RhoA-Rho kinase signaling mediates endothelium- and endoperoxide-dependent contractile activities characteristic of hypertensive vascular dysfunction

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Denniss SG, Jeffery AJ, Rush JWE. RhoA-Rho kinase signaling mediates endothelium- and endoperoxide-dependent contractile activities characteristic of hypertensive vascular dysfunction. Am J Physiol Heart Circ Physiol 298: H1391–H1405, 2010. First published February 12, 2010; doi:10.1152/ajpheart.01233.2009.—Hypertensive vasomotor dysfunction is defined by endothelium-dependent contractions involving prostaglandins and ROS. Since both thromboxane-prostanoid receptor (TPr) signaling and ROS activate RhoA-Rho kinase (ROCK) in vascular smooth muscle (VSM) preparations, we hypothesized that enhanced endothelium-dependent contraction in the common carotid artery (CCA) of spontaneously hypertensive rats (SHRs) is ROCK mediated. ACh-stimulated contractions were approximately twofold greater in SHRs versus normotensive Wistar-Kyoto (WKY) rats, abolished by endothelial denudation or cyclooxygenase (COX)-1 inhibition, and nearly eliminated by TPr blockade. RhoA but not ROCK-II protein expression was increased (~50%) in the SHR CCA. Inhibition of ROCK, but not protein kinase C, caused a dose-dependent reduction in endothelium-dependent contractions to ACh across strains, with the highest dose mirroring the effect of high-dose TPr antagonism. Conversely, ROCK inhibition caused dose-dependent and endothelium- and nitric oxide-independent relaxation in CCAs precontracted with the TPr agonist U-46619. Prostacyclin was the predominant prostaglandin produced by ACh-stimulated CCAs, with greater than twofold more prostacyclin released from SHR versus WKY rats, and its production was unaffected by ROCK inhibition. RhoA activation was approximately twofold higher in quiescent SHR CCAs compared with those from WKY rats and was significantly increased by ACh stimulation. Augmentation of chemical superoxide quenching with tiron or inhibition of the NADPH oxidase-derived superoxide-producing pathway with apocynin reduced ACh-stimulated contractile activity in SHR more than in WKY rats, whereas the SOD mimetic tempol amplified the response. Exposure of CCAs to exogenous H2O2 caused contractions, similar to ACh stimulation, that were greater in SHR than in WKY rats, abolished by COX-1 inhibition, and highly attenuated by TPr blockade or ROCK inhibition. These results indicate that RhoA-ROCK may act as a molecular switch, transducing signals from endothelium-derived prostaglandin(s) and ROS, which are overproduced in SHR CCAs, to “turn on” VSM contractile pathways, thus mediating the enhanced endothelium- and endoperoxide-dependent vascular contractions characteristic of hypertension, among other cardiovascular disease states, such as diabetes and aging.

endothelium-dependent contraction; RhoA activation; Rho kinase; thromboxane-prostanoid receptor; carotid artery

ENDOTHELIUM-DEPENDENT VASOMOTOR (dys)function is dictated by a balance between endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors (EDCF) (10). Although much is known regarding the vascular smooth muscle (VSM) relaxation mechanisms of EDRFs (21, 39), this is not the case for EDCFs. Whether EDCFs activate the well-characterized RhoA-Rho kinase (ROCK) contractile pathway in systemic arteries is uncertain. RhoA activation by G protein-coupled receptor (GPCR) stimulation of G12/13 results in intracellular Ca2+ concentration sensitization via ROCK-mediated phosphorylation of the targeting subunit (MYPT1) of myosin light chain phosphatase (MLCP), leading to its inhibition (46). This, in turn, causes sustained myosin II regulatory light chain (RLC20) phosphorylation, and hence contraction, through the classic intracellular Ca2+ concentration-dependent calmodulin (CaM)-myosin light chain kinase (MLCK) pathway initiated by GPCR stimulation of Gα12/13-linked phospholipase C-β. However, since these mechanisms have largely been studied under conditions of vascular endothelial denudation and/or membrane permeabilization, there is little direct evidence demonstrating how the endothelium or EDCFs may activate/modulate RhoA-ROCK-mediated vasomotor contractile activity.

Several lines of evidence have suggested that vascular RhoA-ROCK signaling plays a pathophysiological role in cardiovascular disease states (24, 39), including both human and animal models of hypertension (21). EDCFs impair endothelium-dependent vasorelaxation, causing vascular dysfunction in spontaneously hypertensive rats (SHRs) compared with normotensive Wistar-Kyoto (WKY) rats (10, 54). The factors responsible for this endothelium-dependent contractile activity appear to be cyclooxygenase (COX)-derived endoperoxides and/or prostaglandin(s) (PGs) and ROS (50, 54). Importantly, EDCF activity is nearly abolished by antagonism of VSM thromboxane-prostanoid receptors (TPr), part of the GPCR family (29). These findings, together with the fact that rodent VSM contraction in response to ROS (18) or agonist stimulation of TPr (59) is mediated predominantly by RhoA activation and ROCK-mediated MYPT1 phosphorylation, gave us compelling reason to hypothesize that the activation of RhoA-ROCK signaling may be a VSM mediator of EDCF actions and, thus, enhanced in arteries from hypertensive animals. Therefore, the purpose of the present study was to test this hypothesis by investigating the effect of ROCK inhibition on EDCF activity in the common carotid artery (CCA) of SHRs, a model in which we recently found marked endothelium-dependent, COX- and TPr-mediated contractile activity (8).
MATERIALS AND METHODS

Materials

See the text below for details. Unless otherwise indicated, all reagents and drugs were purchased from Sigma-Aldrich (St. Louis, MO) or BioShop Canada (Burlington, ON, Canada).

Animals and Vascular Tissue Preparations

All experiments were performed using young adult SHRs and WKY rats aged 18–24 wk (purchased at the age of 7–10 wk from Harlan Laboratories, Indianapolis, IN, or Charles River Laboratories, Wilmington, MA) housed in a reversed 12:12-h light-dark cycle in a temperature-controlled facility (21 ± 1°C) with free access to standard laboratory chow and tap water. All procedures involving rats were approved by the University of Waterloo Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

We chose to study the CCA from young adult SHRs and WKY rats to further develop our past findings (8). Young adult animals were used so that early established alterations in vascular function could be investigated in response to sustained essential hypertension, since the rise in SHR blood pressure with age reaches a relatively stable plateau by 12–20 wk (e.g., see Refs. 11 and 63). The CCA segment of the systemic vasculature was investigated because of its importance both physiologically (the CCA contributes ~80% to total brain perfusion (9)) and clinically (CCA intima-media thickness (51) and hemodynamics (32) are strong predictors of atherosclerosis and cardiovascular disease risk) and because of its high degree of accessibility.

