Aquaporin 1, Nox1, and Ask1 mediate oxidant-induced smooth muscle cell hypertrophy

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Aims	Reactive oxygen species (ROS)-mediated intracellular signalling is well described in the vasculature, yet the precise roles of ROS in paracrine signalling are not known. Studies implicate interstitial ROS hydrogen peroxide (H_2O_2) in vascular disease, and plasma H_2O_2 levels in the micromolar range are detectable in animal models and humans with hypertension. Recently, H_2O_2 was shown to cross biological membranes of non-vascular cells via aquaporin (Aqp) water channels. Previous findings suggest that H_2O_2 activates NADPH oxidase (Nox) enzymes in vascular cells and apoptosis signal-regulating kinase 1 (Ask1) in non-vascular cells. We hypothesized that extracellular H_2O_2 induces smooth muscle cell (SMC) hypertrophy by a mechanism involving Aqp1, Nox1, and Ask1.
Methods and results	Treatment of rat aortic SMCs (rASMC) with exogenous H_2O_2 resulted in a concentration-dependent increase in Nox-derived superoxide ($O_2^{\bullet-}$), determined by L-012 chemiluminescence, cytochrome c and electron paramagnetic resonance. Nox1 was verified as the source of $O_2^{\bullet-}$ by siRNA. Aqp1 siRNA attenuated H_2O_2 cellular entry and H_2O_2-induced $O_2^{\bullet-}$ production. H_2O_2 treatment increased Ask1 activation and induced rASMC hypertrophy in a Nox1-dependent mechanism. Adenoviral-dominant-negative Ask1 attenuated H_2O_2-induced rASMC hypertrophy and adenoviral overexpression of Ask1 augmented it.
Conclusion	Our results demonstrate for the first time that extracellular H_2O_2, at pathophysiological concentrations, stimulates rASMC Nox1-derived $O_2^{\bullet-}$, subsequent Ask1 activation and SMC hypertrophy. The data demonstrate a novel pathway by which H_2O_2 enters vascular cells via aquaporins and activates Nox, leading to hypertrophy, and provide multiple novel targets for combinatorial therapeutics development targeting hypertrophy and vascular disease.
Keywords	NADPH oxidase • Hydrogen peroxide • Aquaporin • Ask1 • Hypertrophy

1. Introduction

Reactive oxygen species (ROS) and their cellular sources have been the focus of intense study in recent years. ROS mediate cell and tissue oxidative stress and damage, as well as participate in key signalling pathways, leading to vascular remodelling, cell migration, and differentiation.[1,2] However, the role of ROS as paracrine modulators of oxidative stress and signalling has largely gone unstudied. Of the various ROS, hydrogen peroxide (H_2O_2) is most likely the best candidate for effecting paracrine signalling attributed to its stability and relative diffusibility.[2] Indeed, previous data from our laboratory indicate H_2O_2-mediated feed-forward signalling between the vascular adventitia and medial smooth muscle cells (SMCs) resulting in local ROS production and SMC hypertrophy.[3,4]

Being a relatively stable ROS, H_2O_2 is thought to partition into and freely cross membranes and thus pervade cells and tissue in a relatively facile manner. This widely accepted notion was recently challenged in a report showing a role for previously defined water channels, aquaporins, in H_2O_2 diffusion into HEK 293 and HeLa cells.[5] Aquaporins are ubiquitously expressed across species from yeast to humans.[6]

It is unknown, however, whether these channels function as conduits for H_2O_2 transport across vascular cells.

The NADPH oxidase (Nox) enzyme family is widely recognized as a robust and critical source of the ROS superoxide anion ($O_2^{\bullet -}$) in the vasculature,[1] and as such, is a plausible target of H_2O_2-mediated feed-forward signalling. However, it is not known whether physiologically relevant concentrations of H_2O_2 (i.e. as low as 5 μM that are found in animal and human studies of vascular disease) activate Nox isozymes.

Interestingly, the mediators of paracrine H_2O_2-induced SMC signalling and dysfunction are also still poorly described. It is well accepted that Nox-derived ROS activate p38 MAPK, which is pivotal in SMC hypertrophy.[7] In this study, we postulated that H_2O_2 induces SMC Nox1, which, in turn, activates apoptosis signal-regulating kinase 1 (Ask1), a heretofore described upstream activator of p38 MAPK.[8] Ask1 is a viable but never before studied activator of vascular hypertrophy. In summary, our findings are the first to our knowledge to draw a link between extracellular H_2O_2 entering vascular SMCs via aquaporin 1 (Aqp1) channels and stimulating vascular Nox-derived ROS, which in turn activate Ask1 and hypertrophy. These findings provide multiple novel therapeutic targets aimed at suppressing or abrogating vascular SMC hypertrophy.

2. Methods

Detailed methods are provided in the Supplementary material online.

2.1 Materials

A detailed list of materials used is included in the Supplementary material online.

2.2 Cell culture

Rat aortic smooth muscle cells (rASMC) were purchased from Lonza (Lonza Cologne GmbH, Cologne, Germany). Cells were maintained in DMEM supplemented with 10% FBS and containing 100 U/mLpenicillin and 100 μg/mL streptomycin (Invitrogen). Cells were cultured at 37°C with 5% CO_2. Cells between passages 2−9 were used.

2.3 $O_2^{\bullet -}$ detection

2.3.1 Electron paramagnetic resonance

Electron paramagnetic resonance (EPR) spin probe CMH (50 μM) was used to examine $O_2^{\bullet -}$ production using a Bruker eScan Table-Top EPR spectrometer (Bruker Biospin, USA). 1×10^6 cells/mL were resuspended in Krebs−HEPES buffer (pH 7.4) treated with Chelex resin and containing 25 μM deferoxamine to minimize the deleterious effects of contaminating metals. Spectra were obtained after 1, 10, and 20 min at 37°C.

2.3.2 L-012 chemiluminescence

Cells on white clear-bottom 96-well culture plates were stimulated with varying concentrations of H_2O_2 for 10−300 min. The media was replaced with PBS (pH 7.4) supplemented with $CaCl_2$ (0.9 mM) and $MgSO_4$ (0.49 mM) and luminol derivative L-012 (400 μM) was added and chemi-luminescence measured in a BioTek Synergy 4 Hybrid Multi-Mode Micro-plate Reader (BioTek, Winooski, VT, USA) for at least 30 min post-H_2O_2 stimulation times. $O_2^{\bullet -}$ production was quantified as relative light units (RLU).

