell to sense shear stress. To act as a mechanosensing molecule, polycystin-1 has to be properly localized in the primary cilium, which may act like a large cantilever to amplify and concentrate stresses at the cell surface.

The presence of cilia in the cardiovascular system has been reported in cultured human umbilical vein endothelial cells and in developing chicken endocardium with low-shear-stress areas.[21,22] In the present study, we show that cilia also can be found in aortic endothelia of embryonic mouse (Figure 1). Although no reported shear stress value in the embryonic mouse has been reported, we calculated that the physiological shear stress values in embryonic mouse aorta would be ≈1.5 to 10 dynes/cm^2 by extrapolating the blood flow data obtained
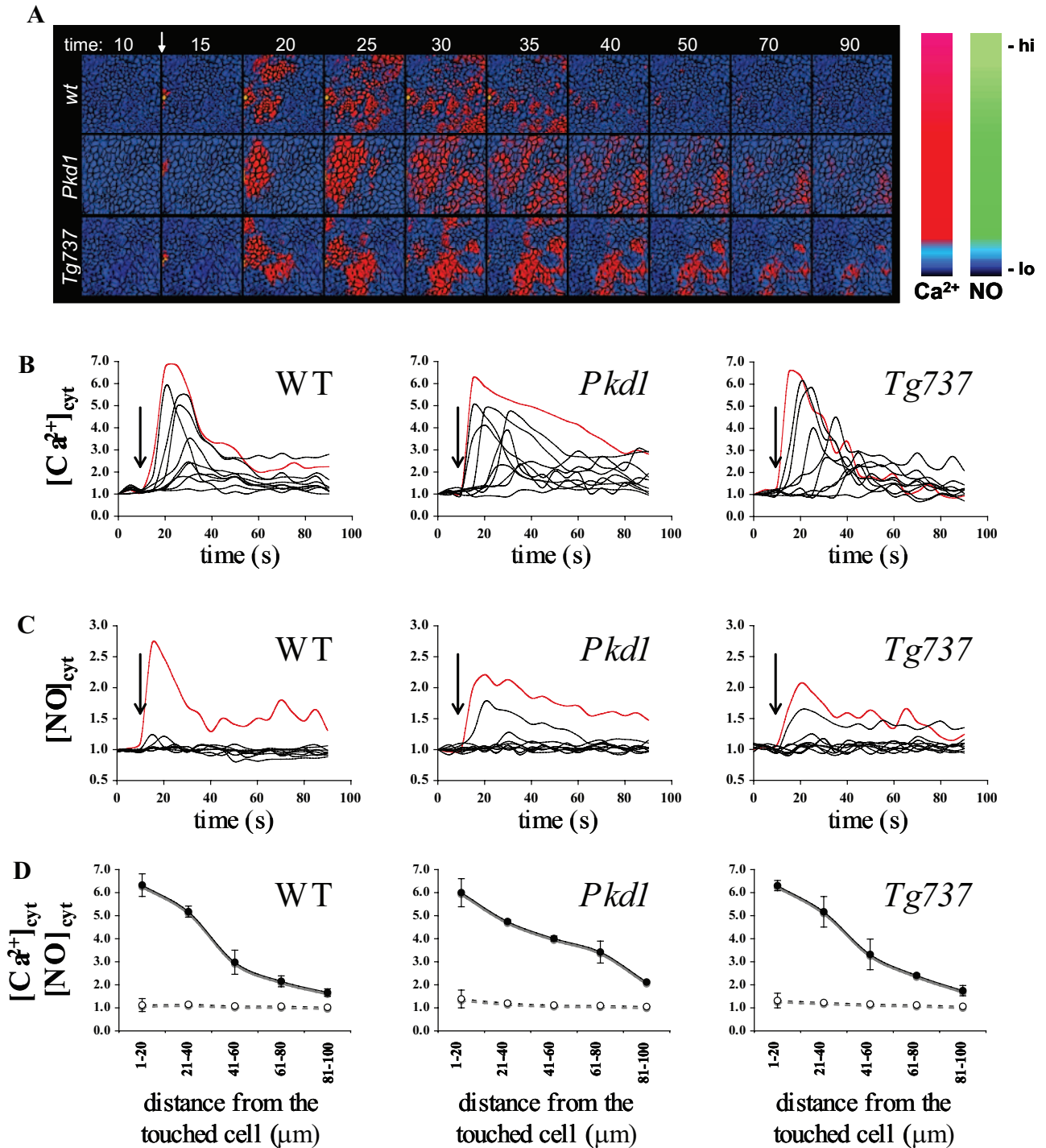
Figure 7. Mechanical touch–induced calcium propagation and NO production are independent of cilia. A, Response of individual wild-type (wt), *Pkd1null/null* (Pkd1), and *Tg737orpk/orpk* (Tg737) cells to a single apical cell pressing is pseudocolored. The calcium (red) and NO (green) are superimposed. The color bar indicates the Ca^{2+} or NO level relative to the corresponding baseline level, with black and blue representing a low (lo) level and red or green denoting a higher (hi) level. The apical membrane of a single cell (far left) was pressed after baseline values of calcium and NO were obtained for at least 14 seconds as indicated by arrows. B, The individual cytosolic calcium ($[Ca^{2+}]_{cyt}$) response to a mechanical stimulus of touching a single cell. C, The individual cytosolic NO ($[NO]_{cyt}$) response. The red line in each graph represents the corresponding calcium and NO values of the touched cell. D, Cells were grouped on the basis of their distance away from the touched cells. The averages of their corresponding cytosolic calcium (●) and NO (○) responses are shown. The units of time and distance are seconds and microns, respectively. n=3.

