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Brief serotonin exposure initiates arteriolar inward remodeling processes in vivo that involve transglutaminase activation and actin cytoskeleton reorganization

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INWARD REMODELING OF THE RESISTANCE VASCULATURE is a hallmark feature of a number of cardiovascular diseases including hypertension (31). The marked increase in the risk for life-threatening cardiovascular events associated with inward remodeling (6, 34, 42) highlights its clinical importance. In addition, inward eutrophic remodeling of the resistance vasculature is believed to play an important role in increasing peripheral vascular resistance and decreasing tissue blood flow during the development and maintenance of essential hypertension (15). Inward remodeling is defined as a decrease in the luminal diameter of blood vessels under passive conditions (38). In essential hypertension, this remodeling is eutrophic; that is, there is no significant change in the cross-sectional area of the vascular wall, which indicates there is no change in the amount of wall material. As for the etiology of the remodeling process, substantial evidence in isolated vessels suggests that prolonged agonist-induced vasoconstriction and not the presence of elevated intravascular pressure is the main stimulus that induces inward remodeling. In accordance with these observations, clinical evidence has shown that pharmacological intervention to ameliorate hypertension also reverses inward remodeling when treatments promote vasodilation, in contrast with interventions that only reduce blood pressure (which do not reverse remodeling), such as those that decrease cardiac output (9, 33). Therefore, it is of particular clinical interest to decipher the biochemical/physiological mechanism(s) that are activated in response to vasoconstriction and initiate the transition from a structurally normal resistance vasculature to an inwardly remodeled one to find new avenues to prevent, treat, or reverse the remodeling process and reduce its life-threatening cardiovascular consequences.

In isolated vessels ex vivo, it has been demonstrated that prolonged (4 h+) exposure of resistance arteries to vasoconstrictors initiates inward remodeling process via pathways that include both the polymerization of actin and the activation of transglutaminases, specifically transglutaminase 2 (5, 32, 45). In vivo studies investigating the role of the actin cytoskeleton on inward remodeling within the resistance vasculature are limited. In comparison, a number of in vivo studies have shown a role for transglutaminases in resistance vessel remodeling in response to changes in flow (1, 3); in response to a
1-wk infusion of the nitric oxide (NO) synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME) (41); and in response to a 1-wk infusion of phenylnephrine (13). An important limitation in the majority of these in vivo studies is that the experimental designs did not allow for differentiation between the effects produced by blood pressure and vasoconstriction on the development of inward remodeling. Nevertheless, the cumulative ex vivo and in vivo evidence suggests that both intracellular changes in the cytoskeleton and modifications of the extracellular matrix (ECM) are associated with transglutaminase activity and represent key early events in the process that leads to inward eutrophic remodeling of the resistance vasculature.

To date, much of our insight into the mechanism(s) that initiate and control the inward remodeling process in resistance vessels comes from both in vitro and ex vivo studies performed on cultured cells and isolated vessels. Although mechanistically informative, these approaches lack the natural dynamics of intact tissues functioning within the body as a whole. The in vivo data are primarily derived from experimental protocols in which animals are exposed to pharmacological agents/vasoactive stimuli, and following a prolonged period of treatment, resistance vessels are excised and analyzed for structural and functional changes. The duration of these treatment protocols extends from days to weeks, thus at the time of tissue isolation, the derived structural/functional data represent but a single snapshot in the progression of vascular structural changes. Moreover, it has been demonstrated ex vivo that remodeling can occur over a relatively short span of time, i.e., over a span of hours. Currently available in vivo data are, therefore, inherently limited in defining the transitional mechanism(s) that occur during onset of the remodeling process.

To more thoroughly investigate the temporal aspects of the early remodeling process as well as verify the applicability of ex vivo studies on the mechanisms of inward remodeling of the resistance vasculature to an in vivo setting, we used an intra-vital microscopy approach. With the use of this technique, vasoactive agents were applied topically and locally to intact resistance vasculature to an in vivo setting, we used an intra-arteriolar segments of 1 mm in length were cannulated onto glass microscope. Rats were initially anesthetized with 5% isoflurane (Vet One, Boise, ID). Mean blood pressure was assessed via catheterization of the left femoral artery and maintained constant in each animal by modulation of percent isoflurane inhalation by using an anesthesia machine (Mouse Anesthesia Suite; Kent Scientific, Torrington, CT). Overall mean arterial pressure was 86 ± 0.6 mmHg. Minute volume respiration was delivered at a constant 230 ml. The left cremaster muscle in anesthetized rats was exteriorized and prepared as previously described (20, 21), with minor modifications.