2.3.3 Cytochrome c reduction assay

The cytochrome c assay was conducted as described previously.[9] Briefly, cells were treated with 50 μM H_2O_2 for 1 h, lysed and $28\,000 \times g$ membrane fraction prepared. Membrane fractions were mixed with

cytochrome c. After 5 min baseline measurement, NADPH was added and $O_2^{\bullet -}$ production calculated from the initial linear rate of superoxide dismutase (SOD)-inhibitable cytochrome c reduction (at 550 nm; extinction coefficient 21.1 $mM^{-1}cm^{-1}$).

2.4 Live-cell imaging confocal microscopy of HyPer fluorescence

Cells on coverslip bottomed dishes (MatTek, Ashland, MA, USA) were mounted in a temperature controlled chamber (Tokai Hit, Japan) atop the motorized stage of a Nikon TiE inverted fluorescent microscope with a ×60, 1.4 NA optic (Nikon, Melville, NY, USA). HyPer was excited by 438 and 513 nm (SpectraX, Lumencor, Beaverton OR, USA) and detected using 525/50 nm bandpass filter (Chroma Technology), C11440−22C camera (Hamamatsu), and NIS Elements software. The ratios (513/438) were calculated for one to three cells per stage position, 10 stage positions per plate per experiment.

2.5 siRNA transfection to suppress Nox1 or Aqp1

Cells were grown to 30−50% confluence on 100 mm, 6-well or 96-well plates and were transfected with scrambled siRNA or three distinct variants of siRNA (5 pmol) against Nox1 or Aqp1 using the transfection reagent Lipofectamine 2000 according to the manufacturer's protocol. Cells were assayed 48 h later.

2.6 Adenoviral transduction for Ask1 gain- and loss-of-function

Adenoviruses for green fluorescent protein (GFP), transgenic (Tg)-, or dominant-negative (DN)-Ask1 were added to 50% confluent cells at a multiplicity of infection (MOI) of 10 and incubated for 24 h at 37°C. For experiments in which siRNA was applied, adenoviruses were added 24 h post-siRNA transfection.

2.7 Quantitative PCR

First cDNA was prepared using 1−2 μg of total RNA (SuperScript™ First-Strand, Invitrogen). Then samples were mixed with primer/probe for Nox1, Nox4, Aqp1, or 18S in 384-well plate (TaqMan® Universal PCR Master Mix, ABI- Applied Biosystems, Inc., Foster City, CA, USA) and qPCR performed in a 7900HT Fast Real-Time PCR System (ABI) for 40 cycles. Relative quantification was obtained using the Ct (threshold cycle) method:

$$\Delta Ct = \Delta Ct_{Nox1/Nox4/Aqp1} - Ct_{18S};$$
$$\Delta\Delta Ct = \Delta Ct_{Nox1/4/Aqp1 \text{ siRNA transfected sample}}$$
$$- \Delta Ct_{Scrambled \text{ siRNA transfected sample}}$$

Relative expression was calculated as $2^{-\Delta\Delta Ct}$.

2.8 Western blot

Cells were lysed with RIPA buffer and SDS−PAGE performed. Membranes were incubated with total Ask1 (Cell Signaling) or phospho-Ask1 (threonine-845, a kind gift from Dr Hidenori Ichijo, University of Tokyo, Japan) antibodies (1:500 dilution). Membranes were probed with goat anti-mouse or goat anti-rabbit secondary antibodies (1:10 000 dilution, Li-Cor Biosciences). Digital imaging was obtained (Odyssey Infra-Red Imaging system, Li-Cor).

2.9 Quantification of cell hypertrophy

Eighty per cent confluent rASMCs were treated with 50 μM H_2O_2 for 24 h then separated into two equal volumes, one was lysed for protein quantification and the other was lysed for DNA quantification

(Hoechst-33258, Invitrogen). Hypertrophy was determined as the protein:DNA ratio normalized to control.

2.10 FACS analysis

Flow cytometry was performed on a BD LSRII cytometer (BD Biosciences). rASMCs were treated as above. Forward scatter (FS) and side scatter (SS) were measured (10 000 events/sample). Quantification was performed using FloJo. Density plots were gated to exclude debris and then quadrant parameters were selected based on the size of 80– 85% of cells from control groups. The percentage of events recorded in Quadrant 2 (FS +, SS +) were quantified for each group and taken as a ratio of Quadrant 2 events percentage in control groups.

2.11 Thymidine incorporation

Radiolabelled thymidine incorporation was performed as previously described.[10] Briefly, 70–80% confluent rASMCs were incubated with 1 μCi/mL [^{3}H] ± 50 μM H_2O_2 for 24 h at 37°C, and exposed to 10% trichloroacetic acid for 10 min. Monolayers were then dissolved in 1 N NaOH for radioactivity measurement.

2.12 Cell size measurement

rASMCs were treated as above with 50 μM H_2O_2 for 24 h at 37°C. Images were captured using a Nikon Eclipse TS100 microscope connected to a Nikon D90 SLR digital camera at an objective lens magnification of ×10. Three to six images were taken per treatment group per experiment. The areas of four individual cells selected at random per image were calculated using Image J (NIH, USA). Areas were then averaged and taken as a ratio of control.

2.13 Statistical analyses

Data are presented as means ± SEM. Data comparisons were performed with Student's *t*-test, one- or two-way-ANOVA including Bonferroni *post hoc* analysis. Differences were deemed statistically significant at $P < 0.05$.