by an innovative high-frequency Doppler ultrasound system of embryonic mouse dorsal aorta.[29,30] These magnitudes of fluid shear stress have been used to induce differentiation of pluripotent embryonic stem cells to endothelium-like pheno-type.[31] Together with the sensitivity of our cultured embryonic endothelial cells to a low fluid shear stress (Figure 4), we hypothesize that cilia might act as specialized low fluid shear–sensing organelles. Consistent with this idea, a much
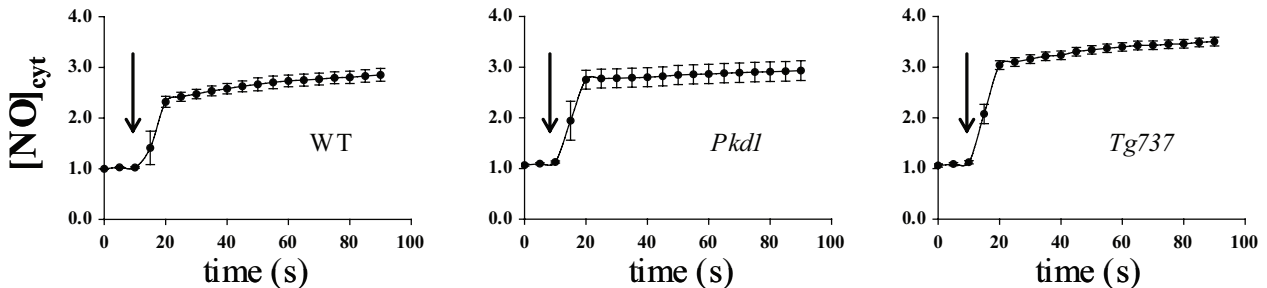
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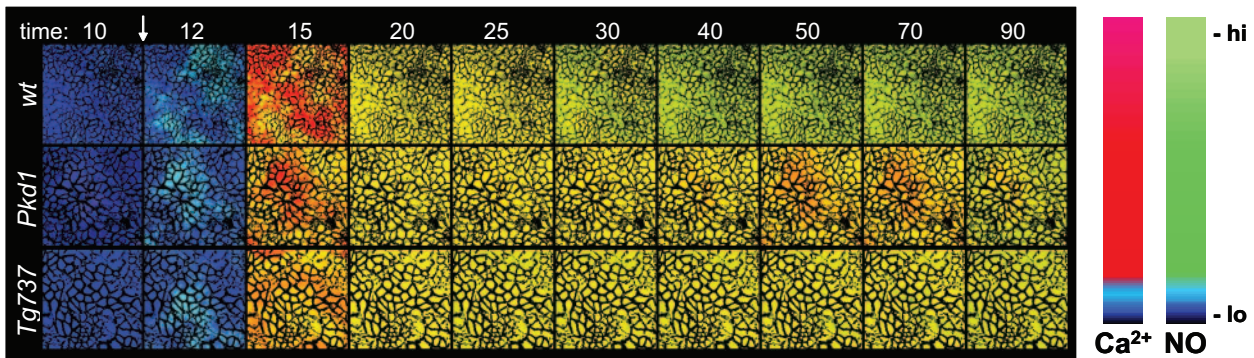
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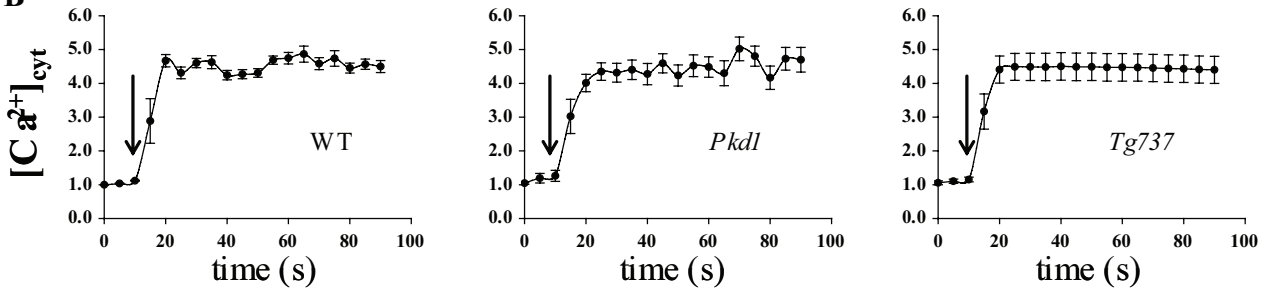
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Figure 8. Agonist-provoked calcium and NO signaling is independent of cilia. A, Response of individual wild-type (wt), $Pkd1^{null/null}$ (Pkd1), and $Tg737^{orpk/orpk}$ (Tg737) cells to 1 μmol/L acetylcholine is pseudocolored. The calcium (red) and NO (green) are superimposed. The color bar indicates the Ca^{2+} or NO level relative to the corresponding baseline level, with black and blue representing a low (lo) level and red or green denoting a higher (hi) level. B, The average cytosolic calcium ($[Ca^{2+}]_{cyt}$) response to acetylcholine. C, Plots of the average cytosolic NO ($[NO]_{cyt}$) response. Acetylcholine was applied to the cells for at least 14 seconds after the baseline values of calcium and NO were obtained as indicated by arrows. All time units are seconds. n=3.

