ANTIOXIDANTS & REDOX SIGNALING
Volume 13, Number 7, 2010

DOI: 10.1089/ars.2009.2908

ORIGINAL RESEARCH COMMUNICATION

Mechanical Strain-Induced RhoA Activation Requires NADPH Oxidase-Mediated ROS Generation in Caveolae

Ying Zhang, Fangfang Peng, Bo Gao, Alistair J. Ingram, and Joan C. Krepinsky

Abstract

Increased intraglomerular pressure leads to kidney fibrosis, and can be modeled by exposing glomerular mesangial cells (MC) to mechanical strain. We previously showed that RhoA mediates strain-induced matrix production. Here we investigate whether reactive oxygen species (ROS) are required for RhoA activation. Maximal RhoA activation (1 min) was inhibited by ROS scavenge or NADPH oxidase inhibition. Strain activated NADPH oxidase, with Rac1, $p47^{phox}$, and $p67^{phox}$ membrane translocation, and Rac1 activation, observed within 30 sec. Epidermal growth factor receptor (EGFR) inhibition blocked RhoA and Rac1 activation, $p67^{phox}$ membrane translocation, and ROS generation. However, EGFR activation was unaffected by ROS inhibitors, placing it upstream of ROS generation. We previously showed, using chemical disruption, that caveolae mediate strain-induced EGFR and RhoA activation. In MC from caveolin-1 knockout mice, which lack caveolae, RhoA and Rac1 activation, $p67^{phox}$ membrane translocation, and ROS generation were absent. These were rescued by caveolin-1 re-expression. ROS generation, Rac1 activation, and $p67^{phox}$ membrane translocation were also prevented by Src inhibition. They were absent in MC stably infected with caveolin-1 Y14A, a mutant resistant to Src phosphorylation. In MC, caveolae are thus important mediators of strain-induced ROS generation through NADPH oxidase, mediating a signaling cascade which results in RhoA activation.
Antioxid. Redox Signal. 13, 959–973.

Introduction

Elevated glomerular capillary pressure (Pgc) is an important determinant of progression in chronic renal diseases of diverse etiologies. Increased Pgc transmits to mesangial cells (MC), which provide architectural support for the glomerular capillary tuft, as mechanical strain (36). *In vitro*, MC subjected to cyclic strain/relaxation increase extracellular matrix protein synthesis (37), thus providing a relevant model system to study mechanical strain-induced signaling in MC. We have shown an important role for the small GTPase RhoA in strain-induced production of the matrix protein fibronectin in MC (20). In studying the mechanism of RhoA activation in this setting, we have also demonstrated that the plasma membrane microdomains caveolae, as well as their marker protein caveolin-1, are required for RhoA activation. Caveolae were recently shown to be involved in localized reactive oxygen species (ROS) production (46, 50). Here we investigate if strain leads to ROS production in MC, if this is required for RhoA activation, and whether caveolae are required for ROS generation.

Caveolae are 50–100 nm plasma membrane omega-shaped invaginations that function in endocytosis, and play an important role in cell signaling (35). They are found in most cell types, including MC (40), and are defined by the presence of caveolin, a 21–24 kDa integral membrane protein that is essential for their formation. Three isoforms of caveolin exist, with MC having been shown to express caveolin-1 (cav-1) and −2 (40). In cells lacking cav-1 either naturally, through genetic manipulation or through downregulation, caveolae are not present. Conversely, expression of cav-1 can induce the *de novo* formation of caveolae in these cells. The role of cav-2 is less clear, possibly functioning to stabilize the cav-1 protein (34, 35).

Cav-1 functions not only in the formation of caveolae, but also interacts with signaling molecules to sequester these proteins within caveolae and modulate their catalytic activities (35). Phosphorylation of cav-1 on tyrosine 14, first identified in v-Src-transformed cells (49), may function to facilitate cav-1 interaction with other proteins in a stimulus-specific fashion (12, 25). Recently, we have shown that Src is activated by stretch in MC and phosphorylates cav-1 on Y14.

Division of Nephrology, McMaster University, Hamilton, Canada.

Stretch-induced RhoA activation is dependent on this phosphorylation event (32). We have also previously demonstrated that transactivation of the epidermal growth factor receptor (EGFR) is required for stretch-induced signal transduction, and its transactivation also requires Src activation (21).

Although mechanical stress has been shown to lead to ROS production in other cell types (24, 26, 45), no studies have addressed the role of ROS in stretch-induced MC signaling. The generation of ROS can occur through several enzyme systems. Most studies have implicated NADPH oxidase as the major source of ROS in response to mechanical stresses in vascular cells (3), and its components have been shown to exist in MC (9, 10, 28). The mitochondrial electron transport chain, a second enzymatic source of ROS, has also been shown in some studies to contribute to stretch-mediated ROS generation (1, 2).

NADPH oxidase, which converts molecular oxygen to superoxide (O_2^-), is an enzyme complex consisting in MC of a) a membrane-associated catalytic core comprising $p22^{phox}$ and a $gp91^{phox}$ homologue (Nox 1 or 4); b) a group of cytosolic proteins ($p47^{phox}$, $p67^{phox}$) that require translocation from cytosol to the membrane core for enzyme activation; and c) the GTPase Rac1 which is also translocated to the membrane during activation (24, 26, 45). Once activated, the various components assemble to form a complex enzyme system. The events required for NADPH oxidase activation include $p67^{phox}$ membrane translocation and association with Nox1, Rac activation and association with $p67^{phox}$, and $p22^{phox}$ functioning as a docking site for $p67^{phox}$ (3). Nox4 association with $p22^{phox}$, however, does not require these cytosolic subunits. Recently, components of the NADPH oxidase system were found to be compartmentalized to caveolae, with localization important to signal transduction and ROS production (41, 46, 50). Indeed, $p67^{phox}$, $p47^{phox}$, and Rac1 have been found in these microdomains, with translocation additionally induced by PMA (46). In vascular smooth muscle cells, Nox1 was found in caveolae at baseline, and angiotensin II induced Rac1 translocation to caveolae (13). Additionally, in endothelial cells, Nox subunits were preassembled in caveolae at baseline, with additional recruitment of $p47^{phox}$ in response to TNFα (48).

In this work, we thus asked whether stretch leads to ROS generation in MC and whether this is dependent on caveolae and Src activation. We investigated whether ROS generation was required for RhoA activation. Our studies demonstrate for the first time that caveolae mediate stretch-induced ROS production and downstream RhoA activation in MC. ROS generation requires Src and phosphorylation of cav-1 at Y14.

Materials and Methods

Cell culture

Sprague-Dawley primary rat and mouse MC were obtained from glomeruli of rats or mice (cav-1 knockout or their corresponding wild-type, B6129SF1/J, both from Jackson Laboratory, Bar Harbour, ME) by differential sieving and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (Invitrogen, Burlington, ON, Canada), streptomycin (100 μg/ml), and penicillin (100 units/ml) at 37°C in 95% air, 5% CO_2. Experiments were carried out using cells between passages 6–15. All experiments use primary rat cells with the exception of those experiments using cav-1 knockout cells (with or without cav-1 reconstituted). These are of murine origin.

Application of strain/relaxation

MC were plated onto 6-well plates with flexible bottoms coated with bovine type I collagen (Flexcell International Corp., Hillsborough, NC). After achieving confluence, cells were rendered quiescent by incubation for 24 h in serum-free medium. Plates were exposed to continuous cycles of strain/relaxation generated by a cyclic vacuum produced by a computer-driven system (Flexercell 4000, Flexcell International Corp.), with each cycle being 0.5 sec of strain (10%) and 0.5 sec of relaxation, for a total of 60 cycles/min.

Pharmacologic inhibitors were added as follows prior to stretch: N-acetylcysteine (NAC, Sigma, Oakville, ON, Canada), 4 m*M* for 30 min, apocynin (Calbiochem, Gibbstown, NJ), 300 μ*M* for 30 min, diphenyleneidodonium chloride (DPI, Calbiochem), 40 μ*M* for 30 min, cyclodextrin (Calbiochem), 10 m*M* for 60 min, AG1478 (Sigma), 1 μ*M* for 30 min, SU6656 (Calbiochem), 10 μ*M* for 30 min, PP2 (Calbiochem), 10 μ*M* for 30 min, pegylated superoxide dismutase (SOD) (Sigma), 10 U/μl for 3 h) and catalase (Calbiochem), 500 U/μl for 1 h.

Protein extraction and Western immunoblotting

Cells were lysed and protein extracted as we have reported previously (20). Briefly, cells were lysed in a buffer containing 20 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 1% Triton X-100, 1 m*M* EDTA, 1 m*M* EGTA, 2.5 m*M* sodium pyrophosphate, 1 m*M* β-glycerophosphate, 2 m*M* DTT, 1 m*M* sodium vanadate, 1 m*M* phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 2 μg/ml aprotinin. Lysates were centrifuged at 4°C, 14,000 rpm for 10 min to pellet cell debris. Supernatant (50 μg) was separated on a 10% SDS-PAGE gel, and Western blotting performed as we have described (20). Antibodies used included monoclonal RhoA (1:500, Santa Cruz, Santa Cruz, CA), monoclonal Rac1 (1:500, Cytoskeleton, Denver, CO), polyclonal $p47^{phox}$ (1:1000, Santa Cruz), goat polyclonal $p67^{phox}$ (1:500, Santa Cruz), polyclonal phospho-EGFR Y1068 (1:1000), polyclonal EGFR (1:1000, both Cell Signaling, Boston, MA), polyclonal phospho-Vav2 Y172 (1:500; GenScript), and polyclonal Vav2 (1:1000; Santa Cruz).

For cytosol-membrane fractionation, cells were harvested in hypotonic lysis buffer (50 m*M* Tris-HCl (pH 7.5), 5 m*M* $MgCl_2$, 10 m*M* EGTA, 2 m*M* EDTA, and inhibitor cocktail), homogenized by 25 *g* needle passage, and centrifuged at 100,000 *g* for 60 min. Supernatant was taken as cytosol and the pellet resuspended in regular lysis buffer (20) with 60 m*M* N-octyl-glucopyranoside. After centrifugation at 100,000 *g* for 60 min, the supernatant was collected as the membrane fraction.

For immunoprecipitation, MC were lysed with buffer containing 60 m*M* N-octyl-glucopyranoside, clarified and equal amounts of lysate incubated overnight with 1 μg of $p67^{phox}$ polyclonal antibody (goat, Santa Cruz), rotating at 4°C. Subsequently, 25 μl of protein G-agarose slurry was added for 1.5 h at 4°C. Immunoprecipitates were washed three times with lysis buffer, resuspended in 2X sample buffer, boiled, and analyzed by Western blot analysis as above using a monoclonal anti-phosphoSer/Thr antibody (BD Transduction, 1:1000).