Briefly, an incision was made through the skin to expose the muscle, and the surrounding connective tissue was gently peeled from the muscle. A cautery (Geiger Model 150; Geiger Medical Technologies, Council Bluffs, IA) was used to open the cremaster longitudinally along its ventral surface, from the apex to the inguinal canal. The deferential arteriole and venule were then gently separated from the cremaster using the cautery, and care was taken to minimize bleeding. The testis and epididymis were moved into the abdominal cavity. The cremaster muscle was gently stretched radially, across a flat pedestal, and pinned at its edges. Grease (Dow Corning High Vacuum Grease; Dow Corning, Midland, MI) was used to encircle the cremaster and form a dam to facilitate continual perfusion of the tissue with a Krebs buffer containing (in mM) 111 NaCl, 25.7 NaHCO3, 4.9 KCl, 2.1 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 11.5 glucose, 10 HEPES (pH = 7.4 at 34°C) equilibrated with 5% CO2-95% N2. Tubing, carrying the perfusate, was fed through an aluminum block, heated with a ribbon heater connected to an Omega Temperature Controller (Omega, Stamford, CT) to maintain a cremastatic temperature of 34°C. Perfusion flow rate was 2 ml/min. All treatments were performed in this buffer.

Vessel isolation for ex vivo experiments.

Animals. Male Sprague-Dawley rats ~200 g (Jackson Laboratories, Bar Harbor, ME) were used in all experiments. The Animal Care Quality Assurance office and the Animal Care and Use Committee at the University of Missouri-Columbia approved all animal protocols and procedures used during these studies. Before experimentation, rats were housed in pairs under a 12-h per day light/dark cycle and provided with ad libitum access to standard rat chow and water.

Intravital microscopy. Rats were initially anesthetized with 5% isoflurane (Vet One, Boise, ID). Mean blood pressure was assessed via catheterization of the left femoral artery and maintained constant in each animal by modulation of percent isoflurane inhalation by using an anesthesia machine (Mouse Anesthesia Suite; Kent Scientific, Torrington, CT). Overall mean arterial pressure was 86 ± 0.6 mmHg. Minute volume respiration was delivered at a constant 230 ml. The left cremaster muscle in anesthetized rats was exteriorized and prepared as previously described (20, 21), with minor modifications.

The following experimental protocol was used to determine the duration and magnitude of vasoconstriction observed in response to different vasoactive agonists and to assess remodeling over the course of the experiment. An initial 10-ml bolus of 10−4 M adenosine + 10−4 M papaverine + 10−5 M sodium nitroprusside (SNP) was added to the perfusate to determine maximum passive diameter. This was washed away with a 10-min perfusion in Krebs buffer, followed by a 4-h perfusion with vasoconstrictors and a subsequent 10-min Krebs wash. Then, a 10-ml bolus of 10−4 M adenosine + 10−4 M papaverine + 10−5 M SNP was administered to reassess maximal passive diameter and remodeling for 10 min. Vessels that did not constrict to at least 65% of their maximum passive diameter after exposure to the vasoconstrictor agonists were excluded from the study.

The same protocol was used to determine whether the vasoconstrictor cocktail, capable of inducing inward remodeling, after 4 h, was able to induce remodeling after only 10 min of exposure. In this acute exposure protocol, the final determination of passive diameter following the Krebs wash to remove the vasoconstrictors consisted of a continuous perfusion of 10−4 M adenosine + 10−4 M papaverine + 10−5 M SNP for 30 min. Maximal passive diameter in this vasodilator cocktail was continuously monitored, and remodeling was determined at 10 and 30 min into the vasodilator cocktail perfusion. To determine the role of transglutaminase activity on the remodeling process, a series of experiments was performed with 10−3 M cyanate (transglutaminase inhibitor) added to all solutions. For comparisons of treatment regimes, vessel diameters were normalized to maximum pretreatment passive diameter. Percent remodeling was calculated using the following equation: 1 − (post-treatment passive diameter/pretreatment passive diameter) × 100%. Percent constriction was calculated as follows: 1 − (smallest diameter in vasoconstrictor/pretreatment passive diameter) × 100%.