higher shear stress of 15 dynes/cm^{2} on endothelial cells has been shown to disassemble cilia.[21]

To enable us to study cilia function, we generated aortic endothelial cells derived from mouse embryos because both $Pkd1^{null/null}$ and $Tg737^{orpk/orpk}$ mouse models are prenatally lethal.[3,16] The division and differentiation of these cells are controlled by the SV40 gene, which is regulated by temperature and INF-γ (Figure 2). Cells undergoing division retract their cilia from the apical cell surface,[5] and differentiated cells generate better-developed, functional cilia.[5,8] Therefore, it is imperative for us to be able to control cellular proliferation and differentiation of cultured cells to study cilia function. These fully differentiated embryonic endothelial cells were characterized by positive expressions of eNOS, ICAM-2, and CD31 (Figures 2 and 3).

To study the molecular functions of polaris and ciliary polycystin-1, wild-type and mutant cells were first subjected to no (0 dynes/cm^{2}), low (1.1 dynes/cm^{2}), or high (7.2

dynes/cm^{2}) fluid shear stress for 30 minutes. A step increase in a higher fluid shear stress was then applied to the cells (Figure 4). In all cases, mutant cells did not show any cytosolic calcium increase in response to a step increase in various magnitudes of fluid shear stress. This suggests that cilia and polycystin-1 are important mechanosensory components in endothelial cells. Furthermore, polycystin-1 is localized to the basal body in $Tg737^{orpk/orpk}$ endothelial cells (Figure 3). This further indicates that subcellular localization of polycystin-1 at designated microcompartments is indeed essential for fluid shear sensing in endothelial cells.

Interestingly, wild-type embryonic endothelial cells preexposed to different fluid shear stresses could modify their sensitivity in response to a step increase in fluid shear stress (Figure 4). The highest increase in cytosolic calcium is seen in endothelial cells in a step increase from static (no shear) to 1.1 and 7.2 dynes/cm^{2}; no significant increase in cytosolic calcium was observed in cells preexposed to 7.2 dynes/cm^{2}.

Although cells with no shear (0 dynes/cm^2) compared with those with an initial low shear (1.1 dynes/cm^2) generated a higher cytosolic calcium increase in response to shear stress, cells preexposed to a low shear could respond to a broader range of fluid shear stresses. We have previously shown that the magnitude of shear stress that optimally increases cytosolic calcium varies with the developmental stage of the animal from which the cells were isolated and the cell type.[9]

Surprisingly, cells preexposed to a higher shear stress (7.2 dynes/cm^2) became mechanoinsensitive to further increases in fluid shear stresses (Figure 4B). Our immunostaining studies ruled out the absence of cilia in cells equilibrated at 7.2 dynes/cm^2 (Figure 5). Consistent with previous studies,[26,27] we suggest that polycystin-1 would undergo a proteolytic cleavage if cilia were activated. Although a shear stress of 1.1 dynes/cm^2 also would induce a secondary cleavage product of polycystin-1, cells equilibrated at 1.1 dynes/cm^2 would still maintain a high amount of functional full-length polycystin-1. This, in turn, would allow cells equilibrated at 1.1 dynes/cm^2 to remain responsive to further increases in fluid shear stress. These variations in shear sensing could possibly be parts of a complex mechanical behavior of cilia and the downstream mechanosignal transduction system that warrant thorough study.