Animals were anesthetized via a 60–70 mg/kg body wt intraperitoneal injection of pentobarbital sodium (Vetoquinol N-A, Lavaltrie, QC, Canada). In some cases, CCA hemodynamics were measured using a Millar catheter-tip pressure transducer and a Transonic perivascular flow probe as we have previously described in detail (8). Animals were then killed by removal of the heart. The left and right ventricles were dissected and weighed. The right and left CCAs were excised and cleaned of surrounding connective tissue in 4°C buffer solution containing (in mM) 131.5 NaCl, 13.5 NaHCO₃, 11.2 glucose, 5.0 KCl, 2.5 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgCl₂, and 0.025 EDTA, which was prepared fresh daily in ultrapure water at pH 7.40. Some CCA segments were blotted dry, flash frozen, and stored at −80°C for later homogenization as detailed in the Supplemental Material for this article (see SUPPLEMENTAL METHODS). Other CCA segments were immediately prepared into arterial rings; those cut to 2.0 mm in axial length were used for testing CCA vasomotor functions and those cut to 3.0 mm in axial length were used for measuring CCA PG production and RhoA activation, as explained below. Digital images of CCA rings were captured through the calibrated eyepiece of a dissection microscope and processed using ImageJ software (version 1.37, standard toolbar, National Institutes of Health, Bethesda, MD) to quantify their dimensions. Care was taken not to damage the endothelium upon CCA excision and processing, as evidenced by a robust endothelium-dependent vasorelaxation response to the muncaricin receptor agonist ACh [maximal WKY CCA relaxation, >90% of precontraction to the α₁-adrenergic receptor agonist phenylephrine (PE); see Supplemental Fig. S1]. To test the vasomotor functions of CCAs without an intact endothelium, arterial rings were first denuded mechanically as previously described (8).

We found that, compared with endothelium-intact rings, this consistently resulted in the loss of ACh-stimulated CCA relaxation and a ~80% reduction in CCA endothelial nitric oxide (NO) synthase (eNOS) protein expression assessed via Western blot analysis (data not shown).

Vascular Mechanical and Molecular Functions

Vasomotor activities, PG production, and RhoA activation were examined to characterize CCA function. Each CCA ring was mounted and equilibrated at an optimal length for drug-stimulated isometric tension production in a 37°C tissue bath containing 5 ml of 95% O₂-5% CO₂-gassed buffer solution, as we have previously described in detail (8). Next, rings were stimulated to contract twice with exposures to 60 mM KCl and washed thoroughly, after which rings were preincubated for 30–90 min in buffer without or with enzyme/protein inhibitor(s), mimetic(s), or receptor antagonist drugs (as described in RESULTS) and then exposed to one of four protocols.

In protocol 1, Y-27632-stimulated relaxation responses were examined, where dose-dependent relaxation to the selective ROCK inhibitor Y-27632 (Calbiochem, San Diego CA) and then to a maximal dose of the NO donor sodium nitroprusside (SNP) was measured in CCAs that had been stimulated to contract near maximally with one of three distinct agents: KCl, PE, or the TPr agonist U-46619 (Cayman Chemical, Ann Arbor, MI). In protocol 2, ACh-, Ca²⁺ ionophore A-23187-, and H₂O₂-stimulated contrac-tile responses were examined, where dose-dependent contraction to these vasomotor stimulants was measured in quiescent (basal, nonprecontracted) CCAs. In protocols 3 and 4, ACh-stimulated PG production (protocol 3) and RhoA activation (protocol 4) were examined, where a maximal ACh dose (determined to be 10−4 M) was used to stimulate quiescent CCAs. In protocol 3, the bathing buffer was collected at the time of peak contraction (4–5 min after ACh stimulation), snap frozen in liquid N₂, stored at −80°C, and later used to assess PG production by measuring the stable metabolites of thromboxane A₂ (TXA₂) and/or PGL₃ using competitive EIA kits according to the manufacturer’s instructions (Cayman Chemical). PG concentrations were normalized to CCA protein content, which was measured in homogenized rings via a standard bicinchoninic acid protein assay (see SUPPLEMENTAL METHODS in the Supplemental Material). In protocol 4, CCA rings were snap frozen in liquid N₂ at the time of peak contraction, quickly homogenized (~5 min), stored at −80°C, and then used within 12–18 h to assess RhoA activation by measuring GTP-bound RhoA using an absorbance-based G-LISA kit according to the manufacturer’s instructions (Cytoskeleton, Denver, CO). Rings were homogenized in the lysis buffer and protease inhibitor cocktail of the kit in addition to 1X Halt Phosphatase Inhibitor Cocktails (Thermo Scientific, Rockford, IL), after which the homogenate supernatant protein concentration was immediately determined using the Precision Red protein assay of the kit and then equalized to 0.5 μg/μl protein. RhoA activation was measured using 30 μg protein homogenate loaded in equal volume. Prolonged (>24 h) storage of either CCA rings or ring homogenates at −80°C was found to significantly degrade the RhoA activation signal (data not shown).

Vascular Protein Expression

Homogenization and Western blot analysis as previously described (8) were used to quantify the relative expression of RhoA and ROCK-II protein in WKY and SHR CCAs. See SUPPLEMENTAL METHODS of the Supplemental Material for details. The ROCK-II (i.e., ROCK-α) isoform of the protein was investigated because it is most associated with MYPT1 phosphorylation and VSM Ca²⁺ sensitization (31).

Data Analyses and Statistics

Data are expressed as means ± SE. For details of data collection and curve fitting analysis, see SUPPLEMENTAL METHODS of the Supplemental Material. Statistical analyses were performed using statistical
analysis software (version 9.1, SAS Institute, Cary, NC). Differences were considered statistically significant if $P$ values were <0.05. Two-tailed, independent-sample Student’s $t$-tests were used for single-point comparisons, and one- or two-way ANOVAs with Bonferroni posttests, when necessary, were used for multiple within and/or between group comparisons.