3. Results

3.1 H_2O_2 activates $O_2^{\bullet-}$ production in rASMC

Treatment for 1 h with concentrations as low as 5 μM H_2O_2 significantly stimulated production of $O_2^{\bullet-}$ as demonstrated by an increase in L-012 chemiluminescence. The other concentrations of H_2O_2 used (10, 50, and 100 μM) also resulted in a significant increase in L-012 chemiluminescence, with the maximal signal observed with 50 μM treatment (*Figure 1A* and *B*). Treatment with 50 μM H_2O_2 for 10– 300 min revealed a rise in $O_2^{\bullet-}$ levels as early as 10 min posttreatment that was sustained for 3 h (data not shown). SOD treatment (300 U/mL) confirmed this signal as $O_2^{\bullet-}$ dependent (*Figure 1B*). These results were also confirmed by EPR studies using the spin probe CMH, where an increase in CM-radical intensity, indicative of $O_2^{\bullet-}$, was seen in response to 50 μM H_2O_2 when compared with vehicle-treated cells and this was abolished with the addition of 300 U/mL SOD (*Figure 1C*). Interestingly, treatment of rASMC with 1 μM H_2O_2 for 1 h did not stimulate $O_2^{\bullet-}$ production (cytochrome c assay; *Figure S1*, Supplementary material online).

3.2 H_2O_2-induced $O_2^{\bullet-}$ in rASMC is Nox1 derived

To identify the source of $O_2^{\bullet-}$ produced in response to H_2O_2, we used a spectrum of pharmacological inhibitors for cellular sources of $O_2^{\bullet-}$. Prior to 50 μM H_2O_2 treatment, cells were incubated for
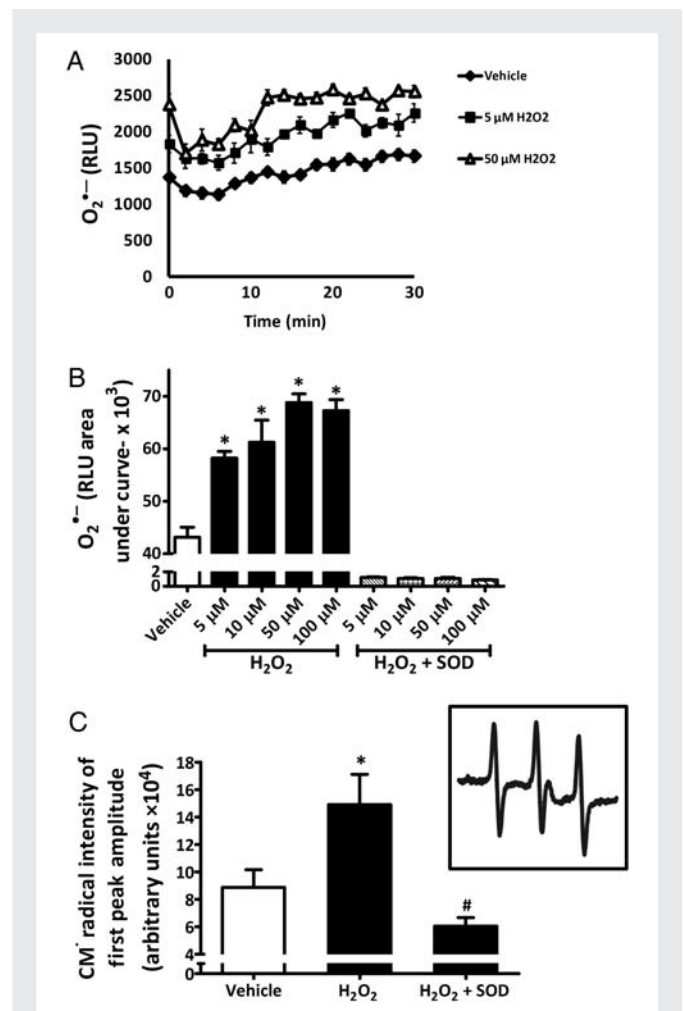

Figure 1 Exogenous H_2O_2 increases $O_2^{\bullet-}$ production in rASMC. (*A*) Representative trace of L-012 chemiluminescence over time after rASMC treatment with indicated concentrations of H_2O_2 ± superoxide dismutase (SOD, 300 U/mL). (*B*) Quantification of all the areas under the curve (AUC) in (*A*) in relative light units (RLU). (*C*) Quantification of first-peak amplitudes of CM$^{\bullet}$ radical EPR spectra of rASMC treated with vehicle or 50 μM H_2O_2 ± SOD (300 U/mL). Inset: representative EPR spectrum of rASMC treated with 50 μM H_2O_2. Means ± SEM, $n = 3$, *$P < 0.05$ vs. vehicle; #$P < 0.05$ vs. H_2O_2.

30 min with mitochondrial complex I inhibitor rotenone (50 μM), nitric oxide synthase (NOS) inhibitor L-NAME (100 μM), xanthine oxidase inhibitor febuxostat (100 nM), cyclooxygenase inhibitor indomethacin (10 μM), or flavoprotein inhibitor diphenylene iodonium (DPI) (5 μM). *Figure S2*, Supplementary material online, shows that only DPI was effective at significantly inhibiting $O_2^{\bullet-}$ production in response to H_2O_2 (77 ± 14% inhibition). Treatment with 300 U/mL SOD or cell-permeant SOD mimetic Tiron (10 mM) both resulted in complete inhibition of the signal (98 ± 0.2 and 99 ± 0.1% inhibition, respectively; *Figure S2*, Supplementary material online). No significant inhibition was observed with rotenone, L-NAME, febuxostat, or indomethacin.

The Nox isoforms expressed in rodent aortic vascular SMCs are Nox1 and Nox4 with Nox1 appearing to be the isoform most likely involved in $O_2^{\bullet-}$ production.[1] We acknowledge that some reports have suggested that Nox4 could liberate $O_2^{\bullet-}$ under certain

conditions.[11] However, considering the controversy leaning towards greater H_2O_2 vs. $O_2^{\bullet-}$ production by Nox4, the most likely source of $O_2^{\bullet-}$ in these cells is Nox1. Thus, to interrogate the role of Nox1 in H_2O_2-induced $O_2^{\bullet-}$ in rASMC, three distinct siRNA sequences against Nox1 were used to ensure knockdown of Nox1 mRNA. To test for specificity, rASMCs were transfected with each siRNA and assessed for Nox1 and Nox4 mRNA expression by PCR 48 h later. *Figure S3*, Supplementary material online, shows that both siRNA oligonucleotides (oligo) 1 and 2 were effective at reducing Nox1 mRNA (to 45 ± 6 and $34 \pm 10\%$ from scrambled siRNA-transfected cells, respectively) without affecting Nox4 mRNA levels. Oligo 3 did not result in a significant reduction in Nox1 mRNA when compared with scrambled siRNA-transfected cells. Since Nox1 mRNA knockdown was greatest with the use of oligo 2, this sequence was selected for the remainder of studies in this manuscript (*Figure S3*, Supplementary material online).