Vascular endothelial cells have been known to release NO in response to fluid shear stress.[14] To examine whether our mutant cells could possibly respond to shear stress by NO biosynthesis, we performed a simultaneous measurement of cytosolic calcium and NO (Figure 6). The increase in cytosolic calcium followed by NO was apparent in wild-type cells. In mutant cells, no increase in cytosolic calcium or NO was observed. This suggests that regardless of the readout measurements (calcium or NO), the mutant cells are mechanically insensitive to fluid shear stress. Although both *Pkd1$^{null/null}$* and *Tg737$^{orpk/orpk}$* cells fail to respond to fluid shear, these cells are still able to respond to other stimuli such as apical membrane distortion (Figure 7) and the pharmacological agent acetylcholine (Figure 8). This implies that polycystin-1 and polaris have a rather specific function to decipher extracellular fluid shear signal.

The increase in cytosolic calcium followed by NO was transient in wild-type cells. The transient increase in cytosolic calcium in response to fluid shear stress–induced cilia activation also was observed previously in mouse and human kidney epithelial cells.[8–10] Cilium-specific calcium channels might open for only a short time. We and others have recently shown that mechanosensitive calcium channels such as polycystin-2 also may be involved in fluid shear sensing.[8,32] At least in kidney epithelial cells, calcium entry through the polycystin-2 channel in response to fluid shear also is associated with intraorganellar calcium release.[8,33] With regard to cardiovascular functions, it is possible that cilia were sensory organelles that responded by a short and transient pulse of cytosolic calcium increase and NO release, which might provide not only short but also longer physiological effects to surrounding cells.[34]

One interesting observation is that calcium and NO profiles are very different among the stimuli that we applied. Although calcium is always thought to be the mediator for eNOS activity, we demonstrate that cells have more complex ways to regulate the biosynthesis of NO. Depending on the stimulus, an endothelial cell could regulate the intracellular calcium in a way that allows a short, a prolonged, or no eNOS activity (Figures 6 through 8). We believe that such differences in calcium profiles also would alter the activity of many other calcium-dependent proteins and cascades, and it remains an interesting topic to pursue in the future.

Collectively, the findings of our study suggest that endothelial cells require functional mechanosensory cilia to respond to fluid shear stress. In response to an optimal shear stress, polycystin-1 is cleaved, resulting in nonresponsiveness of cells to further higher fluid shear. Cells with abnormal cilia function or structure are likely to fail to respond to fluid shear stress. Polycystin-1 for cilia function and polaris for cilia structure and maintenance play crucial roles in mediating fluid shear sensing in endothelial cells, as well as transduction of these mechanical signals into changes in calcium signaling and NO synthesis. We propose that cilia are mechanosensory microcompartments and that polycystin-1 is a sensory molecule that must be "housed" in the cilia to exert its mechanosensory role in endothelial cells.

Disclosures

None.

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CLINICAL PERSPECTIVE

Patients with polycystic kidney disease exhibit an enhanced propensity for hypertension. Studies have revealed that mutations which result in abnormal ciliary proteins such as polaris and polycystin-1 lead to the development of polycystic kidney disease in mice. These findings suggest an association between ciliary function and the development of hypertension. Kidney cells that exhibit abnormal ciliary proteins fail to sense fluid shear stress. Within endothelial cells, this hemodynamic fluid flow can regulate blood pressure by altering calcium signaling and nitric oxide production. Here, we show that embryonic mouse aortic endothelial cells possess primary cilia, specialized organelles that sense and convert fluid shear stresses into changes in intracellular calcium and nitric oxide production. Polaris and polycystin-1 were shown to mediate these effects. In addition, prolonged activation of cilia by high shear stress would induce proteolytic cleavage of polycystin-1, thereby desensitizing endothelial cells to these mechanical stimuli. Overall, these findings suggest that dysfunction of endothelial cilia could interfere with normal shear-induced regulatory mechanisms that may contribute to abnormal vascular control in polycystic kidney disease patients and hence may lead to hypertension. Furthermore, hypertensive patients who exhibit ciliary desensitization resulting from continuous exposure to high levels of fluid shear might be unable to respond normally to small but damaging daily fluctuations in blood pressure. This might increase the likelihood of localized blood vessel injury, aneurysm, hemorrhage, edema, atherosclerosis, vascular ectasia, dissection, and other abnormalities. Greater insight into this novel mechanism of endothelial flow sensing by cilia may lead to advanced understanding of focal cardiovascular diseases and development of novel forms of "ciliary therapy" in the future.