RESULTS

Rat Physical Characteristics and CCA Hemodynamics

As shown in Supplemental Table S1, compared with WKY rats, aged-matched young adult SHRs exhibited a markedly increased CCA blood pressure as well as greater left ventricular mass and CCA wall cross-sectional area, indicative of cardiovascular hypertrophy. Furthermore, we confirmed our previous findings (8) that CCA blood flow and conductance are significantly reduced in SHRs versus WKY rats and that CCA pressure augmentation accompanied by late systolic flow augmentation, which are emerging markers of cardiovascular and cerebrovascular damage and risk (17), were already much more exaggerated in this young adult hypertensive state (data not shown).

ROCK Inhibition With Y-27632 Can Reverse SHR and WKY CCA Contractions Independent of NO- and Endothelium-Mediated Signaling

Y-27632-stimulated vasomotor activity in precontracted SHR and WKY CCAs. We first assessed the ability of Y-27632 to reverse tonic contractions (i.e., developed to a plateau over 30–45 min; see Supplemental Fig. S2) stimulated by either electromechanical coupling via KCl, which is thought to be more dependent on Ca$^{2+}$-dependent CaM-MLCK signaling (5), or pharmacomechanical coupling via the $\alpha_1$-adrenergic receptor agonist PE (Sigma-Aldrich) or the TPr agonist U-46619 (Cayman), which are thought to be more dependent on Ca$^{2+}$ sensitization via G$_{12/13}$-RhoA-ROCK-mediated MYPT1 phosphorylation (53). Y-27632 elicited a robust dose-dependent relaxation under all three precontraction conditions in both strains (Fig. 1), indicating that RhoA-ROCK activity contributes significantly to both GPCR- and membrane depolariization-stimulated SHR and WKY CCA tonic contraction, as it does in other vascular beds/animals (5). This Y-27632-stimulated vasorelaxation effect was least potent under KCl precontraction compared with either GPCR-mediated contractile state and most potent under PE precontraction (Fig. 1).

There were no strain-dependent differences found in Y-27632-stimulated vasorelaxation under KCl precontraction, whereas there were a number of small differences under GPCR-stimulated contractile states: the SHR CCA under PE precontraction was slightly more sensitive to Y-27632 ($P = 0.028$) and under U-46619 precontraction had a slightly blunted peak magnitude of relaxation ($P = 0.015$) compared with the WKY CCA (Fig. 1). However, collectively, these differences were relatively modest, suggesting that there is not a striking distinction between young adult SHR and WKY rats regarding the contribution of RhoA-ROCK activity to tonic CCA contraction in response to a given level of membrane depolarization or concentration of $\alpha_1$-adrenergic receptor or TPr agonist.

Effect of NOS inhibition, endothelium removal, and SNP exposure. Because ROCK isoforms are expressed not just in VSM but also in endothelial cells (24), and because RhoA-ROCK activation and NO production/signaling pathways may be interconnected (39), we further assessed whether EDRFs could be mediating the observed Y-27632-stimulated reversal of SHR and WKY CCA tonic contraction. This was accomplished by examining conditions where NOS was inhibited with $N^\omega$-nitro-$\omega$-arginine methyl ester (L-NAME) or the CCA endothelium was removed. There were no differences found among untreated (no drug), L-NAME-treated, and endothelium-denuded groups in Y-27632-stimulated vasorelaxation under KCl precontraction, whereas there were, again, a number of small differences under GPCR-stimulated contractile states: L-NAME or endothelium denudation decreased the sensitivity to Y-27632 under PE precontraction and reduced the magnitude of relaxation to Y-27632 under U-46619 precontraction (Fig. 1). Collectively, these data suggest that “unmasked” EDRF activity, and, in particular, NO, could in fact be mediating a small part of the reversal of tonic contraction stimulated by Y-27632, as has previously been suggested (21); however, this vasorelaxation response stimulated by ROCK inhibition is predominantly independent of NO- and endothelium-mediated signaling, consistent with previous findings in the intact aorta from SHRs and eNOS knockout mice (23).

SNP was able to elicit an additional relaxation response beyond that caused by the maximal Y-27632 dose in both strains under all experimental conditions (Fig. 1).

Endothelium-Dependent, Endoperoxide-Mediated CCA Contractions Were Greater in SHRs and Markedly Attenuated by ROCK Inhibition but not by PKC Inhibition

ACh-stimulated vasomotor activity in precontracted SHR and WKY CCAs. We confirmed our previous findings (8) that ACh, while stimulating a robust endothelium- and NO-mediated vasorelaxation response in PE-precontracted WKY CCAs, in fact stimulates an endothelium-dependent, COX-mediated “retraction” response at greater than or equal to $–6.5$ logM ACh that significantly impairs an otherwise robust, predominantly NO-mediated vasorelaxation response in SHR CCAs (Supplemental Fig. S1).

ACh-stimulated contractile activity in quiescent SHR and WKY CCAs: effect of NOS inhibition and endothelium removal. We further confirmed our previous findings (8) that contraction stimulated by ACh concentrations greater than or equal to $–6.5$ logM in quiescent SHR CCAs was completely abolished by endothelium denudation compared with the no drug condition, whereas contraction was markedly increased (>5-fold) in the presence of L-NAME (Fig. 2A), indicating the endothelium dependence of this effect and that NO strongly inhibits EDCF activity, consistent with findings in the conduit vasculature of SHRs and eNOS knockout mice (8, 10, 54, 62). In quiescent WKY CCAs, whereas no contractile activity was stimulated by cumulative ACh in the no drug condition, there was an ACh-stimulated, endothelium-dependent contractile response unmasked in the L-NAME condition (Fig. 2A) that was about half the magnitude of the corresponding SHR response ($P < 0.001$), again consistent with previous works (10, 54).
Effect of selective COX-1 and COX-2 inhibition as well as TP receptor antagonism. The primary objective of the present study was to investigate the effect of ROCK inhibition on SHR and WKY EDCF activity. Therefore, considering that NO inhibition of EDCF activity in the SHR aorta is mediated to a large extent by soluble guanylyl cyclase (62), and that cGMP/cGMP-dependent protein kinase I signaling can prevent VSM contraction via phosphorylation of RhoA, thereby inhibiting...
RhoA activation (36), the effects of all enzyme/protein inhibitor(s), mimetic(s), and receptor antagonist drugs were evaluated only while coincubated with l-NAME. This approach is consistent with previous works in this area (8, 10, 47).