Scrambled siRNA control-transfected rASMCs treated with H_2O_2 exhibited an approximate doubling of $O_2^{\bullet-}$ levels as measured by L-012 chemiluminescence compared with scrambled siRNA-transfected, vehicle-treated cells (84 ± 3 vs. $44 \pm 3 \times 10^6$ RLU, respectively; *Figure 2A*). Nox1-siRNA (oligo 2)-transfected rASMC displayed a significant 80% reduction in H_2O_2-induced $O_2^{\bullet-}$ ($51 \pm 7 \times 10^6$ RLU) compared with scrambled siRNA-transfected cells treated with H_2O_2. No significant difference was detected between Nox1-siRNA-transfected H_2O_2-treated cells and scrambled siRNA-transfected vehicle-treated cells (*Figure 2A*). Cytochrome c assay of rASMC membrane fractions confirmed these results (*Figure 2B*). That is, $O_2^{\bullet-}$ levels rose from 2 ± 1 to 8 ± 2 pmol/min/mg protein in H_2O_2-treated scrambled siRNA-transfected rASMCs compared with vehicle-treated scrambled siRNA-transfected cells; Nox1 siRNA abolished this stimulation (2 ± 0.3 pmol/min/mg protein). H_2O_2 treatment did not affect Nox1 mRNA levels (*Figure S4A* and *B*, Supplementary material online).

3.3 Aqp1 is involved in mediating H_2O_2-induced $O_2^{\bullet-}$ production by facilitating H_2O_2 entry into rASMC

qPCR demonstrated that only Aqp1 is expressed in rASMC. Neither Aqp3 nor Aqp8 was expressed ($n = 3$ independent experiments, data not shown). To investigate the role of Aqp1, three distinct siRNAs for Aqp1 were tested for the efficacy of mRNA knockdown by qPCR in rASMC (*Figure S5*, Supplementary material online). Two of the three siRNA sequences, oligo 2 and oligo 3, resulted in a significant reduction in Aqp1 mRNA when compared with scrambled siRNA-transfected cells (down to 38 ± 2 and $37 \pm 2\%$ from scrambled siRNA-transfected cells, respectively). Oligo 1 did not result in a significant reduction in Aqp1 mRNA. Aqp1 oligo 3 was chosen for use in the remainder of studies in this manuscript. The Aqp1 siRNAs did not appear to have any effect on the cell shape or size when compared with scrambled siRNA controls.

To evaluate whether Aqp1 facilitates H_2O_2 transport into rASMC, cells were cotransfected with scrambled or Aqp1 siRNA and the highly specific and sensitive genetically encoded intracellular H_2O_2 fluorescent probe HyPer.[5] Live cell imaging was then conducted prior and post-H_2O_2 addition. As can be seen in *Figure 3A*, the addition of H_2O_2 to rASMC resulted in a rapid three-fold increase in HyPer fluorescence, indicating uptake of H_2O_2. Aqp1 siRNA blocked this increase in HyPer fluorescence.
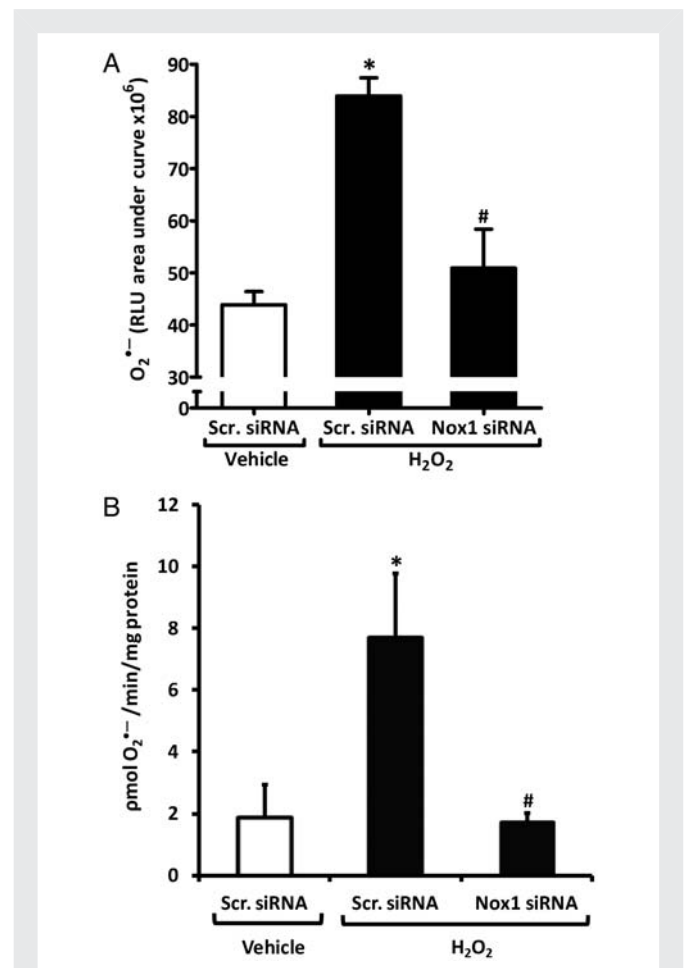

Figure 2 Nox1 is the enzymatic source of H_2O_2-induced $O_2^{\bullet-}$ in rASMC. (*A*) AUC of L-012 chemiluminescence of rASMC transfected with scrambled (Scr.) or Nox1 siRNA and treated with vehicle or 50 μM H_2O_2. Means $\pm$ SEM, $n = 12$, *$P < 0.001$ vs. Scr. siRNA vehicle, #$P < 0.001$ vs. Scr. siRNA H_2O_2. (*B*) $O_2^{\bullet-}$ production in pmol/min/mg protein quantified by SOD-inhibitable cytochrome c reduction in membrane fractions of rASMC transfected and treated as in (*A*). Means $\pm$ SEM, $n = 4$, *$P < 0.05$ vs. Scr. siRNA vehicle, #$P < 0.05$ vs. Scr. siRNA H_2O_2.