Vessel isolation for ex vivo experiments. Rats were anesthetized with an intraperitoneal injection (100 mg/kg) of pentobarbital sodium. After the absence of spinal reflexes was confirmed, cremaster muscles were excised and placed in 4°C physiological saline solution (PSS) containing (in mM) 145.0 NaCl, 4.7 KCl, 2.0 CaCl2, 1.0 MgSO4, 1.2 NaH2PO4, 0.02 EDTA, 2.0 pyruvic acid, 5.0 glucose, and 3.0 MOPS, pH = 7.4. The 1A feed arterioles from each cremaster were isolated, cannulated, and pressurized as previously described (29). Briefly, arteriolar segments of ~1 mm in length were cannulated onto glass micropipettes mounted in an observation chamber (Living Systems Instrumentation, Burlington, VT) and filled with PSS. The arterioles
were pressurized without flow to 60 mmHg using a Pressure Servo System (Living Systems Instrumentation) and PSS containing 0.15 mM bovine serum albumin. The observation chamber containing the cannulated vessel was transferred to an inverted microscope equipped with a video display and video caliper system (Living Systems Instrumentation) to record measurements of wall thickness and luminal diameter. All experiments were performed at 34.5°C. Percent remodeling was calculated as in the intravital experiments.

Assessing transglutaminase activation via fluorescent cadaverine transamidination. To determine relative transglutaminase activity, isolated arteriolar sections were incubated with the transglutaminase substrate Alexa Fluor 488 cadaverine (Invitrogen A30676), as previously described (7), with slight modifications. Briefly, rat cremasteric L-NAME, or 5-HT (Controls and 5-HT) arteriolar sections were incubated with the transglutaminase inhibitor 3M cystamine for 10–30 min at 34°C. All subsequent steps were performed at 4°C. Vessels were washed twice in phosphate buffered saline (PBS) to remove unbound fluorescently labeled cadaverine and incubated overnight at 4°C to ensure that fluorescently labeled cadaverine was able to enter the cells, since we were particularly interested in the intracellular transamidinating activity of transglutaminase. The next day, vessels were warmed to 34°C and incubated in one of the following treatments: PSS + 10−5 M cadaverine (Control), PSS + 10−4 M serotonin (5-HT) + 10−4 M L-NAME + 10−5 M cadaverine (5-HT + L-NAME), PSS + 10−4 M 5-HT + 10−4 M L-NAME + 10−3 M cystamine + 10−5 M cadaverine (5-HT + L-NAME + cystamine) for 10–30 min at 34°C. All subsequent steps were performed at 4°C. Vessels were washed twice in phosphate buffered saline (PBS) to remove unbound fluorescently labeled cadaverine and then fixed for 1 h in 4% paraformaldehyde. Vessels were washed twice in PBS and then incubated for 1 h in 2 μM 4M,6-diamidino-2-phenylindole (DAPI) in PBS + 10% BSA to stain nuclei. Vessels were washed 2× in PBS and subsequently imaged using a Leica SPE confocal microscope with a 63× water objective. Cadaverine was excited with a laser tuned to emit at 488 nm. DAPI was excited at 405 nm. Images were processed and quantitated using Imaris software.

For quantification, a region of interest (ROI = 50 μm × 50 μm × 10 μm) was randomly selected to contain predominantly vascular smooth muscle cells. Two ROIs per vessel were assessed. The mean fluorescence intensity - background per μm1 was determined for the ROI and averaged for each vessel. For publication, all three representative images were equally adjusted for brightness/contrast.

Determining F- to G-actin ratios in isolated vessels. Freshly isolated 1A arterioles were incubated overnight at 4°C in a Ca2+-free PSS solution containing 10−4 M adenosine + 10−6 M verapamil + 100 nM of a silicon rhodamine conjugated jasplakinolide fluorophore (Sir-actin) (SC01; Cytoskeleton) to stain F-actin. The following day, DNase I was added at a final concentration of 300 nM and incubated an additional 2 h at 4°C to stain G-actin. Vessels were cannulated and mounted in PSS, pressurized to 70 mmHg and warmed to 37°C until myogenic tone was established; the PSS buffer was replaced every 15 min. Vessels were imaged with a Leica SP-5 confocal microscope with a 63× water objective. Laser excitation wavelengths used were 488 nm (Alexa Fluor 488 DNase I) and 633 nm (Sir-actin) and acquired simultaneously. Cystained vascular smooth muscle cells within the vessel (~1% of cells contained with both dyes) were identified and imaged to obtain a baseline Sir-actin/DNase I ratio (pretreatment). The buffer was replaced with PSS, and following a 10-min incubation, the same cell was reimaged (Control). The PSS was replaced with PSS + 10−4 M 5-HT + 10−4 M L-NAME, and following a 10-min incubation the same cell was again imaged (5-HT + L-NAME). Acquired images were analyzed with Imaris software to define an ROI encompassing a single costained cell and determine the mean intensity of Alexa Fluor 488 DNase I and 633 Sir-actin. This was achieved by applying a threshold to the 633 channel and masking all values that fell below threshold intensity. The same threshold was used for all three cells (pretreatment, Control, and 5-HT + L-NAME) to define the ROI. The Sir-actin/DNase I ratios following treatment were normalized to the pretreatment ratio. Ratios are expressed as percentage of pretreatment ratio.