We confirmed our previous findings (8) that the nonselective COX inhibitor indomethacin (Indo) and the preferential COX-1 inhibitor valeryl salicylate (VAS; Cayman Chemical) completely abolished ACh-stimulated CCA contractions, whereas the preferential COX-2 inhibitor NS-398 (Cayman Chemical) partly blunted the responses (Fig. 2B); this latter effect was possibly due to a nonselective inhibition of COX-1, as previously discussed (8). VAS has recently been reported to cause mild nonspecific TPR antagonism (44). Thus, we further clarified the COX-1 dependency of endothelium-dependent contractile activity, which is consistent with findings in the conduit vasculature of SHRs and COX-1 knockout mice (8, 10, 54), using the more selective and potent inhibitor SC-560 (Cayman Chemical) (45). Indeed, this compound completely abolished ACh-stimulated CCA contraction (Fig. 2B) but did not affect KCl- or U-46619-stimulated contractions (Supplemental Fig. S3), thus confirming its specificity.

The TPR antagonist SQ-29548 (Cayman Chemical), which highly attenuated contraction stimulated by U-46619 but did not affect KCl-stimulated contraction (Supplemental Fig. S3), abolished ~90% of the ACh-stimulated contractile response in SHRs and completely abolished this response in WKY rats (Fig. 2B).

Effect of selective ROCK inhibitors and PKC-specific inhibition. The selective ROCK inhibitor Y-27632 caused a dose-dependent attenuation of ACh-stimulated contraction in both SHRs and WKY rats (Fig. 2C), which, at the highest dose (~5.0 logM), mirrored the degree of abolishment caused by SQ-29548, consistent with our hypothesis that the vasocontractile signal after COX-1-derived EDCF stimulation of VSM TPr is transduced by RhoA-ROCK activation. Within the dose range used in these experiments, Y-27632 has previously been shown not only to inhibit ROCK but possibly also certain PKC isoforms (59), which, if activated, can phosphorylate PKC-potentiated inhibitor protein of 17 kDa, leading to Ca2+ sensitization via inhibition of MLCP at its protein phosphatase type 1c catalytic site (46). Therefore, to further elucidate the potential roles of these kinases in the EDCF signaling pathway, we wanted to confirm that the attenuation of endothelium-dependent ACh-stimulated contraction by ROCK inhibition was not caused by a possible ROCK-dependent decrease in EDCF production. We found that, although the ACh-stimulated contraction in SHR and WKY CCAs was almost completely eliminated by high-dose H-1152 (Fig. 3B), corroborating Fig. 2C), PGI2 production was unaffected (Fig. 3A), thus implying that the effect of ROCK inhibition on COX-1-and TPR-mediated EDCF signaling is VSM specific and not dependent on decreased EDCF production.
Correlation between ACh-stimulated SHR and WKY CCA PGI2 production and associated contraction. In WKY CCAs, ACh-stimulated contraction was about half the magnitude observed in SHR CCAs ($P < 0.001$; Fig. 3B, corroborating Fig. 2A) and associated with a less-pronounced (~2-fold) increase in PGI2 production (SHRs vs. WKY rats, $P < 0.001$; Fig. 3A). Indeed, within each strain, there was a significant positive correlation between CCA PGI2 production and con-
traction (Fig. 3C), which we were able to demonstrate directly for the first time because of our unique experimental approach, which provided for contemporaneous assessment of both variables.

RhoA but not ROCK-II Protein Was Overexpressed in the CCA of Young Adult SHRs

We confirmed our previous findings (8) that CCA eNOS and COX-1 proteins are overexpressed in young adult SHRs versus WKY rats (data not shown), consistent with the CCA functional data reported here and previously (8). Indeed, there is considerable variability surrounding the expression of RhoA-ROCK signaling components in the vasculature of young adult SHRs and WKY rats (23, 27, 40, 41). Therefore, we used immunoblot analysis to establish the relative CCA expression of RhoA and ROCK-II protein in our SHRs and WKY rats (Fig. 4, A and B), revealing that only RhoA was significantly overexpressed in SHR CCAs (54% vs. WKY CCAs per µg CCA protein).

RhoA Activation Was Greater in the CCA of Young Adult SHRs and Was Increased by ACh Stimulation

RhoA activation in quiescent SHR and WKY CCAs. The function of RhoA depends on its molecular switch from an “inactive” GDP-bound state to an “active” GTP-bound state (46). Compared with normotensive controls, a more than twofold higher GTP-bound RhoA-to-total RhoA ratio has been found in the freshly harvested intact aorta and isolated VSM cultured from SHRs and stroke-prone (SP)-SHRs, among other hypertensive models (27, 40, 41). Congruent with these works, we found that RhoA activation was approximately twofold higher per microgram of protein in quiescent SHR CCAs compared with WKY CCAs (P = 0.033; Fig. 5A). Notably, this effect could not have been caused by a difference in NO bioavailability between strains [thus inhibiting RhoA activation by cGMP-cGMP-dependent protein kinase I-dependent RhoA phosphorylation (36)] since these measurements were made on CCAs incubated with L-NAME. Moreover, the higher level of quiescent CCA RhoA activation measured in SHRs was markedly greater than the increase in SHR RhoA protein expression per microgram of CCA protein (see Fig. 4A), suggesting that total RhoA overexpression could not solely account for the overactivation.

Effect of ACh stimulation on SHR and WKY CCA RhoA activation. In mounted deendothelialized aortas isolated from Sprague-Dawley rats, stimulation with the TPr agonist U-46619 increases both contraction and RhoA activation (42). Moreover, increased RhoA activation has been stimulated by thrombin in cultured VSM isolated from the SHR and WKY aorta (40). However, to our knowledge, no study has investigated the effect of agonist stimulation on RhoA activation in intact arteries from SHRs. We found that CCA RhoA activation was significantly increased in SHRs (83 ± 16%, P = 0.001) and, to a similar extent, in WKY rats (81 ± 26%, P = 0.032) in response to ACh stimulation (Fig. 5), which elicited a greater than twofold greater contraction in SHRs than in WKY rats (P < 0.001; Fig. 4B, corroborating Figs. 2A and 3B).