Transfection of rASMC with Aqp1 siRNA effectively and significantly reduced H_2O_2-induced $O_2^{\bullet-}$ relative to scrambled siRNA-transfected rASMC cells treated with H_2O_2 (1.07 ± 0.08- vs. 1.34 ± 0.06-fold from vehicle-treated scrambled siRNA-transfected cells, respectively; *Figure 3B*). We observed no significant difference between levels of $O_2^{\bullet-}$ in vehicle controls transfected with scrambled siRNA and H_2O_2-treated rASMCs transfected with Aqp1 siRNA. H_2O_2 treatment did not affect Aqp1 mRNA levels (*Figure S4C*, Supplementary material online). Knockdown of Aqp1 mRNA by Aqp1 siRNA had a modest effect ($14 \pm 0.03\%$ reduction) on Nox1 protein (*Figure S6*, Supplementary material online).

3.4 H_2O_2 induces Nox1-dependent phosphorylation of Ask1 in rASMC

Ask1 is activated by phosphorylation at threonine-845.[12,13] To assess Ask1 activation in rASMC, cells were transfected with scrambled or Nox1 siRNA and treated with H_2O_2. H_2O_2 treatment resulted in
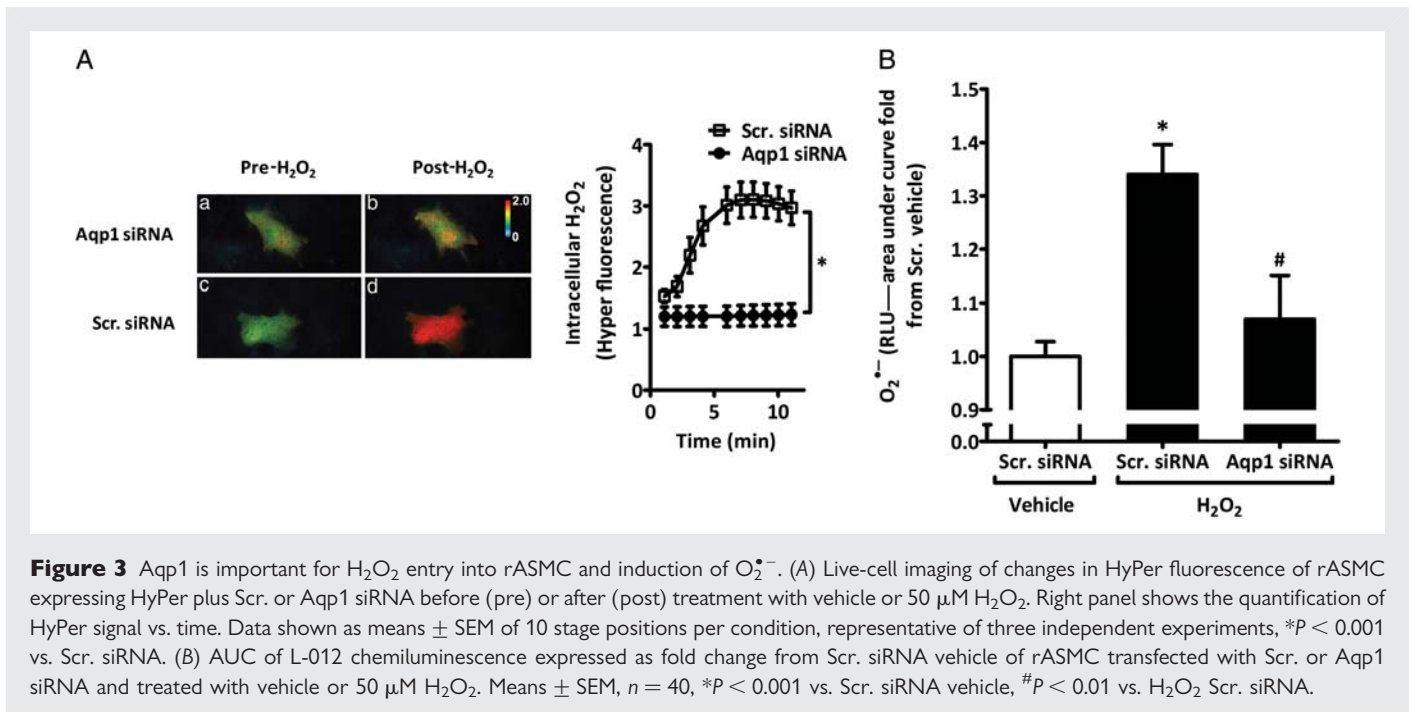
Figure 3 Aqp1 is important for H_2O_2 entry into rASMC and induction of $O_2^{\bullet-}$. (A) Live-cell imaging of changes in HyPer fluorescence of rASMC expressing HyPer plus Scr. or Aqp1 siRNA before (pre) or after (post) treatment with vehicle or 50 μM H_2O_2. Right panel shows the quantification of HyPer signal vs. time. Data shown as means $\pm$ SEM of 10 stage positions per condition, representative of three independent experiments, *$P < 0.001$ vs. Scr. siRNA. (B) AUC of L-012 chemiluminescence expressed as fold change from Scr. siRNA vehicle of rASMC transfected with Scr. or Aqp1 siRNA and treated with vehicle or 50 μM H_2O_2. Means $\pm$ SEM, $n = 40$, *$P < 0.001$ vs. Scr. siRNA vehicle, #$P < 0.01$ vs. H_2O_2 Scr. siRNA.
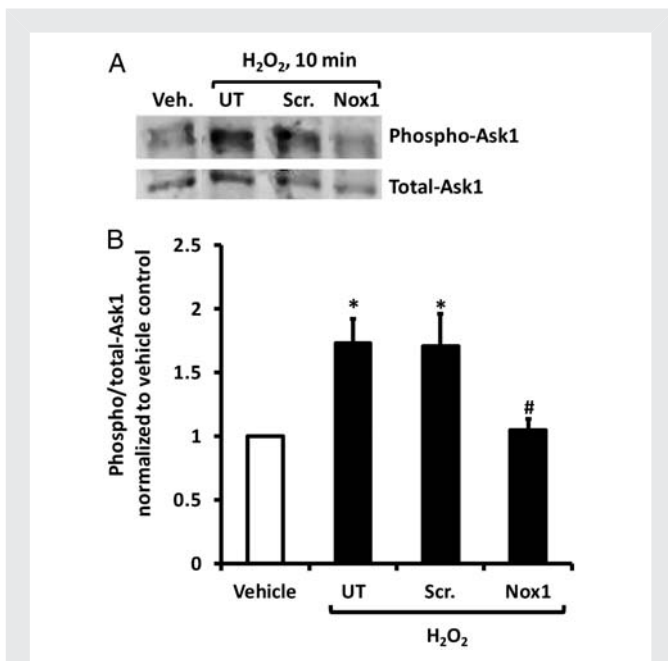