RhoA and Rac1 pull-down assay

This was performed as described previously (20). Briefly, cells were lysed in hypertonic buffer and GTP-bound RhoA or Rac1 was immunoprecipitated from cleared lysate with 30 μg of glutathione-agarose bound GST-tagged rhotekin RhoA binding domain for RhoA or Pak-binding domain (PBD) for Rac1 (both from Cytoskeleton). Beads were washed and the immunoprecipitate resolved on 15% SDS-PAGE. Membranes were probed with anti-RhoA or anti-Rac1 antibody. Lysate (40 μg) was also probed for RhoA or Rac1 to ensure equality across conditions.

Constructs and transfection

Rat cav-1 was amplified from MC cDNA and inserted into the retroviral vector pLHCX (Clontech, Mountain View, CA) with an N-terminal FLAG. Using this as template, Y14 was mutated to alanine. Dominant-negative EGFR K721A (dnEGFR, kindly provided by Dr. S. Parsons, University of Virginia Health System, Charlottesville, VA) was also cloned into pLHCX. Rat MC were infected with empty vector, FLAG-Cav-1Y14A, or dnEGFR and cav-1 KO MC were infected with cav-1 as described previously (20, 21, 51). In brief, competent virus capable of single infection was generated using the vesicular stomatitis virus system (Stratagene, La Jolla, CA), and MC passages 5–12 were exposed to virus concentrated by centrifugation in the presence of polybrene. Seventy-two hours after infection, a 2-week antibiotic selection period was begun. Experiments were performed using a population of pooled, stably infected MC.

Assessment of ROS production

After serum deprivation and treatment with inhibitors, MC were incubated for 30 min with 5 μM of the dihydroethidium (DHE; Invitrogen) that detects the generation of intracellular superoxide anion. After stretch, cells were washed and scraped into ice-cold tricine buffer, pelleted, resuspended in buffer, and sonicated. Fluorescence was measured using 100 μl cell lysate at excitation 585 λ and emission 605 λ in a Biolynx plate reader. Readings were normalized to protein content. As a second measure, ROS production was assessed using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) according to manufacturer's instructions.

RNA interference

Rat Nox1 Silencer® Select siRNA and control nontargeting siRNA were purchased from Applied Biosystems (Foster City, CA). MC were transfected with 100 nM using GeneEraser siRNA reagent (Stratagene) at 60% confluence. After 48 h, cells were serum-deprived for 24 h, then stretched and cell lysate harvested for RhoA activity. Nox1 RNA expression by RT-PCR was used to assess efficacy of downregulation by RNAi.

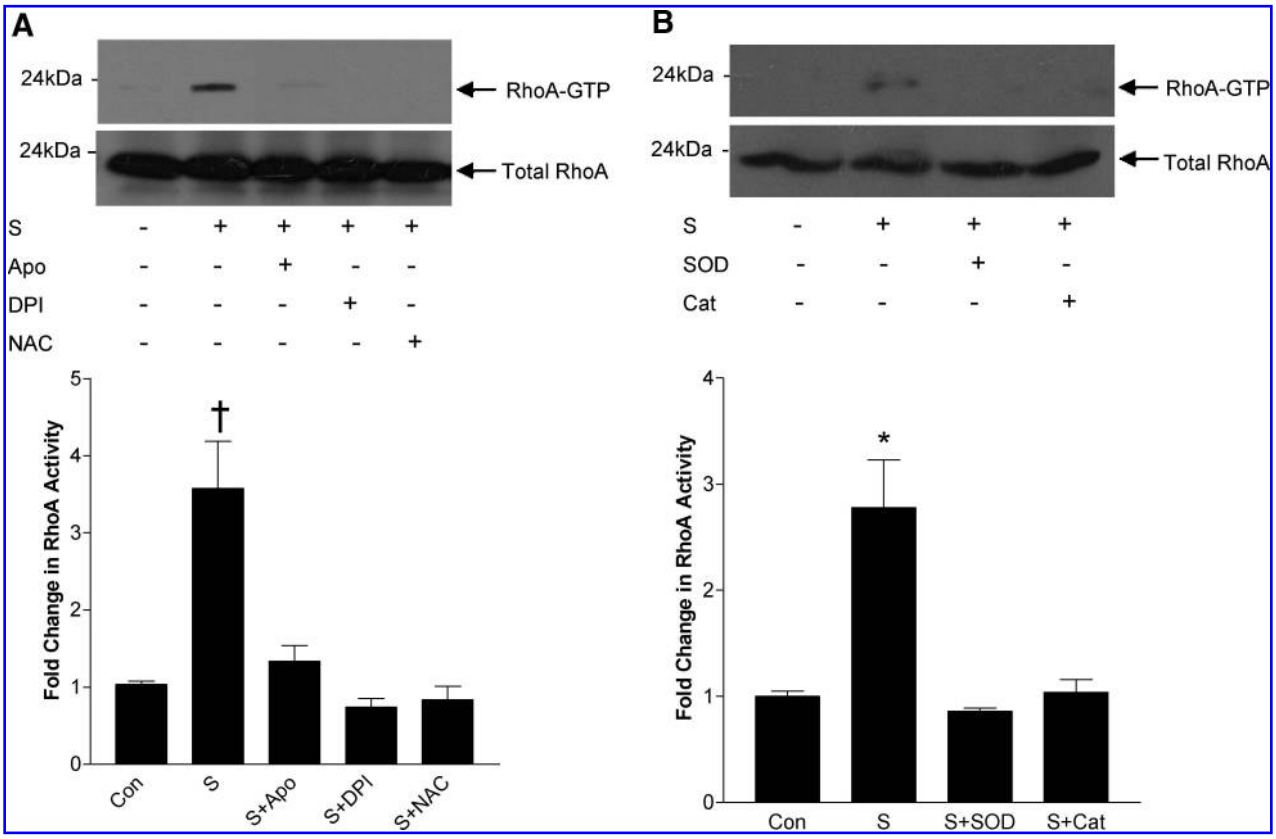

FIG. 1. ROS generation is required for stretch-induced RhoA activation. MC were pretreated with (**A**) the ROS scavenger NAC (4 mM, 30 min) or NADPH oxidase inhibitors apocynin (apo; 300 μM, 30 min) or DPI (40 μM, 30 min), or (**B**) pegylated-superoxide dismutase (SOD; 10 U/μl, 3 h) or catalase (500 U/μl, 1 h) and stretched for 1 min. RhoA activation in response to stretch was blocked by all inhibitors ($^{\dagger}p < 0.01$ S vs. all others, $n = 5$ for **A**, and $^{*}p < 0.05$ S vs. all others, $n = 3$ for **B**).

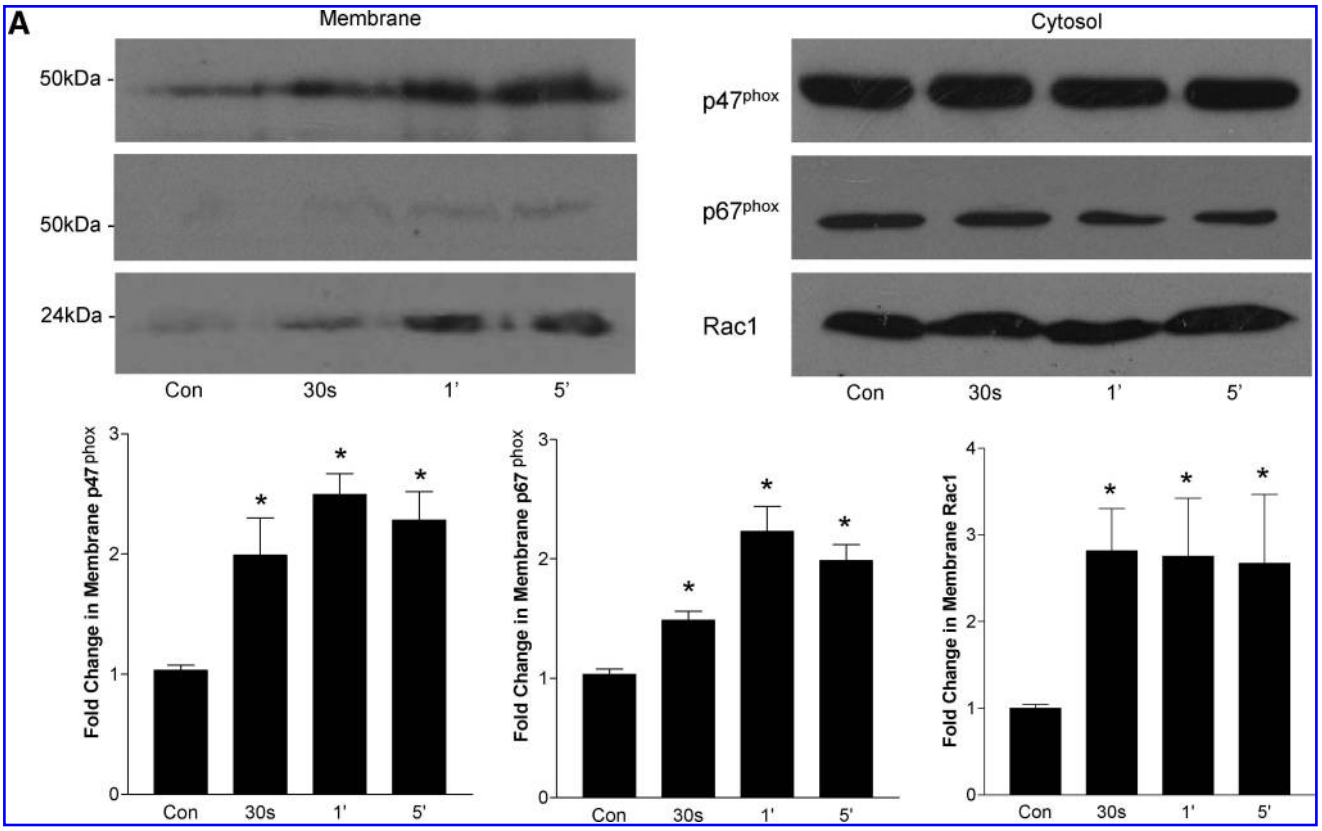

FIG. 2. NADPH oxidase is activated by stretch in MC. MC were stretched for the indicated times. (**A**) Membrane and cytosol proteins were separated and immunoblotted for components of the NADPH oxidase $p47^{phox}$, $p67^{phox}$, and Rac1. Early membrane translocation, by 30 sec, indicative of activation, was seen with all three proteins ($^*p < 0.05$ vs. con for $p47^{phox}$, $n = 3$; $^*p < 0.05$ vs. con for $p67^{phox}$, $n = 3$; $^*p < 0.05$ vs. con for Rac1, $n = 4$). Significantly greater signal was consistently observed in the cytosolic preparations, suggesting that only a small proportion of these three components are translocated to the membrane. (**B**) Rac1 activation by stretch was confirmed with a Rac1-GTP pull-down activity assay ($^*p < 0.05$ vs. con, $n = 5$).