In contrast to the quiescent PG production-RhoA activation status, where each were approximately twofold higher in SHRs (see Fig. 3A and Fig. 5A, respectively), the ~80% increase in ACh-stimulated RhoA activation found in both SHRs and WKY rats (Fig. 5A) did not mirror the differential increase in PGI2 production in response to ACh (Fig. 3A), which was approximately twofold higher in SHRs versus WKY rats. Moreover, no significant correlation could be detected between ACh-stimulated RhoA activation and contraction, as was found for ACh-stimulated PG production and contraction (Fig. 3C), possibly related to the number of animals/rings used.

ROCK Inhibition but not PKC Inhibition Highly Attenuated Endothelium- and NO-Independent, COX-1- and TPr-Dependent CCA Contraction Stimulated by Exogenous H2O2

In addition to PGs, ROS may play a role in EDCF signaling (47, 50). In Sprague-Dawley rats, VSM contraction stimulated by exogenous xanthine-xanthine oxidase, which can produce a mix of O2-, H2O2, and hydroxyl radicals, has been associated with RhoA activation and ROCK-mediated MYPT1 phosphorylation (18). Moreover, exposure of conduit vascular segments from various rodent models to exogenous xanthine-xanthine oxidase or H2O2 itself stimulates contractile activity that is sensitive to COX inhibition and TPr antagonism (50). We confirmed and extended these findings by assessing the effect of COX-1, TPr, ROCK, and PKC inhibition on exogenous H2O2-stimulated contraction of SHR and WKY CCAs.

H2O2-stimulated contractile activity in quiescent SHR and WKY CCAs: effect of NOS inhibition and endothelium removal. Consistent with previous works (50), both endothelium denudation and L-NAME caused a significant augmentation of CCA contraction to exogenous H2O2 in both strains (data not shown). Therefore, similar to the rationale for the approach to assessing ACh-stimulated contractions, we evaluated the effects of all enzyme inhibitors and receptor antagonist drugs using CCAs with an intact endothelium only while coincubated with L-NAME.

Fig. 2. Contraction stimulated by ACh in endothelium-denuded (n = 3–4) or endothelium-intact quiescent SHR and WKY CCA rings incubated with either no drug (n = 4–6), L-NAME (10−4 M, n = 17–18), which acted as a control for all other drug conditions (see RESULTS), or L-NAME coincubated with indomethacin (LN + Indom; 10−5 M, n = 3–4), valeryl salicylate (LN + VAS; 3 × 10−3 M, n = 4–5), SC-560 (LN + SC560; 3 × 10−3 M, n = 7–8), NS-398 (LN + NS398; 10−6 M, n = 8–9), SQ-29548 (LN + SQ29548; 10−6 M, n = 6–8), Y-27632 at 10−6, 10−5, and 10−4 M (LN + Y−6, n = 7–8; LN + Y−5.5, n = 8–9; and LN + Y−5, n = 5–7), H-1112 at 10−7, 10−6, and 10−5 M (LN + H−7, n = 7–8; LN + H−6, n = 8–9; and LN + H−5, n = 5–7), or GS-109203X (LN + GS109203X; 10−5 M, n = 8–9). For clarity, the curve with curve parameters representing the contraction response in L-NAME-treated control rings from SHRs (A, left) and WKY rats (A, right) was duplicated as a dashed line in B–D in their respective graphs. COX, cyclooxygenase; TPr, thromboxane-prostanoid receptor; ROCK, RhoA kinase. Data are means ± SE and are expressed relative to the previous contraction stimulated by KCl. *P < 0.05; †P < 0.05 vs. L-NAME; ‡P < 0.05 vs. L-NAME + SQ-29548 treatment; §P < 0.05 vs. L-NAME + Y-27632 at 10−6 M treatment or L-NAME + H-1112 at 10−7 M treatment; ¶P < 0.05 vs. L-NAME + Y-27632 at 10−5 M treatment or L-NAME + H-1112 at 10−6 M treatment within strain.
Effect of COX-1, TPr, ROCK, and PKC inhibition. Exogenous H$_2$O$_2$-stimulated contraction was almost twofold greater in SHR versus WKY CCAs (P $< 0.001$; Fig. 6). In both strains, the selective COX-1 inhibitor SC-560 completely abolished the contraction to H$_2$O$_2$, as did the TPr antagonist SQ-29548 and the selective ROCK inhibitor H-1152 in WKY rats, whereas in SHRs, SQ-29548 and H-1152 similarly abolished $>90\%$ of the H$_2$O$_2$-stimulated contractile response (Fig. 6), as they did in the ACh-stimulated contractile responses described above (Fig. 2C). Conversely, the selective PKC inhibitor GF-109203X had a minimal effect on H$_2$O$_2$-stimulated contraction in either strain (Fig. 6).

*Fig. 3. Production of PGI$_2$ (A) from quiescent or ACh-stimulated CCA rings isolated from SHRs and WKY rats and incubated with L-NAME (10$^{-4}$ M, n = 6–7) or L-NAME coincubated with SC-560 (3 $\times$ 10$^{-7}$ M, n = 6–7) or H-1152 (10$^{-6}$ M, n = 6–7). Rings were stimulated to contract with 60 mM KCl and then washed. In some cases, rings were incubated in a quiescent state for 45 min before the bathing buffer was collected. In other cases, after 40 min of quiescent incubation, rings were exposed to a maximal dose of ACh, eliciting peak contraction within 4–5 min (B; expressed relative to the previous KCl-stimulated contraction), at which time the bathing buffer was collected. The concentration of 2,3-dinor-6-keto-PGF$_{1\alpha}$ in the collected buffer was used as a marker of CCA PGI$_2$ production and expressed relative to CCA ring protein per milliliter of bathing buffer. C: relationship between individual ring PGI$_2$ production in A and its corresponding ACh-stimulated tension development in B. Data are means $\pm$ SE. $^*$$P < 0.05$ vs. L-NAME treatment of quiescent rings; $^\dagger$$P < 0.05$ vs. L-NAME-treated, ACh-stimulated rings within strain. See RESULTS for SHR versus WKY comparisons.
Effect of catalase treatment. Both polyethylene glycol-conjugated (PEG)-catalase (500 U/ml, from the bovine liver) and unconjugated catalase (1,200 U/ml, from the bovine liver) abolished all contractile activity to exogenous H$_2$O$_2$ (data not shown), confirming the specificity of H$_2$O$_2$ contractile effects. Together, these data suggest that SHR and WKY CCA contractions in response to the potential EDCF H$_2$O$_2$ are dependent on COX-1 and mediated by TPr and RhoA-ROCK signaling.