Figure 4 H_2O_2 induces Ask1 phosphorylation in rASMC in a Nox1-dependent mechanism. (A) Representative Western blot for phospho-Ask1 (threonine-845) and total-Ask1 of lysates of rASMC transfected with Scr. or Nox1 siRNA and treated with 50 μM H_2O_2 (UT, untransfected cells). (B) The quantification of fluorescence intensity of phospho-Ask1 bands obtained in (A) normalized to total-Ask1. Means $\pm$ SEM, $n = 6$, *$P < 0.05$ vs. vehicle; #$P < 0.05$ vs. H_2O_2 Scr. siRNA.

an increase in Ask1 phosphorylation that was reversed by Nox1 siRNA but not by scrambled siRNA (*Figure 4A*). Cumulative results confirm these findings (*Figure 4B*). No difference in the ratio of phospho:total Ask1 was observed between untransfected (UT) and scrambled siRNA-transfected cells treated with H_2O_2. Similarly, no difference was observed in the phospho:total Ask1 ratio between UT vehicle-treated cells and Nox1-siRNA-transfected H_2O_2-treated cells (*Figure 4B*). Transfection with scrambled siRNA had no effect on Ask1 phosphorylation in vehicle-treated cells (data not shown). To support these findings, these experiments were repeated in rASMC adenovirally overexpressing Ask1 yielding similar results (*Figure S7*, Supplementary material online).

3.5 H_2O_2 treatment results in increased hypertrophy, but not proliferation of rASMC, in a mechanism involving Aqp1, Nox1, and Ask1

Hypertrophy in rASMC transfected with Nox1 or scrambled siRNA was assessed after H_2O_2 treatment by two independent methods, quantifying the ratio of the protein:DNA content, and FACS analysis. As seen in *Figure 5A*, H_2O_2 treatment resulted in a 1.4 $\pm$ 0.16-fold increase in hypertrophy in scrambled siRNA-transfected cells compared with scrambled siRNA-transfected vehicle control. Nox1 siRNA inhibited this increase in hypertrophy in response to H_2O_2 treatment (0.9 $\pm$ 0.06-fold from scrambled siRNA vehicle). These findings were confirmed by FACS analysis: H_2O_2 treatment increased the percentage of enlarged cells by 1.3 $\pm$ 0.08-fold compared with vehicle in scrambled siRNA-transfected cells and this increase in response to H_2O_2 treatment was abolished in Nox1 siRNA transfected cells (0.9 $\pm$ 0.02-fold from scrambled siRNA vehicle; *Figure 5B* and *C*). No difference was observed between scrambled siRNA-transfected vehicle-treated cells and Nox1-siRNA-transfected H_2O_2-treated cells. In addition, the quantification of average cell areas in experiments with similar treatment groups further supported these findings (*Figure S8*, Supplementary material online). Furthermore, Aqp1 siRNA inhibited H_2O_2-induced rASMC hypertrophy to a similar extent as Nox1 siRNA, as seen by FACS analysis of the percentage of enlarged cells post-H_2O_2 treatment (fold from scrambled siRNA vehicle: 1.4 $\pm$ 0.06
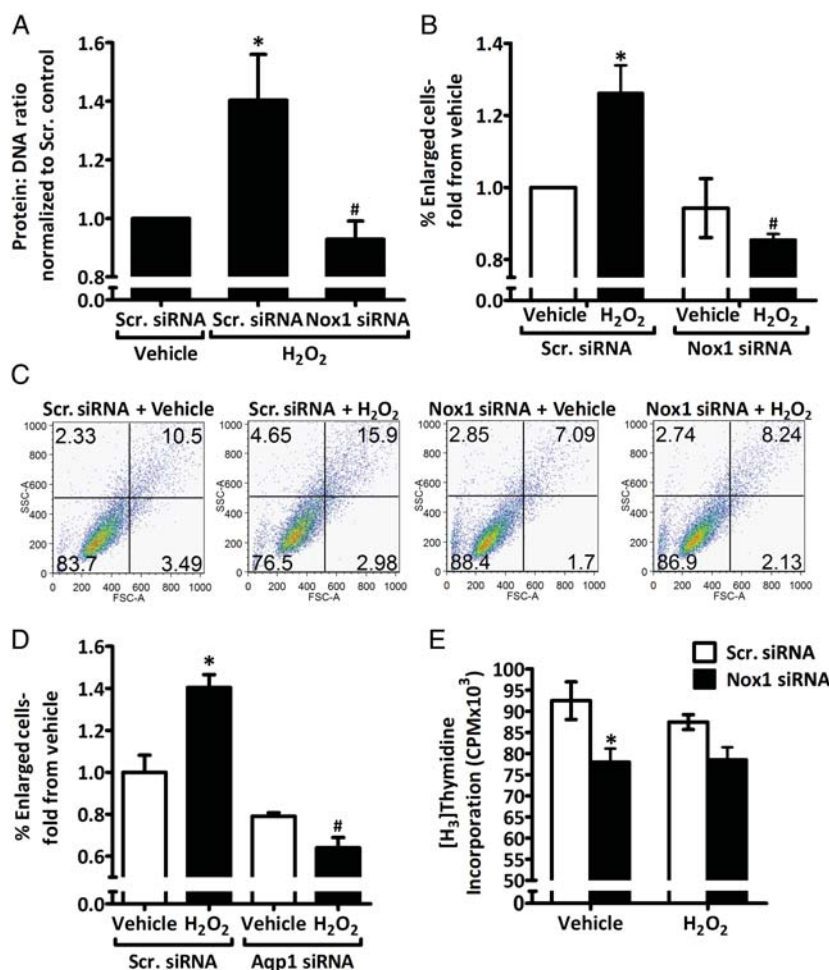
Figure 5 H_2O_2 induces increase in rASMC hypertrophy, but not proliferation, in an Aqp1- and Nox1-dependent mechanism. (A) Quantification of the protein:DNA ratio of lysates from rASMC transfected with Scr. or Nox1 siRNA and treated with vehicle or 50 µM H_2O_2 for 24 h expressed as fold from Scr. vehicle. (B) FACS analysis of % enlarged cells of total rASMC treated as in (A) expressed as fold from Scr. vehicle. (C) Representative FACS analysis image of B. (D) FACS analysis quantified as in (B) of rASMC transfected with Scr. or Aqp1 siRNA and treated with vehicle or 50 µM H_2O_2 for 24 h. (E) [^{3}H]thymidine incorporation measurement in counts per minute (CPM) of rASMC treated as in (A). Means ± SEM, $n = 3-4$. *$P < 0.05$ vs. Scr. siRNA vehicle. #$P < 0.05$ vs. H_2O_2 Scr. siRNA.