Fluorescence microscopy

After stretch, MC were washed, fixed, permeabilized, and blocked as previously described (22). Rac1 monoclonal antibody (Cytoskeleton, 1:25) was applied for 2 h at room temperature, followed by washing and secondary antibody (Alexa Fluor 488, 1:100, 1 h). DAPI-containing mounting medium was applied prior to coverslips, after which images were taken using a fluorescence microscope (Olympus). After capture, images were converted to black and white bitmaps and intensity of membrane Rac1 staining was quantified for 15 to 20 cells per image using Scion Image software. Pixel intensity was normalized to a measure of the perimeter of the membrane for each cell.

Statistical analyses

Statistical analyses were performed using one-way ANOVA and Tukey's HSD was used for post-hoc analysis to determine which groups were significantly different from one another. A p value <0.05 (two-tailed) was considered significant. Data are represented as the mean $\pm$ standard error of the mean. Experiments were repeated multiple times, and the number of repetitions is identified in the figure legends by "$n =$". All analyses used the statistical package SPSS for Windows 14.

Results

ROS generation is required for stretch-induced RhoA activation

We have previously shown that RhoA is activated early by stretch (by 1 min), and have shown the importance of RhoA signaling in matrix elaboration by MC (20). The events leading to RhoA activation by strain, however, have not been identified. Since mechanical stresses have been shown to lead to ROS production in other cell types, primarily through the NADPH oxidase enzyme system (3), we investigated a possible role for ROS in RhoA activation in MC. Cells were stretched for 1 min in the presence or absence of the ROS scavenger NAC, NADPH oxidase inhibitors apocynin or DPI, superoxide scavenger SOD, or hydrogen peroxide scavenger catalase. All of these prevented stretch-induced RhoA activation (Fig. 1).

We next confirmed the activation of NADPH oxidase by strain. MC have been shown to express the subunits $p47^{phox}$, $p67^{phox}$, Rac1, p22phox, Nox1, and Nox4 (9, 10, 28). Several of these, namely $p47^{phox}$, $p67^{phox}$, and Rac1, require translocation from cytosol to membrane for activation. Figure 2A shows that strain leads to very early (30 s) membrane translocation of these subunits. Since Rac1 is a GTPase, we confirmed that its membrane translocation corresponds with activation by immunoprecipitating active (GTP-bound) Rac1 using glutathione-agarose bound GST-tagged Pak-binding domain (PBD). As seen in Figure 2B, Rac1 activation parallels the observed membrane translocation. Furthermore, since Nox1 requires activation of these subunits, we used siRNA to test its role in stretch-induced RhoA activation. Supplemental Figure 1 (*see* www.liebertonline.com/ars) shows successful Nox1 downregulation as well as inhibition of RhoA activation in response to stretch in comparison with control siRNA, supporting a role for this Nox subunit in MC stretch responses.

Caveolae are required for RhoA and NADPH oxidase activation by stretch

We have previously shown that RhoA activation by stretch is dependent on the presence of intact caveolae, as well as phosphorylation of their marker protein cav-1 at Y14 by Src kinase (32). Since caveolae contain components of the NADPH oxidase system as well as NADPH oxidase activity in some cells (13, 46, 50), we determined their role in stretch-induced ROS activation in MC. As we have previously used cholesterol-depleting agents to define a role for caveolae in RhoA activation, we first sought to confirm our findings in MC derived from cav-1 knockout (KO) mice. Tissues and cells from these mice are also devoid of caveolae (34). As compared to MC derived from their wild-type (WT) counterparts, RhoA is not activated by strain in cav-1 KO cells (Fig. 3A). We then assessed the role of caveolae in the activation of NADPH oxidase. As seen in Figure 3B, Rac1 activation was also absent in cav-1 KO cells. Chemical depletion of cholesterol with cyclodextrin, resulting in loss of caveolar structures (43), also prevented stretch-induced $p67^{phox}$ membrane translocation (Fig. 3C). Furthermore, membrane translocation was not seen in cav-1 KO cells (Fig. 3D). We then re-expressed cav-1 in KO cells. Successful expression has previously been demonstrated (51). Both Rac1 activation and $p67^{phox}$ membrane translocation were restored in KO MC with cav-1 as compared to cells infected with the empty vector pLHCX (Figs. 3E and 3F). Similarly, membrane translocation of $p47^{phox}$ was not seen in KO cells, but was restored in cells re-expressing cav-1 (Supplemental Fig. 2A; *see* www.liebertonline.com/ars). Furthermore, $p67^{phox}$ is also regulated by Ser phosphorylation. Supplemental Figure 3A (*see* www.liebertonline.com/ars) shows that stretch leads to $p67^{phox}$ phosphorylation by 30 sec, a time at which membrane translocation was also observed (Fig. 2A). Furthermore, this phosphorylation is absent in cav-1 KO cells (Supplemental Fig. 3B), suggesting that caveolae are required for both membrane translocation and phosphorylation of this subunit.

We directly assessed the production of superoxide anion using DHE. Stretch-induced ROS production was abrogated by chemical disruption of caveolae with cyclodextrin (Fig. 4A). Similarly, cav-1 KO cells did not generate ROS in response to strain (Fig. 4B, Supplemental Fig. 4A; *see* www.liebertonline.com/ars), and this was restored by re-expression of cav-1 in KO cells (Fig. 4C). Taken together, caveolae thus mediate stretch-induced NADPH oxidase activation and ROS production in MC.

EGFR transactivation is required for stretch-induced NADPH oxidase activation, ROS production, and RhoA activation

The epidermal growth factor receptor (EGFR) is known to serve in signal transduction for diverse nonligand-mediated stimuli in a process known as transactivation (53). We and others have shown that its transactivation is involved in various downstream effects of mechanical stress (21, 23). We have also previously shown that EGFR transactivation by strain in MC requires caveolae (51). In Figure 5A, the EGFR inhibitor AG1478 prevented RhoA activation, demonstrating its requirement for stretch-induced RhoA activation. Furthermore, in MC stably infected with a dominant negative (kinase inactive) EGFR K721A (dnEGFR), RhoA activation by cyclic strain was abrogated in comparison with MC expressing the empty vector pLHCX (Fig. 5B). Successful overexpression of this construct in MC has previously been shown (47).

Since ROS is also required for RhoA activation, we next sought to determine whether EGFR transactivation is upstream of NADPH oxidase activation. We first used the EGFR inhibitor AG1478 and show that AG1478 prevents Rac1 activation (Fig. 6A), $p67^{phox}$ membrane translocation (Fig. 6B), and $p47^{phox}$ membrane translocation (Supplemental Fig. 2B). We further confirmed the inhibitory effect of AG1478 on stretch-induced Rac1 membrane translocation using immunofluorescence (Supplemental Fig. 5; *see* www.liebertonline

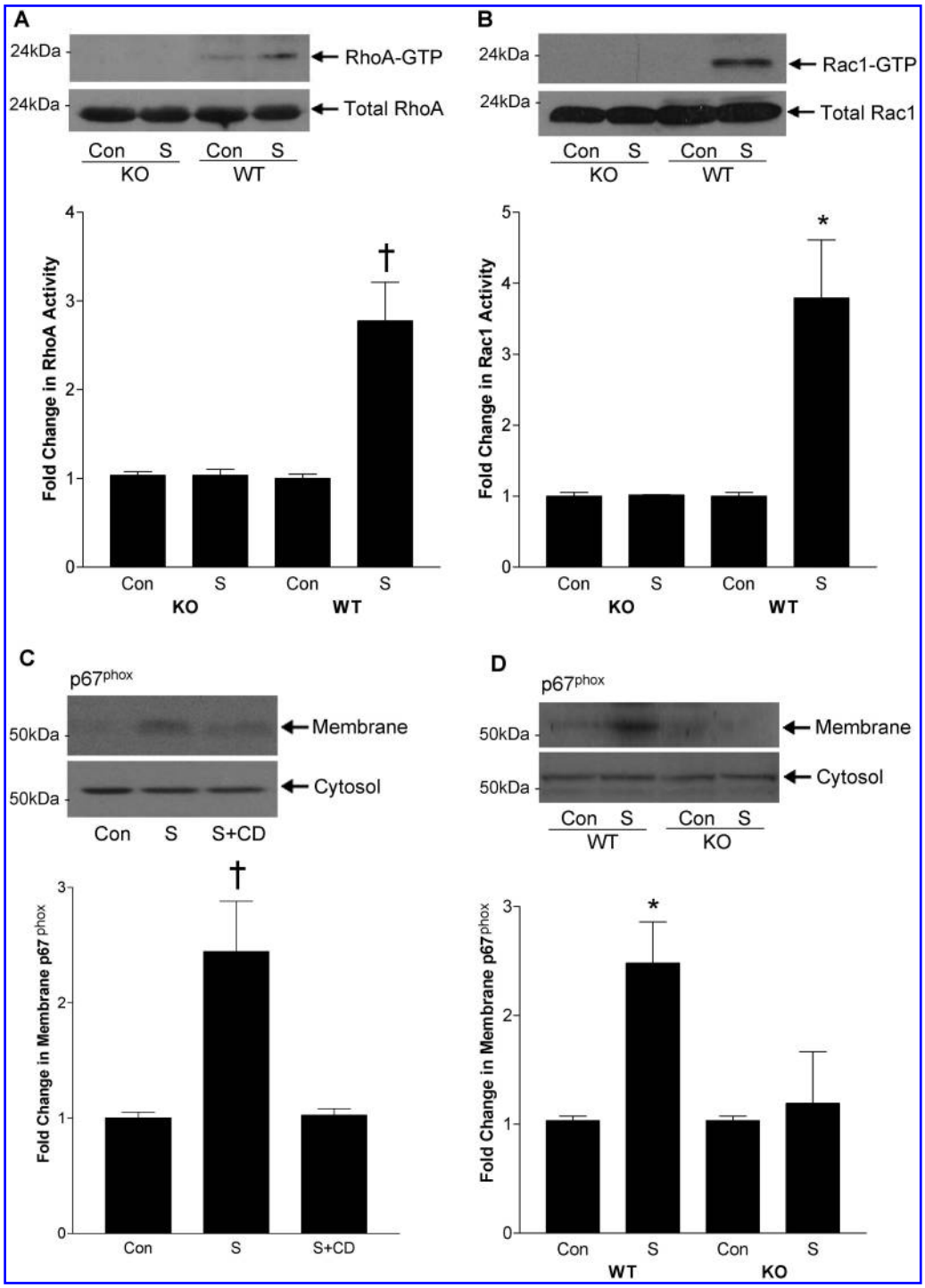

FIG. 3. Caveolae are required for RhoA and NADPH oxidase activation by stretch. MC from cav-1 knockout (KO) mice were compared with their wild-type (WT) counterparts in their response to stretch for 1 min. (**A**) Absence of cav-1 abrogated the stretch-induced RhoA activation seen in WT cells ($\dagger p < 0.01$ vs. others, $n = 4$). (**B**) Stretch-induced Rac1 activation was similarly abrogated in cav-1 KO cells ($^{*}p < 0.05$ vs. others, n = 3). (**C**) Membrane translocation of $p67^{phox}$ in response to stretch was inhibited by chemical disruption of caveolae with cyclodextrin (CD; 10 μM, 60 min) ($\dagger p < 0.01$ vs. others, $n = 3$). (**D**) Translocation of $p67^{phox}$ was also absent in cav-1 KO cells as compared to their WT counterparts ($^{*}p < 0.05$ vs. all others, $n = 3$). (**E**) Cav-1 was stably re-expressed in KO cells. In comparison to KO cells expressing empty vector pLHCX, both Rac1 activation (**E**) and $p67^{phox}$ membrane translocation (**F**) were rescued by re-expression of cav-1 ($^{\ddagger}p < 0.001$ vs. others, $n = 3$ in (**E**) and $^{\dagger}p < 0.01$ vs. others, $n = 4$ in (**F**)).