Augmentation of ACh-stimulated CCA Contraction by Tempol and Attenuation by Tiron and Apocynin Suggest that Endogenous H$_2$O$_2$ Acts as an EDCF

Since we found that ACh- and H$_2$O$_2$-stimulated CCA contractions are each greater in SHRs versus WKY rats, and that each are reliant on a signaling mechanism including COX-1, TPr, and RhoA-ROCK to elicit these contractions, we further explored the effect of modulating ROS-generating or -quenching pathways on ACh-stimulated SHR and WKY CCA contractions.

ACh-stimulated contractile activity in quiescent SHR and WKY CCAs: effect of tiron and apocynin. ROS could affect EDCF signaling either directly, by stimulating VSM contraction, or indirectly, by decreasing NO bioavailability (50). In the presence of l-NAME, thus excluding the latter possibility, both the cell-permeable O$_2^-$ scavenger tiron (Fig. 7A) and the cell-permeable NADPH oxidase inhibitor apocynin (Fig. 7B) moderately reduced the sensitivity and maximal amplitude of ACh-stimulated contraction in SHR CCAs, whereas in WKY CCAs, there was only a small, nonsignificant reduction in sensitivity, consistent with previous work (47) demonstrating a greater burst of endothelial ROS production in the SHR versus WKY aorta after ACh stimulation.

Notably, at the commonly used concentrations employed in our experiments, there is evidence suggesting that tiron can...
bind Ca\(^{2+}\), thereby affecting contraction (14), and apocynin may elicit vasorelaxation by a mechanism possibly involving ROCK inhibition (37). Importantly, we found that neither tiron nor apocynin affected contraction stimulated by KCl (a Ca\(^{2+}\)-dependent mechanism; see Ref. 5) or U-46619 (a ROCK-dependent mechanism; see Ref. 53 and Supplemental Fig. S3), thereby discrediting these potential nonspecific effects in our SHR and WKY CCA preparations.

**Effect of tempol and PEG-catalase.** Tempol is a cell-permeable nitroxide compound that can metabolize O\(_2^-\) to H\(_2\)O\(_2\) and, thus, is considered a SOD mimetic (58). In both SHR and WKY CCAs, tempol significantly augmented ACh-stimulated contractions (Fig. 7A) without exerting nonspecific effects on KCl- or U-46619-stimulated contractions (Supplemental Fig. S3).

Taken together, these data suggest that endogenous H\(_2\)O\(_2\), possibly derived from elevated NADPH oxidase-derived O\(_2^-\) production and made more available by the SOD mimetic properties of tempol, may act as an EDCF in the CCAs of SHRs and WKY rats, congruent with our exogenous H\(_2\)O\(_2\) responses (Fig. 6) and work (50) in other vascular beds and/or animal models using various other inhibitors. As such, it was further hypothesized that catalase, which completely eliminated the contraction to exogenous H\(_2\)O\(_2\) (described above), might inhibit ACh-stimulated contraction at least to the same extent as tiron and apocynin. We exposed SHR and WKY CCAs to a 500 U/ml concentration of PEG-catalase for at least 3 hr before assessing ACh-stimulated contraction as to ensure its penetration into the cells of the vascular wall (58), an unmitigated concern when using nonconjugated catalase (48, 50).

However, contrary to the hypothesis, PEG-catalase actually augmented contraction stimulated by ACh in both strains to the same extent as did tempol (Fig. 7A). As addressed in the **DISCUSSION**, this last finding potentially raises some controversy regarding the precise vasomotor action of endogenous H\(_2\)O\(_2\) that may be produced in response to ACh in the present model.

**DISCUSSION**

The data presented provide important new information related to vascular EDCF signaling and support our hypothesis that EDCF(s) trigger RhoA-ROCK signaling in the arterial wall, particularly in SHRs. Thus, RhoA-ROCK may act as a molecular switch transducing signals from EDCF(s) to turn on VSM pathways related to contractile activity, uniquely implicating RhoA-ROCK in the multifaceted disorder of endothelium-dependent vascular dysfunction hallmarking hypertension. This work contributes to a growing body of evidence elucidating the mechanisms of RhoA-ROCK signaling in VSM contraction (46, 59) and its over-activation and function in cardiovascular disease states, including hypertension (21, 24, 39).

**Methodological Considerations**

Vascular endothelial denudation and/or membrane permeabilization preparations have provided much insight into the regulation of RhoA-ROCK in VSM contraction. However, as recently highlighted by Neppl et al. (30), such preparations by their nature cannot be used to investigate whether endothelial cell signaling affects RhoA-ROCK-mediated VSM activation and function, which they showed can occur at a molecular level in important ways. Studying the intact vasculature, though, carries with it the inherent difficulty of accurately interpreting such findings because of the presence of both endothelial and VSM cells, each expressing RhoA and ROCK (24), and thus the possibility of ROCK inhibition having cell-specific effects. Importantly, it is apparent in the present study that the prevention of endothelium-dependent, COX-1- and TPr-mediated CCA contractions can be attributed to the action of ROCK inhibition on the VSM exclusive of the endothelium. Indeed, alterations in EDRF production/activity can be excluded because TPr-stimulated contraction could be reversed by ROCK inhibition in a manner independent of an intact endothelium...
or the EDRF NO and because ROCK inhibition in the presence of L-NAME could prevent TPr-stimulated contraction, together with the fact that L-NAME was coincubated while the inhibitory effect of ROCK inhibition on ACh and H$_2$O$_2$ stimulation was tested. Moreover, alterations in EDCF production can be excluded because ACh-stimulated PG production was not affected by ROCK inhibition.

The specificity of ROCK inhibitors, with respect to their activity toward PKC isoform(s) (46), presents another difficulty in studying the role of RhoA-ROCK in vascular functions. However, by demonstrating that contraction stimulated by PKC activation was unaffected by the two structurally distinct ROCK inhibitors used, and that broad-spectrum PKC inhibition did not affect ACh- or H$_2$O$_2$-stimulated contraction, it is apparent that the effect of the ROCK inhibitors on endothelium-dependent CCA contractions cannot be attributed to their previously reported nonspecific effects (59).