Chemicals. All chemicals and drugs used in this study were purchased from Sigma (St. Louis, MO), except for LimKi, which was acquired from EMD Millipore (EMD Millipore, San Diego, CA), and Sir-actin, which was purchased from Cytoskeleton (Denver, CO). A stock solution of LimKi was made in DMSO at a concentration of 20 mM and diluted in the buffer solution used as superfusate (i.e., PSS or Ca2+-free PSS). A concentration-response curve was assessed to determine a concentration (10−6 M) that did not affect constriction to 10−4 M 5-HT + 10−4 M L-NAME (data not shown). The final concentration was reported refer to final concentrations in the superfusate. Sir-actin was dissolved in DMSO to generate a 1 mM stock solution. All other drugs were prepared the day of the experiment and diluted into the appropriate buffer at the indicated concentrations. Antibodies for P-cofilin (c8736) and total-cofilin (c8992) were from Sigma. Alexa Fluor 488 cadaverine was from Invitrogen (Life Technologies, Grand Island, NY).
RESULTS

Topical exposure to 5-HT + l-NAME for 4 h maintains vasoconstriction and induces arteriolar inward remodeling in vivo. Topical application of 10^{-5.5} M norepinephrine (NE) + 10^{-7} M Ang II for 4 h to exposed cremasters in vivo was unable to maintain vasoconstriction in 1A arterioles over the course of exposure to the agonists. After an initial constriction of 44 ± 7%, vessels dilated back to original diameters in the presence of these vasoconstrictor agonists. To determine whether vessels failed to maintain constriction due to vasodilation induced by NO or prostaglandins, vessels were incubated with the vasoconstrictor agonists in the presence of l-NAME (10^{-4} M) to inhibit NOS or indomethacin (5 × 10^{-5} M) to inhibit cyclooxygenase activity. Inhibition of NOS or prostaglandin production increased vasoconstriction to 55 ± 6% and 65 ± 2%, respectively, but did not result in sustained constriction for the full 4-h exposure to the agonists. In contrast, exposure to 10^{-4} M 5-HT + 10^{-4} M l-NAME caused a 50 ± 4% vasoconstriction in 1A arterioles that was consistently maintained for 4 h (Fig. 1A) and caused vessels to remodel inwardly. The mean time for vessels to return to their passive diameter in the presence of 10^{-5.5} M NE + 10^{-7} M Ang II, 10^{-5.5} M NE + 10^{-7} M Ang II + 10^{-4} M l-NAME, or 10^{-5.5} M NE + 10^{-7} M Ang II + 10^{-4} M l-NAME + 5 × 10^{-5} M indomethacin was 88 min, 113 min, and 111 min, respectively (Fig. 1B). Vessels exposed to 10^{-4} M 5-HT + 10^{-4} M l-NAME for 4 h inwardly remodeled, with a reduced passive diameter of 10.7 ± 2.95% (Fig. 1C).

Topical exposure to 5-HT + l-NAME for 10 min induces arteriolar inward remodeling in vivo. To determine whether a brief exposure to 5-HT + l-NAME was also able to reduce the passive diameter of arterioles, we exposed the cremaster muscle of rats in vivo to 10^{-4} M 5-HT + 10^{-4} M l-NAME for 10 min and subsequently measured the maximal passive diameter of arterioles for up to 30 min after removal of the vasoconstrictor. Topical application of 5-HT + l-NAME for 10 min caused a significant reduction in the maximal passive diameter of 1A arterioles as measured after up to 30 min of exposure to 10^{-4} M adenosine + 10^{-4} M papaverine + 10^{-5} M SNP. The reduction in passive diameter was prevented by the presence of 10^{-3} M cystamine, a broad inhibitor of transglutaminase activity. Moreover, the inward remodeling was specific to 5-HT, since exposure to 10^{-5.5} M NE + 10^{-7} M Ang II + 10^{-4} M l-NAME for 10 min did not reduce the passive diameter of arterioles (Fig. 2A). Vessels exposed to 10^{-4} M 5-HT + 10^{-4} M l-NAME had an 11.2 ± 1.9% reduction in passive diameter after 10 min in the vasodilators (Fig. 2C) and a 6.5 ± 1.1% reduction after 30 min under passive conditions (Fig. 2D). The degree of constriction was not significantly different between 10^{-4} M 5-HT + 10^{-4} M l-NAME and 10^{-4} M 5-HT + 10^{-4} M l-NAME + 10^{-3} M cystamine. Also, 10^{-5.5} M NE + 10^{-7} M Ang II + 10^{-4} M l-NAME significantly constricted vessels to a greater degree than the other two treatment regimens (Fig. 2B).