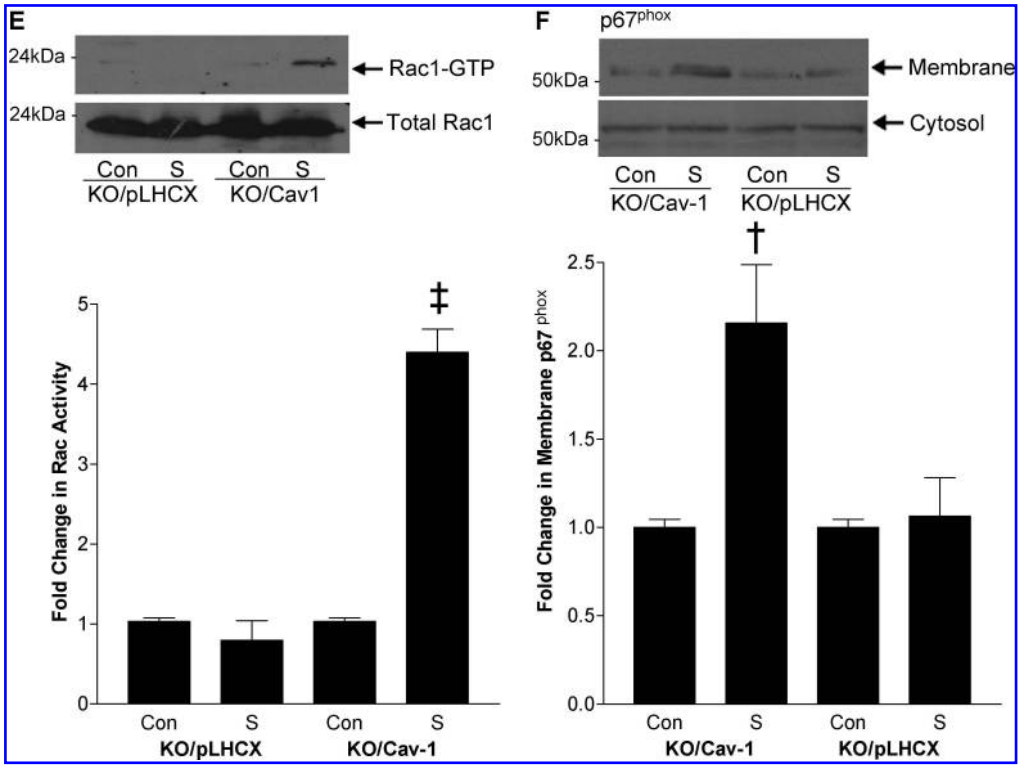

FIG. 3. (Continued).

.com/ars). Finally, we confirmed the role of EGFR transactivation with the use of MC overexpressing dnEGFR. As compared with cells overexpressing the empty vector pLHCX, both Rac1 activation (Fig. 6C) and $p67^{phox}$ membrane translocation (Fig. 6D) were prevented in cells with dnEGFR.

To confirm that ROS production was also prevented with EGFR inhibition, we measured superoxide anion using dihydroethidium. Strain led to superoxide production and this was prevented by EGFR inhibition with AG1478 (Fig. 7A) or by overexpression of dnEGFR (Fig. 7B). Similarly, Amplex Red assessment of stretch-induced ROS production showed its inhibition by AG1478 (Supplemental Fig. 4B). Finally, to determine whether EGFR transactivation in response to strain itself requires the presence of ROS, we stretched MC in the presence or absence of various ROS inhibitors, as in Figure 1. As seen in Figure 7C, none of these altered stretch-induced EGFR transactivation. Thus, EGFR transactivation is an upstream event leading to NADPH oxidase activation, ROS production and consequent RhoA activation.

Src and phosphorylation of cav-1 at Y14 are required for NADPH oxidase activation and ROS generation in response to stretch

We have previously demonstrated that Src-induced cav-1 phosphorylation at Y14 was required for both stretch-induced EGFR and RhoA activation (32, 51). Indeed, very early stretch-induced activation of Src (within 0.3 sec) has been directly demonstrated by FRET analysis in smooth muscle cells (30). We thus assessed whether Src activation was also required for NADPH oxidase activation and ROS generation. We first used two distinct inhibitors of Src, SU6656 and PP2, to examine the requirement for Src in NADPH oxidase activation. As seen in Figure 8A, both inhibitors prevented stretch-induced Rac1 activation. Membrane translocation of $p67^{phox}$ was similarly prevented by both inhibitors (Fig. 8B), as was membrane translocation of $p47^{phox}$ by SU6656 (Supplemental Fig. 2B). PP2 also prevented stretch-induced superoxide anion generation as determined by DHE (Fig. 8C). Since SU6656 itself has strong emission at 605λ, we were unable to use this inhibitor for DHE studies, but we did confirm its inhibition of ROS production by Amplex Red assessment (Supplemental Fig. 4C).

We next investigated the role of cav-1 Y14 phosphorylation, a downstream target of Src activation. We used a pooled population of MC stably infected with the nonphosphorylatable mutant cav-1 Y14A. We have previously shown successful overexpression and functionality of this construct (32). As seen in Figure 8D, stretch-induced Rac1 activation was not seen in cav-1 Y14A overexpressing MC as compared with MC infected with the empty vector pLHCX. Furthermore, stretch-induced ROS production was also abrogated in MC overexpressing the nonphosphorylatable mutant (Fig. 8E). Thus, Src activation and phosphorylation of cav-1 at Y14 are required for activation of NADPH oxidase and ROS generation upstream of RhoA activation in stretched MC.

Finally, since RhoA activation is regulated in part by guanine nucleotide exchange factors (GEFs), and we have recently shown that the RhoA GEF Vav2 is required for stretch-induced RhoA activation (33), we investigated the effects of ROS on its activation. Supplemental Figure 6 (*see* www.liebertonline.com/ars) shows that stretch-induced Vav2 activation, assessed by its phosphorylation of Y172, is abrogated by preincubation with the superoxide scavenger

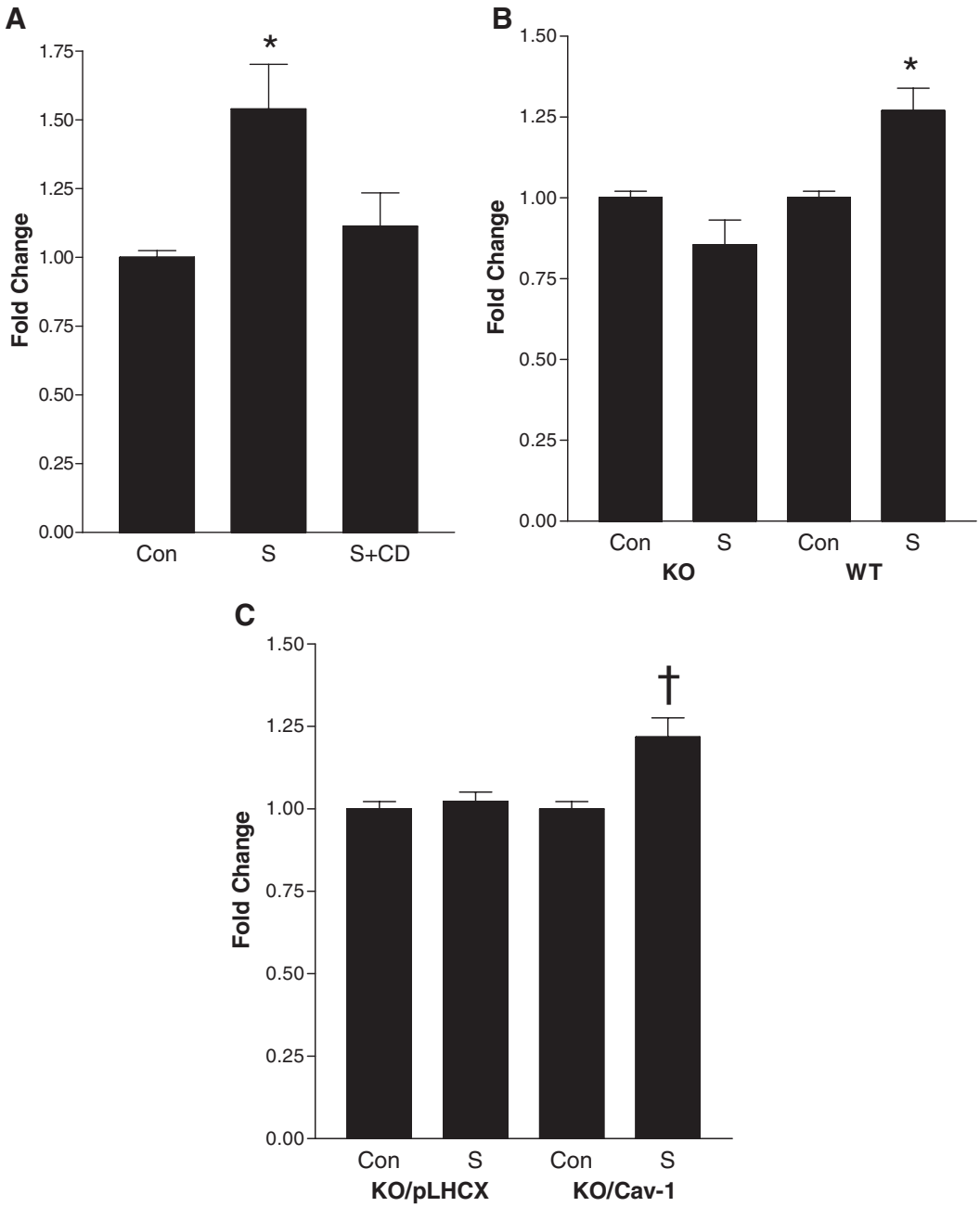

FIG. 4. Stretch-induced generation of ROS requires caveolae. ROS production was measured fluoroscopically by dihydroethidium (DHE) oxidation. (**A**) Stretch-induced ROS production was inhibited by chemical disruption of caveolae with cyclodextrin (CD; 10 μM, 60 min) (*$p < 0.05$ vs. others, $n = 6$). (**B**) Stretch did not lead to ROS production in cav-1 KO cells as compared to their WT counterparts (*$p < 0.05$ vs. others, $n = 4$). (**C**) Re-expression of cav-1 in KO cells restored the increase in ROS production seen with stretch (†$p < 0.01$ vs. others, $n = 5$).