**Evidence for a Direct Role of EDCF in the Activation of Vascular RhoA-ROCK Signaling**

Some reports in the pulmonary vasculature have suggested that EDCF(s) may play a significant role in mediating hypoxia-stimulated pulmonary vasoconstriction by activating VSM RhoA-ROCK signaling, although the exact identity of the potential EDCF(s) remains elusive (33, 34). While major inherent differences exist between the pulmonary and systemic vasculature, the data from the present study may help to elucidate the mechanism(s) by which EDCF(s) activate RhoA-ROCK in the pulmonary vasculature and possibly provide important insight into the potential mechanisms of established and emerging therapeutic interventions used to treat pulmonary hypertension (1, 3), including prostacyclin analogs (e.g., epoprostenol and iloprost) and ROCK inhibitors (e.g., fasudil).
As the present manuscript was in preparation for submission, Chan et al. (7) published a study that evaluated the effect of the ROCK inhibitors Y-27632 and HA-1077 (fasudil) on contractions in the aorta of SHRs and WKY rats. The data from this independently conceived and executed work, although generated using a distinct segment of the systemic conduit arterial system from differently aged animals, eloquently supports our findings that high-dose ROCK inhibitors can almost abolish ACh- and A-23187-stimulated contractile activity, in addition to corroborating our findings that this effect must be mainly due to a direct inhibition of VSM ROCK. Notably, the present study both strengthens and extends the work by Chan et al., as well as other studies evaluating the effects of ROCK inhibitors on vascular function, in a number of important regards. Our well-controlled functional experiments established that the inhibition of endothelium-dependent contractions was indeed concentration dependent and maximally effective in both strains at the typically used high dose of Y-27632 as well as HA-1077, a more potent and specific ROCK inhibitor than either Y-27632 or HA-1077. Moreover, our control experiments discredit the possibility of any PKC-related nonspecific effects previously reported for Y-27632, thus solidifying the interpretation of vascular function data sets relying on the effect of ROCK inhibitors.

As an obvious major extension of the study by Chan et al., we demonstrate not only that quiescent RhoA activation is elevated in SHR CCAs compared with WKY CCAs but also show, for the first time, that RhoA activation is stimulated by ACh, thus providing molecular support of the functional ACh-stimulated findings. Furthermore, beyond confirming with different pharmacological tools previous work (50) suggesting that ROS contributes to conduit vascular endothelium-dependent contractions, we established, for the first time, that H2O2, which is speculated to be an EDCF (50), elicits a COX-1- and TPr-dependent contraction in both SHR and WKY CCAs that is indeed mediated by ROCK. Thus, our data provide strong evidence that the two most likely candidate species for EDCFs, COX-1-derived PGs and ROS, both trigger VSM contraction by TPr-dependent RhoA-ROCK signaling.

Noteworthy, using tempol and PEG-catalase for the first time in ACh-stimulated SHR and WKY vascular preparations, we found that both these two pharmacological tools, in fact, augmented ACh-stimulated CCA contraction. A potential explanation to reconcile these unexpected and seemingly paradoxical results could emerge from considering that H2O2 has also been shown to elicit relaxation, acting presumably as an endothelium-derived hyperpolarizing factor (EDHF) endogenously, in many vascular beds and animal models, including intermediate-sized arteries from SHRs and WKY rats (6, 8, 12), and that tempol can act also as a catalase mimetic (58). Thus, it appears possible that tempol, like catalase, augmented ACh-stimulated contractile activity not by enhancing (as conventionally suggested) but by diminishing the bioavailability of H2O2 (58), which endogenously may have in fact been acting not as an EDCF (as suggested by our exogenous H2O2 data) but as an EDRF, thus curtailling contraction, akin to the effect of NO demonstrated here and by others (8, 10, 54, 62). Because these findings were contrary to our hypothesis of how these pharmacological tools (in particular PEG-catalase) would act, we have no additional data to directly substantiate this possible caveat put forth. Therefore, it is clear that further functional, biochemical, and pharmacological exploration is required to definitively support or refute this controversial hypothesis.

Proposed Mechanism: Overproduction of EDCF(s) Causes Overactive RhoA-ROCK Signaling in the Intact SHR Vasculature

Although it is well established that both endothelium-dependent contractions (8, 10, 50, 54) and RhoA-ROCK activation (21, 27, 28, 39–41, 56) are greater in the hypertensive vasculature, the important root causes of these pathological characteristics have not been fully elucidated. Reconciling findings from the present study with previous literature, a novel mechanism accounting for conduit artery endothelium-dependent RhoA-ROCK activation and contractile activity can be hypothesized to better understand what may account for triggering the overactivation of this signaling pathway in hypertension (Fig. 8). We propose that overproduction of EDCFs could be a main cause of overactive RhoA-ROCK signaling in the intact vascular wall of SHRs.

To our knowledge, it is currently unknown whether RhoA, ROCK, and their regulatory proteins are differentially expressed in the SHR endothelium. We (present study) and others (40) have found greater RhoA protein in the intact CCA or aorta and cultured aortic VSM from young adult SHRs or SP-SHRs versus WKY rats, whereas others found no differences in expression (27, 41). Similarly, we (present study) and others (7, 23, 27, 40, 41) have found no difference in ROCK-II protein or mRNA, whereas others have found greater ROCK-II or ROCK-I expression (2, 28), in the intact, denuded, or aged CCA or aorta or cultured aortic VSM from SHRs or SP-SHRs. Likewise, the expression of Rho-GDIα, a RhoA-ROCK regulatory factor, has been found to be no different (27, 40) or greater (4) in the aorta from SHRs or SP-SHRs. From these conflicting results, it is not clear whether overexpression of VSM RhoA- and ROCK-associated proteins per se could itself be a significant cause of greater membrane-bound RhoA-GTP (i.e., activated RhoA) (27, 40, 41) and/or ROCK-mediated MYPT1 phosphorylation (i.e., activated ROCK) (27, 28, 56) found in intact conduit arteries or cultured VSM from hypertensive models (41). Certainly it could play a role (28, 40), as reflected in reports of SHR VSM contractile hyperresponsiveness to agonists such as PGH2 (13); however, in the present study and in previous works (27, 40, 41), the level of RhoA expression does not appear to account for the increase in SHR RhoA activation or the increase in MYPT1 phosphorylation (27).