vs. 0.64 ± 0.05 for scrambled siRNA H_2O_2 vs. Aqp1 siRNA H_2O_2 groups, respectively; Figure 5D). There was a trend towards a smaller percentage of enlarged cells in both H_2O_2- and vehicle-treated Aqp1 siRNA-transfected cells compared with vehicle scrambled siRNA cells, but these were not statistically significant (Figure 5D).

Treatment of cells with H_2O_2 elicited no effect on rASMC proliferation compared with vehicle treatment in scrambled siRNA-transfected cells as measured by radio-labelled thymidine incorporation (Figure 5E). Similarly, H_2O_2 treatment had no effect on proliferation compared with vehicle treatment in Nox1-siRNA-transfected cells. There was a significant inhibition of proliferation by Nox1 siRNA when compared with scrambled siRNA in vehicle-treated cells (78 ± 3 vs. $92 \pm 4 \times 10^3$ CPM, respectively).

To test the role of Ask1 on hypertrophy, rASMCs were transfected with adenovirus expressing dominant negative Ask1 (Ad-DN-Ask1) or GFP (Ad-GFP). As shown in Figure 6A, H_2O_2 caused a 1.35 ± 0.16-fold rise in the protein:DNA ratio compared with vehicle treatment in cells transduced with GFP control adenoviruses. In the

presence of Ad-DN-Ask1, this increase was abolished. No difference was detected between vehicle and H_2O_2 treatments in cells transduced with Ad-DN-Ask1. Ask1 overexpression (transgenic Ask1, Ad-Tg-Ask1) in rASMC resulted in an enhanced increase in hypertrophy in response to H_2O_2 compared with Ad-GFP cells treated with H_2O_2 (1.97 ± 0.24- vs. 1.35 ± 0.16-fold from Ad-GFP vehicle control, respectively; Figure 6B). No difference was detected under vehicle treatment between Ad-GFP and Ad-Tg-Ask1-transduced cells.

Co-transfection of Nox1 siRNA into Ad-Tg-Ask1-transduced rASMC abolished H_2O_2-induced hypertrophy when compared with H_2O_2-treated scrambled siRNA-transfected Ad-Tg-Ask1-tansduced cells (0.78 ± 0.09- vs. 1.91 ± 0.34-fold from scrambled siRNA Ad-Tg-Ask1 vehicle control, respectively; Figure 6C). No significant difference was detected between H_2O_2-treated Nox1 siRNA-transfected Ad-Tg-Ask1 cells and vehicle-treated scrambled siRNA-transfected Ad-Tg-Ask1 cells. Similarly, there was no significant difference under vehicle treatment of Ad-Tg-Ask1 cells between scrambled and Nox1 siRNA transfections.
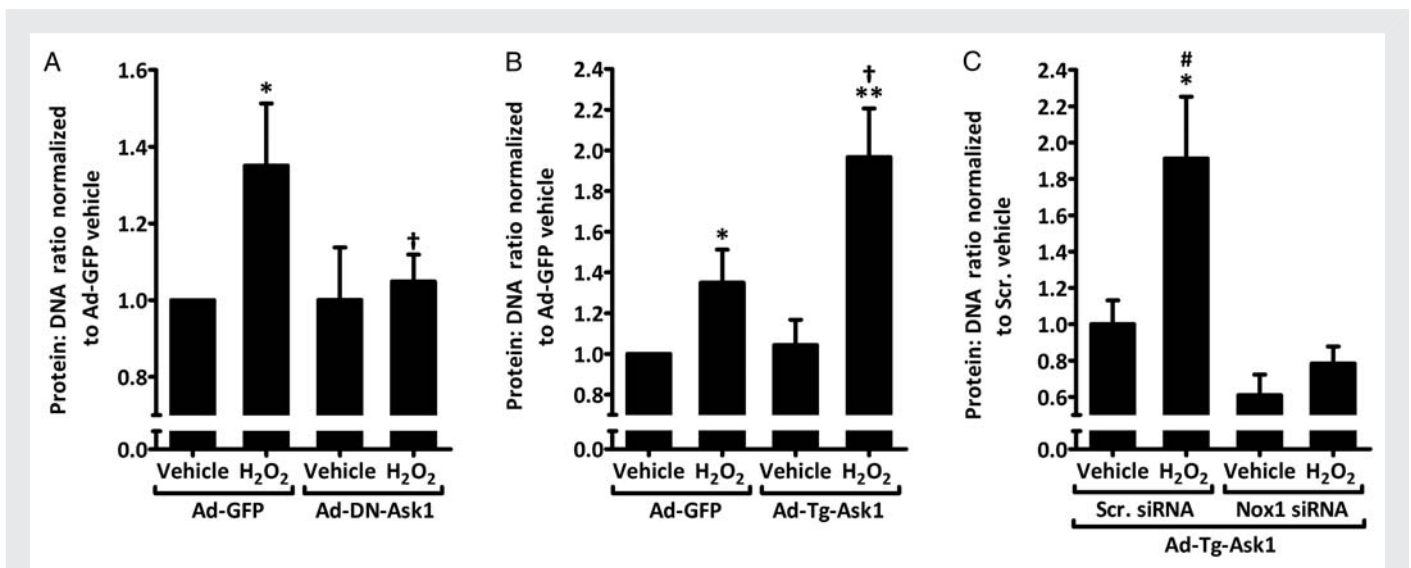
Figure 6 H_2O_2-induced Nox1-dependent increase in rASMC hypertrophy is augmented by Ask1 overexpression and attenuated by dominant-negative Ask1 expression. (*A* and *B*) Quantification of the protein:DNA ratio of lysates from rASMC transduced with adenoviruses expressing GFP control (Ad-GFP), dominant-negative Ask1 (Ad-DN-Ask1), or transgenic Ask1 (Ad-Tg-Ask1) and treated with vehicle or 50 μM H_2O_2 for 24 h expressed as fold from Ad-GFP vehicle. Means ± SEM, $n = 5$, *$P < 0.05$ vs. Ad-GFP vehicle. **$P < 0.001$ vs. Ad-Tg-Ask1 vehicle. †$P < 0.05$ vs. Ad-GFP H_2O_2. (*C*) Quantification of the protein:DNA ratio of lysates from rASMC transduced with transgenic Ask1 (Ad-Tg-Ask1), transfected with Scr. or Nox1 siRNA and treated with vehicle or 50 μM H_2O_2 for 24 h expressed as fold from Scr. siRNA vehicle Ad-Tg-Ask1. Means ± SEM, $n = 5$, *$P < 0.01$ vs. Scr. siRNA vehicle Ad-Tg-Ask1, #$P < 0.001$ vs. H_2O_2 Nox1 siRNA Ad-Tg-Ask1.