SOD. This suggests that ROS also affect the regulators of RhoA. However, how Vav2 activation is altered by ROS remains to be studied.

Discussion

A greater understanding of the mechanisms whereby mechanical stresses transmit signals to MC is important in elucidating the pathophysiology of hypertension-induced glomerular sclerosis. Although we and others have shown caveolae to be important signal transducers in response to mechanical stress (32, 38, 51), their contribution to stretch-induced ROS generation in MC has not been investigated. In expanding on our previous work in which we identified an important role for caveolae in stretch-induced RhoA activation, we now make the following novel observations in MC: a) stretch-induced NADPH oxidase-dependent ROS generation requires caveolae, b) stretch-induced RhoA activation is

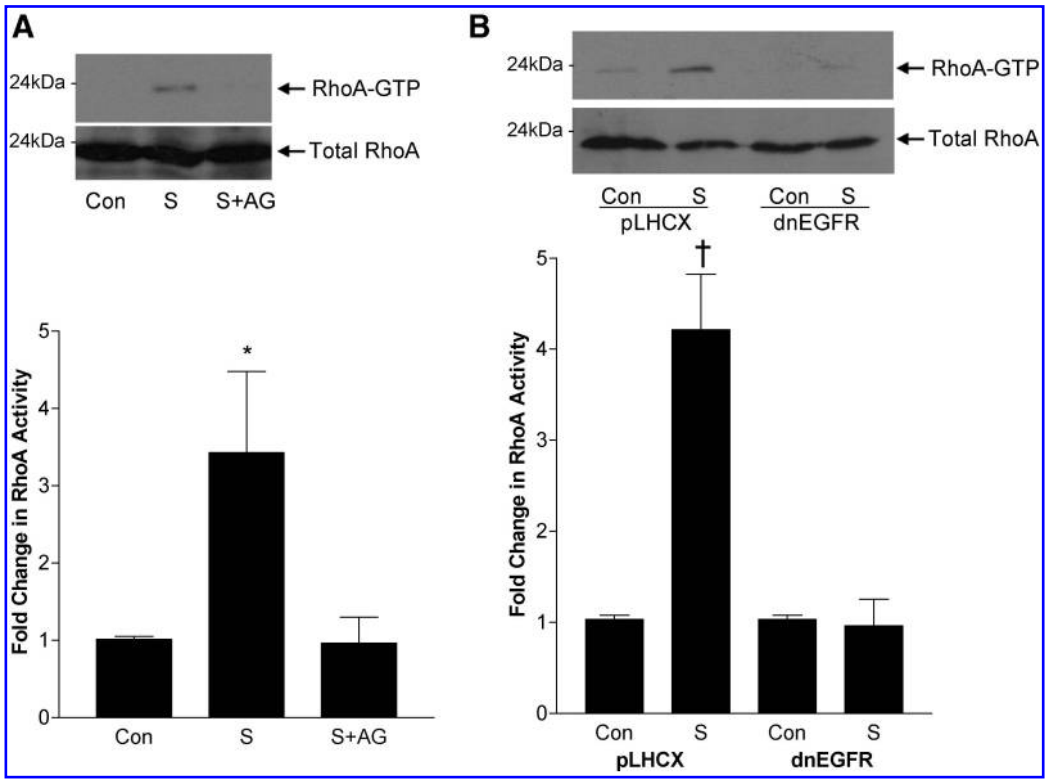

FIG. 5. The EGFR is required for stretch-induced RhoA activation. (**A**) Treatment of MC with the EGFR inhibitor AG1478 (1 μM, 30 min) prior to stretch for 1min prevented RhoA activation ($^{*}p < 0.05$, $n = 4$). (**B**) A pooled population of MC stably expressing dominant-negative EGFR K721A (dnEGFR) or empty vector pLHCX were stretched for 1 min. RhoA activation was prevented by dnEGFR expression ($^{\dagger}p < 0.01$ vs. all others, $n = 3$).

mediated by ROS, c) EGFR transactivation, regulated by Src activity and previously shown to require caveolae (51), is necessary for stretch-induced NADPH oxidase activation and ROS generation, and d) Src-induced phosphorylation of cav-1 at Y14 is required for NADPH oxidase-induced ROS generation in response to stretch. Thus, both caveolae and EGFR are key signal transducers in stretch-induced redox signaling in MC and may serve as potential modifiable targets in the treatment of hypertensive renal disease.

Our data show for the first time that ROS mediate stretch-induced RhoA activation. Redox regulation of RhoA was also shown in other systems. In vascular smooth muscle cells, aldosterone and angiotensin II functioned synergistically to activate RhoA through NADPH oxidase-derived ROS (29). In a human adenocarcinoma cell line, hypoxia led to mitochondrial ROS release which mediated RhoA activation, and hydrogen peroxide itself activated RhoA (7). Conversely, others have shown that Rac1/NADPH oxidase signaling downregulates RhoA activity in Ras-transformed normal rat kidney cells (39). The effects of ROS on RhoA activation thus appear to be cell and/or stimulus-specific.

ROS generation in response to mechanical stresses has been demonstrated in both cell and organ culture (5, 8, 11, 31). Although most studies showed ROS production at significantly later time points (hours) than we have assessed, stretch-induced ROS production in vascular smooth muscle cells was also seen as early as 1–5 min (11). Most studies addressing contribution of enzyme systems to ROS production in response to mechanical stress have implicated NADPH oxidase as the major source. Its activity was specifically measured and found increased by mechanical stress in pulmonary epithelial cells and endothelial cells (5, 8, 14), and membrane translocation of $p47^{phox}$ was observed in response to stretch as early as 2.5 min (11, 31). Use of NADPH oxidase inhibitors has also suggested a specific role for this enzyme in mechanical stress signaling (5, 11, 17, 31). This was supported by the lack of mechanical stress-induced ROS generation in $p47^{phox}$ null vascular smooth muscle or endothelial cells (11, 16). Our studies now demonstrate the generation of ROS in MC by mechanical stress, and implicate the NADPH oxidase enzyme complex through the use of both NADPH oxidase inhibitors as well as analysis of activation of several of its subunits.

Recent evidence suggests that components of the NADPH oxidase system are compartmentalized, with localization important to signal transduction (41). Some components have been found in caveolae, including Nox1, Nox2, and $p22^{phox}$ (13, 46, 50). In response to phorbol esters, NADPH oxidase activation included the translocation of $p47^{phox}$, $p67^{phox}$, and Rac1 from cytosol to caveolae, and this localization as well as superoxide production were inhibited by cholesterol-depleting agents (46). Similar observations were made in endothelial cells, where NADPH oxidase activity was increased by Fas ligand within isolated caveolae, and inhibited by cholesterol depletion (50). Angiotensin II-induced NADPH oxidase activation and ROS generation were also dependent on cav-1/caveolae (52). Our data show for the first time that caveolae are required for stretch-induced NADPH oxidase

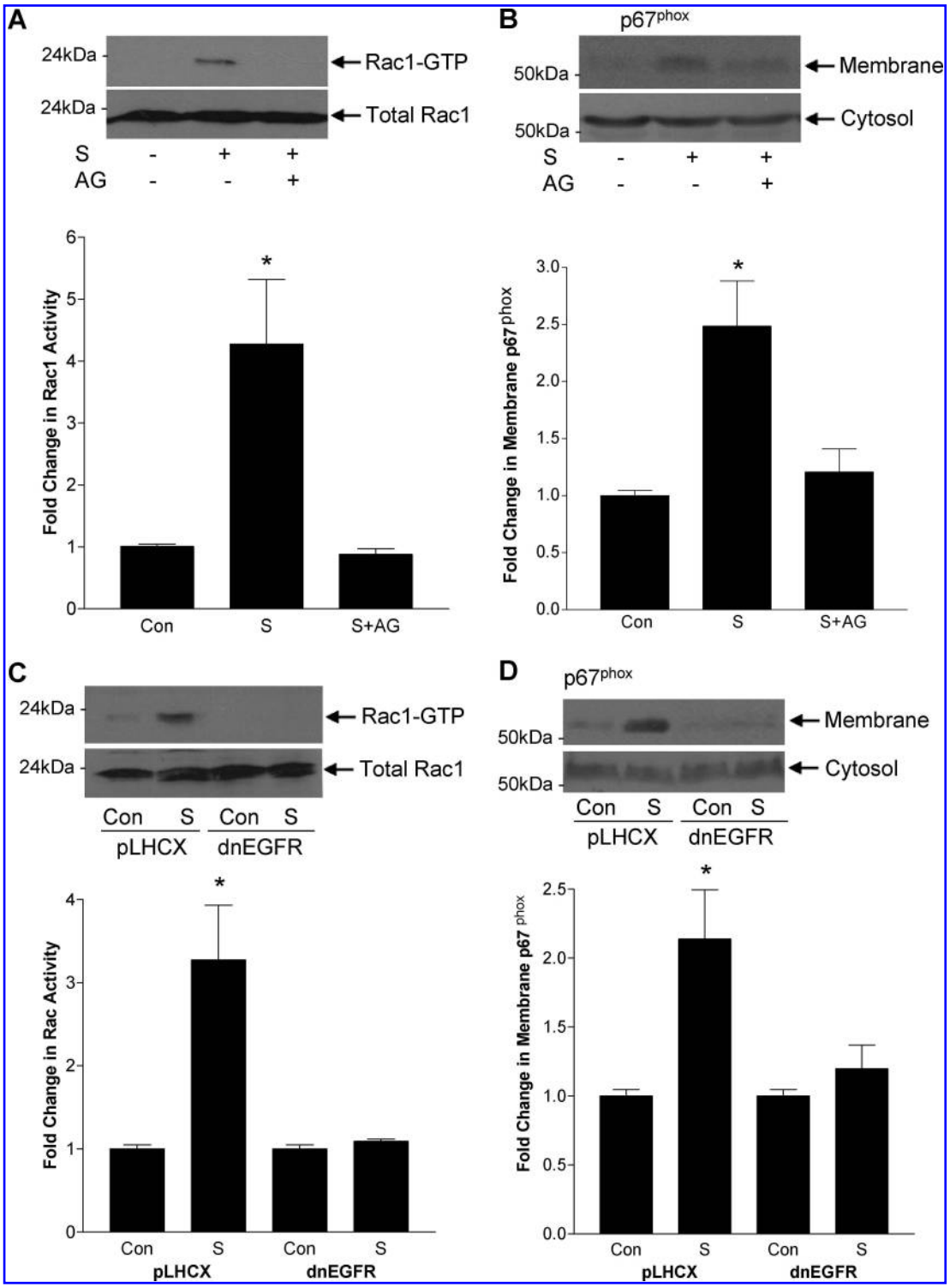

FIG. 6. EGFR transactivation is required for stretch-induced NADPH oxidase activation. MC were stretched for 1 min in the presence or absence of the EGFR inhibitor AG1478 (1 μM, 30 min). (**A**) Stretch-induced Rac1 activation was abrogated by EGFR inhibition (*$p < 0.05$ vs. others, $n = 3$), as was (**B**) $p67^{phox}$ membrane translocation (*$p < 0.05$ vs. others, $n = 3$). MC were stably infected with the dominant negative EGFR K721A (dnEGFR) or empty vector pLHCX. Stretch-induced Rac1 activation (**C**) and $p67^{phox}$ membrane translocation (**D**) were prevented by dnEGFR overexpression (*$p < 0.05$, $n = 3$ vs. others in (**C**) and *$p < 0.05$ vs. others, $n = 4$ in (**D**)).