Alternatively, the main cause of overactive RhoA-ROCK signaling in the SHR vasculature may be overproduction of GPCR agonists. As we propose, for the first time, those agonists could include EDCFs. Endothelium-derived PGs (50) and ROS (47) are both produced in greater amounts in the SHR versus WKY conduit vasculature. This may be best explained by a greater rise in endothelial intracellular Ca2+ upon agonist stimulation (47), coupled with overexpression of endothelial cell COX-1 and PGH2 and TXA2 synthases (49) as well as subunits of NADPH oxidase (16). Indeed, ACh elicits a large endothelium-dependent increase in SHR and WKY vascular PGH2 production, which, as the present study established, has a strong direct correlation with contraction magnitude; con-
versely, the endothelial Ca\textsuperscript{2+} mobilizers A-23187 and ADP elicit a large increase in TXA\textsubscript{2} production (50). COX-1 activation appears to be the source of these endoperoxide derivatives in SHRs and WKY rats, whereas, in other vessel beds (55) and/or animal models and in humans (60), various PGs derived from either overexpressed COX-1- or COX-2-specific activation, including PGF\textsubscript{2\alpha} and 8-isoprostane, have been reported to be associated with endothelium-dependent contractions. In response to endothelial Ca\textsuperscript{2+} mobilizers, COX (47) and NADPH oxidase, as indicated for the first time in the present study, may be sources of ROS that might possess EDCF action.

Stimulation of the VSM TPr is essential in eliciting endothelium-dependent, endoperoxide-mediated contractions (8, 10, 50, 54, 55, 60). As clearly demonstrated by the present findings, ROCK signaling activated via TPr stimulation by PGs, and possibly ROS, plays an obligatory role in transducing these endothelium-derived signals within the VSM. Certainly, future investigation is necessary to elucidate exactly how EDCFs promote the RhoA-GTP membrane association shown in the present study, in turn activating ROCK, and to establish exactly what effect this has on MYPT1 and RLC\textsubscript{20} phosphorylation status (18, 30, 42, 46, 59). In addition, it will be important to establish at this molecular level how the production of EDRFs, including NO (21, 36, 39) and also EDHF(s) [e.g., via K\textsuperscript{+} channels (19)], counteract these EDCF-triggered RhoA-ROCK-mediated signaling events, and vice versa.

In the SHR VSM, the IP receptor, which typically transduces a vasorelaxation signal from PGI\textsubscript{2}, is equally expressed (49) but dysfunctional (15) compared with WKY VSM. Instead, PGI\textsubscript{2} is transduced, like TXA\textsubscript{2} and PGF\textsubscript{2\alpha}, as a vasocontractile signal via the TPr, which is also equally expressed across strains (49). Accordingly, in contrast to the evidence suggesting hyperresponsiveness to PGH\textsubscript{2} (13), we (present study) and others (7) have shown that SHR VSM contraction to the ROCK-dependent TPr agonist U-46619 is no different from that of WKY rats. Therefore, taken together, it appears that both endothelium-dependent contractions and RhoA-ROCK activation in SHR conduit arteries may be best explained by EDCF overproduction. Moreover, the SHR VSM contractile hyperresponsiveness to exogenous H\textsubscript{2}O\textsubscript{2} shown here and by others (50) may best be explained not necessarily by overexpression of VSM RhoA and ROCK associated proteins but by H\textsubscript{2}O\textsubscript{2} stimulation of VSM COX-1 (20), which is overexpressed in SHR VSM (49), thus resulting in PG overproduction that, via VSM TPr, elicits RhoA activation and ROCK-mediated MYPT1 phosphorylation (18).

Fig. 8. Proposed mechanism accounting for the endothelium-dependent contractile activity prominent in the CCA of SHRs. See DISCUSSION for details. [Ca\textsuperscript{2+}], intracellular Ca\textsuperscript{2+} concentration; PLA\textsubscript{2}, phospholipase A\textsubscript{2}; eNOS, endothelial NOS; AA, arachidonic acid; CAT, catalase; TXA\textsubscript{2}, thromboxane A\textsubscript{2}; EDRF, endothelium-derived relaxing factor; PLC-\beta, phospholipase C-\beta; sGC, soluble guanylyl cyclase; SR, sarcoplasmic reticulum; CaM, calmodulin; MLCK, myosin light chain; MLCP, MLC kinase; MLCP, MLC phosphatase.
Pathophysiological and Clinical Significance

Impaired vasorelaxation caused by endothelium-dependent contractions occurs not only in SHRds but also various other hypertensive animal models and in human essential hypertension (54) as well as in aging (60) and diabetic (43) models. Our present data collectively signify that this vasomotor dysfunction characteristic may be a biomarker of overactive vascular RhoA-ROCK signaling, which is supported by the fact that biomolecular evidence exists for vascular RhoA-ROCK overactivation in all of these risk factor cohorts (22, 26, 41, 61). Beyond its contractile effects, overactive RhoA-ROCK stimulates cytoskeletal reorganization, proliferation/differentiation, migration, inflammation, and apoptosis (24), all cellular processes implicit in cardiovascular disease states, including hypertension. Thus, small artery/arteriolar EDCF activity, as recently shown in SHRds to be prevented by statin treatment (55), which is known to have a pleiotropic RhoA-ROCK inhibition-related effect (22), may be responsible for both active/functional (endothelium-dependent vasomotor tone) and passive/structural (vascular remodeling and rarefaction) resistances, thereby directly contributing to elevated blood pressure. Furthermore, since EDCF activity in the conduit vasculature, in which the present experiments were performed, beyond potentially exerting a mechanical consequence (8), may also impart a biological consequence (e.g., inflammatory mediators and VSM migration/activation), this could help explain at a cellular/molecular level why hypertension conveys such a high risk of developing large vessel diseases related to RhoA-ROCK overactivation, such as atherogenesis (25) and aortic aneurysm (57), which also occur with aging and diabetes. Accordingly, a better understanding of the mechanisms governing endothelium-dependent contraction and RhoA-ROCK signaling in both the conduit and resistance vasculature should uncover new prevention and/or treatment strategies, beyond COX or TPR blockade (38), to combat vascular pathologies characteristic of hypertension and other cardiovascular disease disorders.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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