Exposure to 5-HT + l-NAME induces transglutaminase activation in isolated vessels. To confirm that exposure to 5-HT + l-NAME induced transglutaminase activation, isolated cremasteric 1A arterioles were exposed to these compounds in the presence of fluorescently labeled cadaverine (Fig. 3A). Exposure of vessels to 5-HT + l-NAME for 10 (Fig. 3B) or 30 (Fig. 3C) min caused a significant incorporation of cadaverine compared with vessels not exposed to the vasoconstrictors. To confirm that the incorporation of cadaverine was transglutaminase specific, the transglutaminase inhibitor cystamine was added to the treatment protocol. Vessels exposed to
$10^{-4}$ M 5-HT + $10^{-4}$ M l-NAME + $10^{-3}$ M cystamine had significantly reduced levels of cadaverine incorporation compared with vessels treated with 5-HT + l-NAME, similar to control vessels (Fig. 3, B and C).

**Exposure to 5-HT + l-NAME increases cofilin phosphorylation in isolated vessels.** To determine the effects of 5-HT + l-NAME on the activation of cofilin, we measured P-cofilin and total-cofilin in isolated 1A cremaster arterioles after 10 min of exposure to $10^{-4}$ M 5-HT + $10^{-4}$ M l-NAME. The normalized ratio of P-cofilin to total-cofilin was significantly increased in isolated 1A cremaster vessels exposed to $10^{-4}$ M 5-HT + $10^{-4}$ M l-NAME than in control vessels. The increase in P-cofilin relative to total-cofilin in the presence of $10^{-4}$ M 5-HT + $10^{-4}$ M l-NAME was attenuated by the addition of $10^{-3}$ M cystamine. Presence of $10^{-6}$ M LimKi, an inhibitor of the kinase (LIMK) that phosphorylates cofilin, also significantly reduced the ratio of P-cofilin to total cofilin in the presence of $10^{-4}$ M 5-HT + $10^{-4}$ M l-NAME (Fig. 4).

**Exposure to 5-HT increases the ratio of filamentous F-actin to globular G-actin in cell culture and in live vessels.** To determine the effect of 5-HT on actin polymerization in smooth muscle cells isolated from cremaster 1A arterioles, we measured the fluorescence intensity of DNAse I and Phalloidin staining in control cells and those treated with $10^{-4}$ M 5-HT (Fig. 5A). Exposure of cells to $10^{-4}$ M 5-HT resulted in a significant increase in the ratio of Phalloidin to DNAse I fluorescence intensity compared with control cells (Fig. 5B). We also assessed changes in actin polymerization dynamics in response to 5-HT + l-NAME in isolated vessels by measuring the fluorescent intensity of the cell permeable F-actin dye, SiR-actin, and DNAse I. Cannulated and pressurized vessels were imaged at 34.5°C and then treated with PSS for 10 min and imaged a second time as a control measurement (Fig. 5C). This was followed by a 10-min treatment with $10^{-4}$ M 5-HT + $10^{-4}$ M l-NAME, and a third image was obtained (Fig. 5C).

The ratio of SiR-actin intensity to DNAse I intensity was significantly higher following the 5-HT + l-NAME treatment than the control treatment (Fig. 5D).

**Exposure to 5-HT + l-NAME induces inward remodeling in isolated vessels.** To corroborate that 5-HT + l-NAME also induce inward remodeling in isolated vessels, pressurized 1A cremaster vessels were exposed for 10 min to $10^{-4}$ M 5-HT + $10^{-4}$ M l-NAME alone or in the additional presence of $10^{-3}$ M cystamine or $10^{-6}$ M LimKi. Remodeling was assessed by comparing maximal passive diameters obtained in the presence of Ca$^{2+}$-free PSS + 2 mM EGTA + $10^{-4}$ M adenosine before and after the exposure to the vasoconstrictor agonists (Fig. 6). Only the 5-HT + l-NAME treatment resulted in significant inward remodeling of 7.6 ± 1.1% (Fig. 6D). Presence of either $10^{-3}$ M cystamine or $10^{-6}$ M LimKi effectively blocked the 5-HT + l-NAME induced remodeling (Fig. 6D). Similar to the intravital data, the inhibitors had a negligible effect on the degree of constriction caused by 5-HT + l-NAME (Fig. 6, A–C).