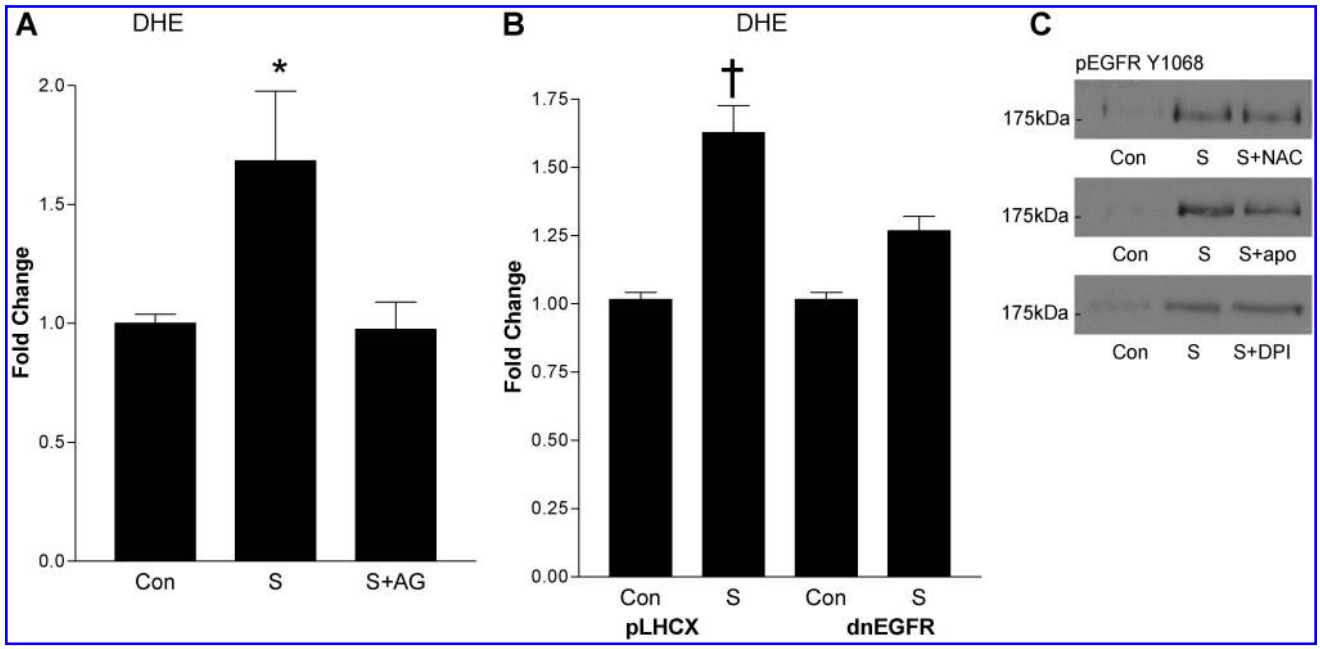

FIG. 7. Stretch-induced ROS production requires EGFR transactivation. (**A**) Stretch increased ROS production by 1 min, as assessed by fluoroscopic quantification of dihydroethidium (DHE). This was prevented by AG1478 (1 μM, 30 min, $^*p < 0.05$ vs. others, $n = 6$). (**B**) ROS production was also significantly attenuated in MC overexpressing dnEGFR ($\dagger p < 0.01$ vs. all others, $n = 6$). (**C**) Stretch-induced activation of the EGFR, as assessed by autophosphorylation of Y1068, was not prevented by the ROS scavenger NAC (4 mM, 30 min) or NADPH oxidase inhibitors apocynin (apo; 300 μM, 30 min) or DPI (40 μM, 30 min).

activation and ROS production. Both disruption of caveolae by cholesterol depletion with cyclodextrin, as well as absence of caveolae in cav-1 knockout MC, abrogated translocation of NADPH oxidase subunits and superoxide generation. Cav-1 re-expression rescued the defects, implicating cav-1 and caveolae in NADPH oxidase activation. It is of interest that in a model of ischemia comprising abrupt reduction of shear stress in pulmonary vascular endothelial cells, NADPH oxidase-induced ROS production was prevented by cholesterol depletion and in cav-1 knockout cells (27). This suggests a role for caveolae in ROS generation by more diverse alterations in mechanical stresses.

We have previously shown that stretch-induced EGFR transactivation is dependent on cav-1 and caveolae, as is RhoA activation (32, 51). Although the EGFR is well known to be activated by ROS in various settings such as with angiotensin II or aldosterone (15, 42), a description of its role as an upstream mediator of NADPH oxidase activation has been limited to a few studies (6, 19). However, the role of the EGFR in stretch-induced ROS signaling has not as yet been assessed. Our studies now demonstrate that EGFR transactivation by stretch is independent of ROS, lying upstream of NADPH oxidase activation. Inhibition of the EGFR effectively prevented translocation of the various NADPH oxidase subunits, Rac1 activation, and superoxide generation, as well as RhoA activation. These data clearly implicate EGFR transactivation as an early event in stretch-induced redox signaling and activation of pathways leading to matrix upregulation.

We previously showed that stretch-induced Src activation requires caveolae and mediates cav-1 Y14 phosphorylation. This phosphorylation is necessary for RhoA-cav1 association, RhoA activation, and EGFR transactivation (32, 51). Given the role of Src upstream of EGFR-RhoA activation, one would expect Src inhibition to prevent NADPH oxidase activation and superoxide production. Indeed, our data demonstrate that Src inhibition with two distinct compounds (SU6656, PP2) prevented Rac1 activation, $p67^{phox}$ membrane translocation, and stretch-induced superoxide generation. Here we show for the first time that Src-induced cav-1 Y14 phosphorylation is also required for NADPH oxidase activation and ROS generation, since these were prevented in MC overexpressing the nonphosphorylatable cav-1 Y14A. Although Src-induced cav-1 phosphorylation at this site was shown to occur in response to ROS (4), its role in NADPH oxidase activation was only indirectly suggested in studies investigating angiotensin II signaling. Here, cav-1/Rac co-association required Src-mediated cav-1Y14 phosphorylation, and cav-1 downregulation prevented Rac1 activation and ROS generation. Furthermore, the Rac1 activator Sos-1 localized to caveolae, associated with cav-1 and cav-1 downregulation prevented its phosphorylation (44, 52). Hence, caveolae may function to localize not only NADPH oxidase subunits, but also their activators, and this may be dependent on the phosphorylation status of cav-1. It is also of interest that phosphorylated cav-1 was found to associate with an activated EGFR and this interaction was ROS dependent (18). Taken together, in stretched MC, Src-induced phosphorylation of cav-1 may function to recruit EGFR, enable EGFR transactivation with downstream activation of the NADPH oxidase enzyme complex. Subsequent ROS production leads to RhoA activation through an as yet unidentified mechanism, although recruitment of these signaling components to a localized compartment

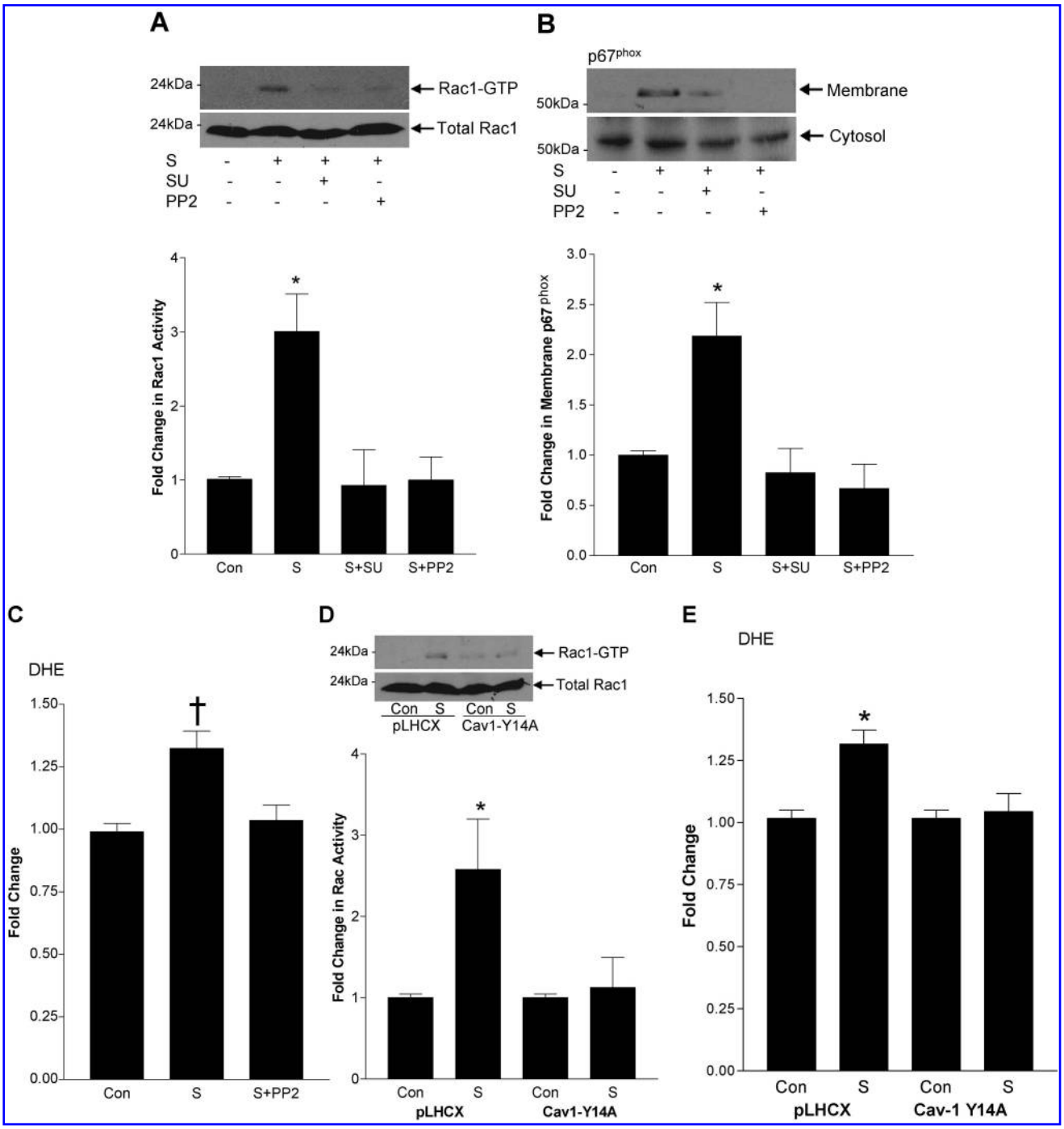

FIG. 8. Src and phosphorylation of cav-1 at Y14 are required for NADPH oxidase activation and ROS generation in response to stretch. (**A**) MC were stretched in the presence or absence of the Src inhibitors SU6656 (SU, 10 μM, 30 min) or PP2 (10 μM, 30 min). Rac1 activation was prevented by both Src inhibitors (*$p < 0.05$ vs. others, $n = 4$). (**B**) Stretch-induced p67^{phox} membrane translocation was also prevented by SU6656 and PP2 (*$p < 0.05$ vs. others, $n = 3$). (**C**) Similarly, ROS production, measured by DHE oxidation, was abrogated by Src inhibition with PP2 (†$p < 0.01$ vs. others, n = 5). (**D**) Stretch-induced Rac1 activation was not seen in MC stably overexpressing the nonphosphorylatable cav-1 mutant Y14A (cav-1 Y14A) as compared with MC infected with the empty vector pLHCX (*$p < 0.05$ vs. others, $n = 4$). (**E**) ROS production, as assessed by DHE oxidation, was also absent in MC overexpressing cav-1 Y14A (*$p < 0.05$ vs. others, $n = 3$).

(caveolae) may enable efficient signal transduction. This is summarized in Figure 9.

In conclusion, we have made several new observations which contribute to our understanding of the mechanism of stretch-induced RhoA activation in MC. We have shown that NADPH oxidase-dependent ROS generation is required, that this is activated downstream of EGFR transactivation and that cav-1/caveolae are critical to these events. We have previously shown the importance of both the EGFR and RhoA to matrix upregulation in stretched MC (20; 21), suggesting that

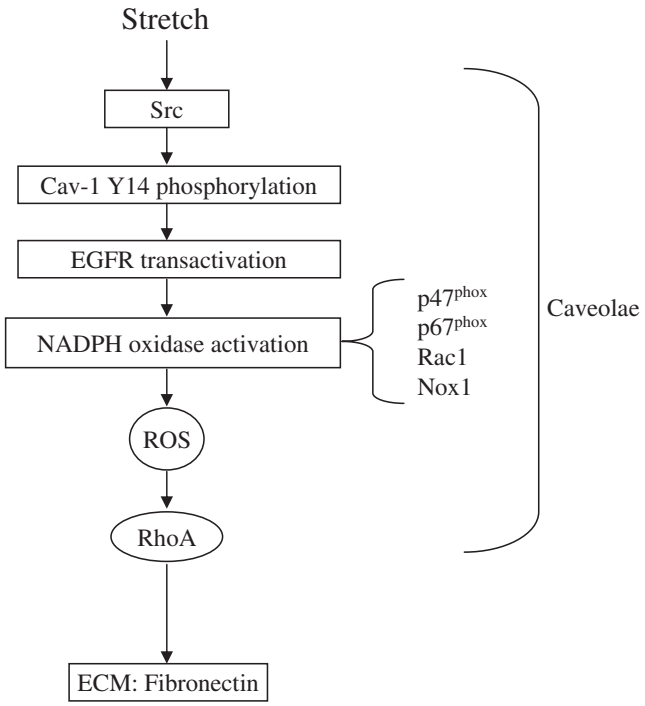

FIG. 9. Diagram showing our proposed schematic for stretch-induced activation of RhoA in MC. Mechanical stress leads to the activation of Src, which phosphorylates cav-1 on Y14. This requires an intact caveolar microenvironment. Cav-1 Y14 phosphorylation is necessary for subsequent transactivation of the EGFR, as we have shown previously (51). EGFR transactivation leads to activation of the NADPH oxidase system with subsequent ROS generation and RhoA activation. Our previous work has shown that RhoA activation mediates the stretch-induced upregulation and secretion of the matrix protein fibronectin (20).

blockade of early events in this pathway may be of benefit in the prevention of hypertension-associated renal disease.

Acknowledgments

We are grateful for the support of the Kidney Foundation of Canada and the Canadian Institutes of Health Research (JK). We thank Dr. S. Parsons (Charlottesville, VA) for kindly providing pcDNA-EGFR K721A. We also thank the St. Joseph's Hospital Foundation for support of Nephrology Research.

Author Disclosure Statement

The authors have no competing financial interests.

References

1. Aikawa R, Nagai T, Tanaka M, Zou Y, Ishihara T, Takano H, Hasegawa H, Akazawa H, Mizukami M, Nagai R, and Komuro I. Reactive oxygen species in mechanical stress-induced cardiac hypertrophy. *Biochem Biophys Res Commun* 289: 901–907, 2001.
2. Ali MH, Mungai PT, and Schumacker PT. Stretch-induced phosphorylation of focal adhesion kinase in endothelial cells: Role of mitochondrial oxidants. *Am J Physiol Lung Cell Mol Physiol* 291: L38–L45, 2006.
3. Brandes RP and Kreuzer J. Vascular NADPH oxidases: Molecular mechanisms of activation. *Cardiovasc Res* 65: 16–27, 2005.
4. Cao H, Sanguinetti AR, and Mastick CC. Oxidative stress activates both Src-kinases and their negative regulator Csk and induces phosphorylation of two targeting proteins for Csk: caveolin-1 and paxillin. *Exp Cell Res* 294: 159–171, 2004.
5. Chapman KE, Sinclair SE, Zhuang D, Hassid A, Desai LP, and Waters CM. Cyclic mechanical strain increases reactive oxygen species production in pulmonary epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 289: L834–L841, 2005.
6. Chen X, Abair TD, Ibanez MR, Su Y, Frey MR, Dise RS, Polk DB, Singh AB, Harris RC, Zent R, and Pozzi A. Integrin alpha1beta1 controls reactive oxygen species synthesis by negatively regulating epidermal growth factor receptor-mediated Rac activation. *Mol Cell Biol* 27: 3313–3326, 2007.
7. Dada LA, Novoa E, Lecuona E, Sun H, and Sznajder JI. Role of the small GTPase RhoA in the hypoxia-induced decrease of plasma membrane Na,K-ATPase in A549 cells. *J Cell Sci* 120: 2214–2222, 2007.
8. De Keulenaer GW, Chappell DC, Ishizaka N, Nerem RM, Alexander RW, and Griendling KK. Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: Role of a superoxide-producing NADH oxidase. *Circ Res* 82: 1094–1101, 1998.
9. Gill PS and Wilcox CS. NADPH oxidases in the kidney. *Antioxid Redox Signal* 8: 1597–1607, 2006.
10. Gorin Y, Ricono JM, Wagner B, Kim NH, Bhandari B, Choudhury GG, and Abboud HE. Angiotensin II-induced ERK1/ERK2 activation and protein synthesis are redox-dependent in glomerular mesangial cells. *Biochem J* 381: 231–239, 2004.
11. Grote K, Flach I, Luchtefeld M, Akin E, Holland SM, Drexler H, and Schieffer B. Mechanical stretch enhances mRNA expression and proenzyme release of matrix metalloproteinase-2 (MMP-2) via NAD(P)H oxidase-derived reactive oxygen species. *Circ Res* 92: e80–e86, 2003.
12. Han DC and Guan JL. Association of focal adhesion kinase with Grb7 and its role in cell migration. *J Biol Chem* 274: 24425–24430, 1999.
13. Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, and Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 24: 677–683, 2004.
14. Howard AB, Alexander RW, Nerem RM, Griendling KK, and Taylor WR. Cyclic strain induces an oxidative stress in endothelial cells. *Am J Physiol* 272: C421–C427, 1997.
15. Huang S, Zhang A, Ding G, and Chen R. Aldosterone-induced mesangial cell proliferation is mediated by EGF receptor transactivation. *Am J Physiol Renal Physiol* 296: F1323–F1333, 2009.
16. Hwang J, Saha A, Boo YC, Sorescu GP, McNally JS, Holland SM, Dikalov S, Giddens DP, Griendling KK, Harrison DG, and Jo H. Oscillatory shear stress stimulates endothelial production of O2- from p47phox-dependent NAD(P)H oxidases, leading to monocyte adhesion. *J Biol Chem* 278: 47291–47298, 2003.
17. Inoue N, Kawashima S, Hirata KI, Rikitake Y, Takeshita S, Yamochi W, Akita H, and Yokoyama M. Stretch force on vascular smooth muscle cells enhances oxidation of LDL via superoxide production. *Am J Physiol* 274: H1928–H1932, 1998.
18. Khan EM, Heidinger JM, Levy M, Lisanti MP, Ravid T, and Goldkorn T. Epidermal growth factor receptor exposed to