**DISCUSSION**

The primary finding of this study is that a brief topical application of 5-HT + l-NAME initiated arteriolar inward remodeling processes in vivo. The reduction in passive diameter of the resistance vessels occurred in the absence of changes in systemic blood pressure. Ex vivo experiments further indicate that the inward remodeling process induced by 5-HT was dependent on the activity of transglutaminases, LIMK, and the phosphorylation of cofilin.

In accordance with what we and others have previously reported ex vivo in isolated vessels (2, 5, 29, 30), our results demonstrate that in vivo a brief (minutes to hours) exposure to vasoconstrictors is also able to initiate inward remodeling processes in the absence of changes in intravascular pressure.
However, one caveat is that the rapid induction of inward remodeling in vivo appears to be specific to certain vasoconstrictor agonists, at least with respect to the cremasteric vasculature. Thus far, our results indicate remodeling occurs in response to 5-HT, and not in response to NE/Ang II. We used NE/Ang II because we have previously shown that a 4-h incubation in this combination of vasoconstrictors consistently induces inward remodeling in isolated arterioles (32, 45). Topical application of this combination of vasoconstrictors in vivo was unable to maintain arteriolar constriction over the duration of the experiment, even when supplemented with L-NAME or indomethacin. This suggests that mechanisms other than NO- and prostacyclin-induced dilations prevent topical NE + Ang II from sustaining vasoconstriction for more than ~2 h. The only combination of vasoconstrictors tested that maintained a sustained 4-h constriction was 5-HT + L-NAME (Fig. 1A). In addition to maintaining prolonged vasoconstriction, 5-HT + L-NAME also induced inward remodeling following a 4-h incubation (Fig. 1C). We used a 10^-4 M concentration of 5-HT to induce near maximal constriction. A literature search revealed that submaximal constriction of cremasteric arterioles to topically applied 5-HT was achieved with a concentration of 10^-4 M 5-HT (25).

We also report that just a 10-min superfusion with 5-HT + L-NAME induced a significant inward remodeling of 11.2% when measured after 10 min under passive conditions and 6.50% after 30 min (Fig. 2). These results are comparable with the remodeling we observed following a 4-h incubation with 5-HT + L-NAME at the equivalent time following the removal of the vasoconstrictors, indicating that structural changes to the wall occur relatively early in the remodeling process induced by these agonists. This rapid induction of inward remodeling appeared specific to 5-HT + L-NAME, since a 10-min exposure to NE/Ang II/L-NAME did not induce remodeling (Fig. 2). We have also previously shown that a brief 5- to 10-min or 4-h exposure to a high K^+ solution, NE alone (5 μM), or endothelin-1 (10^-8 M) does not induce inward remodeling in isolated arterioles (19, 29). Others, however, have found that longer than 4-h incubations with endothelin-1 induce inward remodeling in isolated arterioles (2). This suggests that vasoconstrictor agonists vary in their capacity and rate to initiate inward remodeling processes. Furthermore, it would appear that the degree of constriction is not a primary factor in inducing remodeling, since NE + Ang II + L-NAME significantly constricted vessels to a greater degree than 5-HT + L-NAME, yet those vessels did not remodel (Fig. 2, B–D).

It is our contention that the presence of an incomplete vasodilation capacity following vasoconstriction represents early stages of the inward remodeling process (19, 31). Cumulative data support our concept of a remodeling continuum that
allows blood vessels to modify their structural characteristics to accommodate for changes in their neurohumoral and/or mechanical environments (4, 29). The incomplete relaxation response we observed upon exposure to the vasodilator cock-tail in vivo, or the calcium-free buffer ex vivo, is in accordance with the definition of inward remodeling introduced by Mulvany et al. (38) and therefore indicates that inward remodeling processes have been initiated in vessels exposed to 5-HT + L-NAME. We have also shown that the inward remodeling induced by a 4-h endogenous activation of transglutaminases is able to withstand a 1-h exposure to calcium-free conditions in the presence of 2 mM EGTA and 10⁻⁴ M adenosine. During this time frame, only active disruption of the actin cytoskeleton was able to reverse the inward remodeling (7), which further supports our view that the incomplete vasodilation capacity observed after a 10-min exposure to 5-HT + L-NAME represents the early stages of the inward remodeling process.