oxidative stress undergoes Src- and caveolin-1-dependent perinuclear trafficking. *J Biol Chem* 281: 14486–14493, 2006.
19. Kim Y, Lee YS, Choe J, Lee H, Kim YM, and Jeoung D. CD44-epidermal growth factor receptor interaction mediates hyaluronic acid-promoted cell motility by activating protein kinase C signaling involving Akt, Rac1, Phox, reactive oxygen species, focal adhesion kinase, and MMP-2. *J Biol Chem* 283: 22513–22528, 2008.
20. Krepinsky JC, Ingram AJ, Tang D, Wu D, Liu L, and Scholey JW. Nitric oxide inhibits stretch-induced MAPK activation in mesangial cells through RhoA inactivation. *J Am Soc Nephrol* 14: 2790–2800, 2003.
21. Krepinsky JC, Li Y, Chang Y, Liu L, Peng F, Wu D, Tang D, Scholey J, and Ingram AJ. Akt mediates mechanical strain-induced collagen production by mesangial cells. *J Am Soc Nephrol* 16: 1661–1672, 2005.
22. Krepinsky JC, Li Y, Tang D, Liu L, Scholey J, and Ingram AJ. Stretch-induced Raf-1 activation in mesangial cells requires actin cytoskeletal integrity. *Cell Signal* 17: 311–320, 2005.
23. Kudoh S, Komuro I, Hiroi Y, Zou Y, Harada K, Sugaya T, Takekoshi N, Murakami K, Kadowaki T, and Yazaki Y. Mechanical stretch induces hypertrophic responses in cardiac myocytes of angiotensin II type 1a receptor knockout mice. *J Biol Chem* 273: 24037–24043, 1998.
24. Lacy F, O'Connor DT, and Schmid–Schonbein GW. Plasma hydrogen peroxide production in hypertensives and normotensive subjects at genetic risk of hypertension. *J Hypertens* 16: 291–303, 1998.
25. Lee H, Volonte D, Galbiati F, Iyengar P, Lublin DM, Bregman DB, Wilson MT, Campos–Gonzalez R, Bouzahzah B, Pestell RG, Scherer PE, and Lisanti MP. Constitutive and growth factor-regulated phosphorylation of caveolin-1 occurs at the same site (Tyr-14) *in vivo*: Identification of a c-Src/Cav- 1/Grb7 signaling cassette. *Mol Endocrinol* 14: 1750–1775, 2000.
26. Manning RD, Jr., Tian N, and Meng S. Oxidative stress and antioxidant treatment in hypertension and the associated renal damage. *Am J Nephrol* 25: 311–317, 2005.
27. Milovanova T, Chatterjee S, Hawkins BJ, Hong N, Sorokina EM, Debolt K, Moore JS, Madesh M, and Fisher AB. Caveolae are an essential component of the pathway for endothelial cell signaling associated with abrupt reduction of shear stress. *Biochim Biophys Acta* 1783: 1866–1875, 2008.
28. Miyata K, Rahman M, Shokoji T, Nagai Y, Zhang GX, Sun GP, Kimura S, Yukimura T, Kiyomoto H, Kohno M, Abe Y, and Nishiyama A. Aldosterone stimulates reactive oxygen species production through activation of NADPH oxidase in rat mesangial cells. *J Am Soc Nephrol* 16: 2906–2912, 2005.
29. Montezano AC, Callera GE, Yogi A, He Y, Tostes RC, He G, Schiffrin EL, and Touyz RM. Aldosterone and angiotensin II synergistically stimulate migration in vascular smooth muscle cells through c-Src-regulated redox-sensitive RhoA pathways. *Arterioscler Thromb Vasc Biol* 28: 1511–1518, 2008.
30. Na S, Collin O, Chowdhury F, Tay B, Ouyang M, Wang Y, and Wang N. Rapid signal transduction in living cells is a unique feature of mechanotransduction. *Proc Natl Acad Sci USA* 105: 6626–6631, 2008.
31. Oeckler RA, Kaminski PM, and Wolin MS. Stretch enhances contraction of bovine coronary arteries via an NAD(P)H oxidase-mediated activation of the extracellular signal-regulated kinase mitogen-activated protein kinase cascade. *Circ Res* 92: 23–31, 2003.
32. Peng F, Wu D, Ingram AJ, Zhang B, Gao B, and Krepinsky JC. RhoA activation in mesangial cells by mechanical strain depends on caveolae and caveolin-1 interaction. *J Am Soc Nephrol* 18: 189–198, 2007.
33. Peng F, Zhang B, Ingram AJ, Gao B, Zhang Y, and Krepinsky JC. Mechanical stretch-induced RhoA activation is mediated by the RhoGEF Vav2 in mesangial cells. *Cell Signal* 22: 34–40, 2010.
34. Razani B, Engelman JA, Wang XB, Schubert W, Zhang XL, Marks CB, Macaluso F, Russell RG, Li M, Pestell RG, Di Vizio D, Hou H, Jr., Kneitz B, Lagaud G, Christ GJ, Edelmann W, and Lisanti MP. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *J Biol Chem* 276: 38121–38138, 2001.
35. Razani B and Lisanti MP. Caveolins and caveolae: Molecular and functional relationships. *Exp Cell Res* 271: 36–44, 2001.
36. Riser BL, Cortes P, and Yee J. Modeling the effects of vascular stress in mesangial cells. *Curr Opin Nephrol Hypertens* 9: 43–47, 2000.
37. Riser BL, Cortes P, Zhao X, Bernstein J, Dumler F, and Narins RG. Intraglomerular pressure and mesangial stretching stimulate extracellular matrix formation in the rat. *J Clin Invest* 90: 1932–1943, 1992.
38. Rizzo V, Sung A, Oh P, and Schnitzer JE. Rapid mechanotransduction in situ at the luminal cell surface of vascular endothelium and its caveolae. *J Biol Chem* 273: 26323–26329, 1998.
39. Shinohara M, Shang WH, Kubodera M, Harada S, Mitsushita J, Kato M, Miyazaki H, Sumimoto H, and Kamata T. Nox1 redox signaling mediates oncogenic Ras-induced disruption of stress fibers and focal adhesions by down-regulating Rho. *J Biol Chem* 282: 17640–17648, 2007.
40. Tamai O, Oka N, Kikuchi T, Koda Y, Soejima M, Wada Y, Fujisawa M, Tamaki K, Kawachi H, Shimizu F, Kimura H, Imaizumi T, and Okuda S. Caveolae in mesangial cells and caveolin expression in mesangial proliferative glomerulonephritis. *Kidney Int* 59: 471–480, 2001.
41. Ushio–Fukai M. Localizing NADPH oxidase-derived ROS. *Sci STKE* 2006: re8, 2006.
42. Ushio–Fukai M, Griendling KK, Becker PL, Hilenski L, Halleran S, and Alexander RW. Epidermal growth factor receptor transactivation by angiotensin II requires reactive oxygen species in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 21: 489–495, 2001.
43. Ushio–Fukai M, Hilenski L, Santanam N, Becker PL, Ma Y, Griendling KK, and Alexander RW. Cholesterol depletion inhibits epidermal growth factor receptor transactivation by angiotensin II in vascular smooth muscle cells: Role of cholesterol-rich microdomains and focal adhesions in angiotensin II signaling. *J Biol Chem* 276: 48269–48275, 2001.
44. Ushio–Fukai M, Zuo L, Ikeda S, Tojo T, Patrushev NA, and Alexander RW. cAbl tyrosine kinase mediates reactive oxygen species- and caveolin-dependent AT1 receptor signaling in vascular smooth muscle: Role in vascular hypertrophy. *Circ Res* 97: 829–836, 2005.
45. Vaziri ND. Roles of oxidative stress and antioxidant therapy in chronic kidney disease and hypertension. *Curr Opin Nephrol Hypertens* 13: 93–99, 2004.
46. Vilhardt F and van Deurs B. The phagocyte NADPH oxidase depends on cholesterol-enriched membrane microdomains for assembly. *EMBO J* 23: 739–748, 2004.
47. Wu D, Peng F, Zhang B, Ingram AJ, Gao B and Krepinsky JC. Collagen I induction by high glucose levels is mediated by epidermal growth factor receptor and phosphoinositide 3-kinase/Akt signalling in mesangial cells. *Diabetologia* 50: 2008–2018, 2007.

48. Yang B and Rizzo V. TNF-alpha potentiates protein-tyrosine nitration through activation of NADPH oxidase and eNOS localized in membrane rafts and caveolae of bovine aortic endothelial cells. *Am J Physiol Heart Circ Physiol* 292: H954–H962, 2007.
49. Zacharias DA, Violin JD, Newton AC, and Tsien RY. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science* 296: 913–916, 2002.
50. Zhang AY, Yi F, Zhang G, Gulbins E, and Li PL. Lipid raft clustering and redox signaling platform formation in coronary arterial endothelial cells. *Hypertension* 47: 74–80, 2006.
51. Zhang B, Peng F, Wu D, Ingram AJ, Gao B, and Krepinsky JC. Caveolin-1 phosphorylation is required for stretch-induced EGFR and Akt activation in mesangial cells. *Cell Signal* 19: 1690–1700, 2007.
52. Zuo L, Ushio–Fukai M, Ikeda S, Hilenski L, Patrushev N, and Alexander RW. Caveolin-1 is essential for activation of Rac1 and NAD(P)H oxidase after angiotensin II type 1 receptor stimulation in vascular smooth muscle cells: Role in redox signaling and vascular hypertrophy. *Arterioscler Thromb Vasc Biol* 25: 1824–1830, 2005.
53. Zwick E, Hackel PO, Prenzel N, and Ullrich A. The EGF receptor as central transducer of heterologous signalling systems. *Trends Pharmacol Sci* 20: 408–412, 1999.

Address correspondence to:
Joan C. Krepinsky
St. Joseph's Hospital
50 Charlton Ave East, Rm T3311
Hamilton, ON L8N 4A6
Canada
E-mail: krepinj@mcmaster.ca

Date of first submission to ARS Central, September 19, 2009; date of final revised submission, March 29, 2010; date of acceptance, April 11, 2010.

Abbreviations Used

Cav-1 = caveolin-1
DHE = dihydroethidium
EGFR = epidermal growth factor receptor
KO = knockout
MC = mesangial cell
ROS = reactive oxygen species
WT = wild-type

This article has been cited by:

1. Isabel Mercedes García, Luciana Mazzei, María Eugenia Benardón, Liliana Oliveros, Fernando Darío Cuello-Carrión, Andrea Gil Lorenzo, Walter Manucha, Patricia G. Vallés. 2012. Caveolin-1–eNOS/Hsp70 interactions mediate rosuvastatin antifibrotic effects in neonatal obstructive nephropathy. *Nitric Oxide* **27**:2, 95-105. [CrossRef]
2. Hongqiao Zhang, Honglei Liu, Zea Borok, Kelvin J.A. Davies, Fulvio Ursini, Henry Jay Forman. 2012. Cigarette smoke extract stimulates epithelial–mesenchymal transition through Src activation. *Free Radical Biology and Medicine* **52**:8, 1437-1442. [CrossRef]
3. Mark F. McCarty. 2011. Marinobufagenin and cyclic strain may activate endothelial NADPH oxidase, contributing to the adverse impact of salty diets on vascular and cerebral health. *Medical Hypotheses* . [CrossRef]
4. Volker Vallon, Radko KomersPathophysiology of the Diabetic Kidney . [CrossRef]
5. Hong Pyo Kim, Hyun-Ock Pae, Sung Hun Back, Su Wol Chung, Je Moon Woo, Yong Son, Hun-Taeg Chung. 2011. Heme oxygenase-1 comes back to endoplasmic reticulum. *Biochemical and Biophysical Research Communications* **404**:1, 1-5. [CrossRef]
6. Radko Komers. 2011. Rho kinase inhibition in diabetic nephropathy. *Current Opinion in Nephrology and Hypertension* **20**:1, 77-83. [CrossRef]