A number of reports have implicated transglutaminases in the inward remodeling process. Ex vivo, the activation of TG2 by dithiothreitol induces inward eutrophic remodeling in resistance vessels from mice (1) and rats (7). In addition, previous reports have shown that 5-HT is a potent transglutaminase activator in vascular tissues (40, 49). Therefore, we examined the involvement of transglutaminase activation in the rapid inward remodeling we observed in response to 5-HT + L-NAME exposure. Our evidence suggests that transglutaminases facilitate the remodeling process in vivo, as the addition of 10⁻³ M cystamine to the superfusate effectively blocked the inward remodeling induced by 5-HT + L-NAME (Fig. 2). Note that although the presence of cystamine dampened the level of constriction induced by 5-HT + L-NAME, this reduction in

Fig. 4. Exposure of isolated arterioles to 5-HT + L-NAME induces cofilin phosphorylation. A: representative Western blot image of lysates from cremaster 1A arterioles following a 10-min exposure to control conditions or 10⁻⁴ M 5-HT + 10⁻⁴ M L-NAME in the presence or absence of 10⁻³ M cystamine or 10⁻⁶ M LIM kinase inhibitor (LimKi). B: ratio of P-cofilin to total-cofilin, expressed as means ± SE, n = 4 for all treatments. *P ≤ 0.05 vs. Control.

Fig. 5. Exposure of vascular smooth muscle cells, in culture or in situ, to 5-HT increases the ratio of F-actin to G-actin. A: representative image of cultured arteriolar smooth muscle cells untreated (top; Control) or treated (bottom) with 10⁻⁴ M 5-HT for 10 min and stained with fluorescent DNase I, phalloidin, and DAPI. Scale bar = 20 μm. B: ratio of mean phalloidin intensity to DNase I intensity, expressed as means ± SE; n = 4 for both treatments. *P ≤ 0.05 vs. Control. C: representative image of a vascular smooth muscle cell within a living isolated cremasteric arteriole after 10-min treatment with vehicle (top: Control) and the same cell following a 10-min treatment with 10⁻³ M 5-HT + 10⁻⁴ M L-NAME (bottom: 5-HT + L-NAME), stained with fluorescent DNase I and SiR-actin. Scale bar = 15 μm. D: ratio of SiR-actin intensity to DNase I intensity, expressed as mean percentage normalized to pretreatment ratio, ± SE; n = 4 for both treatments. *P ≤ 0.05 vs. Control.
Infusion (4 wk) of L-NAME increased transglutaminase activity, whereas in vivo a chronic treatment increased Ca²⁺ levels, due, in part, to 5-HT receptor-mediated increases in intracellular calcium. 5-HT-induced vasoconstriction was severely blunted in the presence of L-type Ca²⁺ blockers. The role of transglutaminases in vascular remodeling has previously been attributed to modifications of the ECM: during exposure to vasoconstrictors, transglutaminases are activated and secreted from vascular smooth muscle cells and form cross-links that reorganize the ECM around a smaller constricted vessel. Under passive conditions, the newly reorganized ECM constrains the vessel to a smaller diameter. Data presented in this study suggest that transglutaminases also function intracellularly to mediate remodeling. Both in vivo and ex vivo, a 10-min exposure to 5-HT + L-NAME was sufficient to induce remodeling, which was inhibited by cystamine, thus indicating a role for transglutaminases in the process (Figs. 2 and 6). We hypothesize that transglutaminases are acting intracellularly in processes that modify the actin cytoskeleton and constrain the vessel to a smaller passive diameter. We have previously demonstrated that the actin cytoskeleton plays an important role in inward remodeling, and its severing activity is involved in actin depolymerization, and its phosphorylation by Lim Kinase(s) prevents its association with actin and inhibits its severing activity.

To assess if 5-HT affects actin polymerization/dem polymerization equilibrium in vascular smooth muscle cells, we...
costained cells with Phalloidin, to quantitate F-actin, and DNase I, to quantitate G-actin. We demonstrate that the ratio of Phalloidin to DNase I fluorescence is increased in vascular smooth muscle cells exposed to 5-HT compared with untreated cells (Fig. 5, A and B). This result is in accordance with previously published data demonstrating that stimulation of vascular smooth muscle cells with a number of distinct vasoconstriction agonists increases the ratio of Phalloidin to DNase I staining (24). We used a similar approach to examine actin polymerization dynamics in vascular smooth muscle cells in intact live vessels following 5-HT + L-NAME exposure. In lieu of fluorescently labeled phalloidin, which is not cell permeable, we used a silicon rhodamine derivative, SiR-actin, that is reported to increase its fluorescence ~100-fold when bound to F-actin (28). We were able to repeatedly image vascular smooth muscle cells costained with both SiR-actin and DNase I within an intact pressurized vessel following a 10-min control treatment and then after a 10-min exposure to 5-HT + L-NAME (Fig. 5C). Similar to the cell culture data, we observed a significant increase in the ratio of the fluorescent probe binding F-actin versus the probe for G-actin following treatment with 5-HT + L-NAME compared with the ratio following control treatment (Fig. 5D). These results support the hypothesis that vasoconstrictors mediate remodeling of the actin cytoskeleton, in part, via mechanism(s) that promote actin polymerization.

Together, our results suggest that modifications to the actin cytoskeleton occur following 5-HT exposure and that these changes are contingent on transglutaminase activity. To further assess the role of the actin cytoskeleton on remodeling, we examined inward remodeling, ex vivo, in vessels exposed to 5-HT + L-NAME + LimKi (an inhibitor of Lim Kinases). Remodeling was effectively blocked when LIN kinases were inhibited compared with vessels treated with 5-HT + L-NAME in the absence of LimKi (Fig. 6). In addition, the ratio of P-cofilin to total-cofilin was also reduced to control levels in the presence of LimKi (Fig. 4). Thus two distinct pharmacological approaches that decrease the phosphorylation state of cofilin also block remodeling. The data support a model in which signaling pathway(s), triggered by 5-HT exposure, intersect. The RhoA-Rho Kinase-Lim Kinase pathway merits particular investigation, since transglutaminases have been demonstrated to activate RhoA in response to 5-HT treatment (16). It is well established that this pathway increases actin polymerization, and we present data that inhibition of Lim Kinases, similar to cofilin addition, attenuates cofilin phosphorylation and blocks remodeling. We postulate that a brief exposure to vasoconstrictors induces the transition from a normal vessel to an inwardly remodeled one via the transglutaminase-dependent formation of persistent actin cytoskeleton structures. Moreover, it is this intracellular actin cytoskeletal alteration, within vascular smooth muscle cells, that stiffens the vascular smooth muscle cell so that it resists expansion under passive conditions, thus contributing to the remodeling process of the vascular wall around a smaller luminal diameter.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

The pathophysiological implications of our findings rest on the role that 5-HT at the concentration we used has or may have on reducing the structural diameter of blood vessels during the process of wound healing and/or vasospasm. Although our studies were performed in vessels from skeletal muscle, it has been demonstrated in rat middle cerebral arteries that 5-HT increases the phosphorylation state of cofilin and reduces the concentration of G-actin, indicating that 5-HT-induced remodeling of the actin cytoskeleton also occurs in the cerebral circulation (36), where localized vasoconstriction or vasospasm following subarachnoid hemorrhage or hemorrhagic stroke hinders blood flow re-establishment (8, 12, 39). Moreover, in a rat model of subarachnoid hemorrhage, inward remodeling of middle cerebral arteries, following exposure to hemolyzed blood, is blocked by inhibition of transglutaminases (17). In addition, presence of reversible cerebral vasoconstriction syndrome has been associated with intake of serotonergic drugs (23, 35, 43, 44), which suggest that 5-HT-induced vasoconstriction and acute inward remodeling, via changes to the actin cytoskeleton and upregulation of transglutaminases, may be implicated in the developmental process of this pathological condition.

In conclusion, our results provide novel evidence that suggest exposure of arterioles to 5-HT initiates inward remodeling processes that are linked to transglutaminase activation and affect the actin polymerization/dem polymerization equilibrium via alterations of the phosphorylation state of cofilin. Additional experiments are necessary to determine the points at which transglutaminase activities and the actin polymerization signaling pathway(s) intersect. The RhoA-Rho Kinase-Lim Kinase pathway merits particular investigation, since transglutaminases have been demonstrated to activate RhoA in response to 5-HT treatment (16). It is well established that this pathway increases actin polymerization, and we present data that inhibition of Lim Kinases, similar to cofilin addition, attenuates cofilin phosphorylation and blocks remodeling. We postulate that a brief exposure to vasoconstrictors induces the transition from a normal vessel to an inwardly remodeled one via the transglutaminase-dependent formation of persistent actin cytoskeleton structures. Moreover, it is this intracellular actin cytoskeletal alteration, within vascular smooth muscle cells, that stiffens the vascular smooth muscle cell so that it resists expansion under passive conditions, thus contributing to the remodeling process of the vascular wall around a smaller luminal diameter.

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