4. Discussion

Previously our laboratory and others demonstrated that ROS are important autocrine mediators of medial SMC signalling and hypertrophy. Previous findings suggested a paracrine feed-forward ROS signalling mechanism in the vascular wall; however, the signalling pathways involved in this response are not known. The findings of this study elucidate a novel pathway by which ROS effect medial hypertrophy and demonstrate for the first time that: (i) low micromolar concentrations of H_2O_2 induce $O_2^{\bullet-}$ production in rASMCs; (ii) Nox1 oxidase is the source of H_2O_2-induced $O_2^{\bullet-}$ production; (iii) the water channel Aqp1 is involved in H_2O_2-mediated $O_2^{\bullet-}$ production in rASMC through facilitating H_2O_2 transport into the cells; (iv) Ask1 is activated in vascular cells in response to pathophysiologically relevant concentrations of H_2O_2; and (v) Ask1 activation is involved in the propagation of rASMCs hypertrophy. Thus, these data support a mechanism by which vascular ROS can enter SMC via Aqp1, stimulate Nox1 and $O_2^{\bullet-}$ production, leading to Ask1-mediated vascular SMC hypertrophy.

We demonstrate that low micromolar concentrations of H_2O_2 (in the physiological range in plasma) are capable of increasing rASMC $O_2^{\bullet-}$ production. For ease of use in initial comprehensive measurements of concentration response and time course experiments, the highly sensitive L-012 chemiluminescence method was used. The data demonstrated a stimulation of vascular SMC $O_2^{\bullet-}$ production by concentrations of H_2O_2 as low as 5 μM in a concentration-dependent manner. We confirmed these data using two widely accepted methods for $O_2^{\bullet-}$ detection, EPR and cytochrome *c* assays. $O_2^{\bullet-}$ detection was confirmed by two $O_2^{\bullet-}$ scavengers, cell-impermeant SOD and cell-permeant Tiron. The inhibition by SOD demonstrated a trend towards lower $O_2^{\bullet-}$ production even when

compared with vehicle (*Figure 1C*). This effect is in line with low-level basal ROS production in these cells, which have been deemed operant in normal cellular signalling. Interestingly, our data suggest that $O_2^{\bullet-}$ production in vascular SMC is predominantly extracellular since non-cell-permeant SOD was capable of completely ablating $O_2^{\bullet-}$ detection (*Figure 1B* and *C*, and *Figure S2*, Supplementary material online). This difference in localization of $O_2^{\bullet-}$ production from that which was previously reported[14,15] could, however, be specific to the means by which H_2O_2 elicits Nox1 activation in comparison with receptor-mediated agonists. One hypothesis as to how this Nox1-derived extracellular $O_2^{\bullet-}$, which cannot easily cross biological membranes, carries out its action on Ask1 inside the cell is that it initiates a propagated response via H_2O_2. Extracellular $O_2^{\bullet-}$ dismuted to H_2O_2 is expected to enter the cell and activate Ask1. It should be noted also that undetectable intracellular $O_2^{\bullet-}$ production in response to exogenous H_2O_2 cannot be entirely excluded. Studies are ongoing to delineate the precise mechanism by which H_2O_2 activates Nox1 in these cells.

Interestingly, plasma levels of 3–5 μM H_2O_2 have been reported in patients with hypertension,[16] with some studies showing plasma levels up to 50 μM.[17,18] Moreover, studies have suggested that vascular cells produce H_2O_2, accumulating micromolar levels of the oxidant under stress stimuli, which are likely exacerbated by infiltrating leukocytes in inflammation.[19,20] Previous studies using exogenous H_2O_2 typically employed concentrations >100 μM,[19,21,22] and, to our knowledge, none used concentrations lower that 50 μM in vascular SMC. Thus our studies are the first to demonstrate that H_2O_2 concentrations, increasing from physiological to pathophysiological levels (*Figure 1* and *Figure S1*, Supplementary material online) in the cardiovascular system, are capable of propagating ROS production and hypertrophy.

Our findings indicate that Nox1 is the source of H_2O_2-stimulated $O_2^{\bullet-}$ in rASMC. Using a broad spectrum of pharmacological inhibitors of cellular oxidases we were able to preliminarily conclude that Nox-like activity was responsible for the SMC $O_2^{\bullet-}$ production since only iodonium compound DPI abolished $O_2^{\bullet-}$ levels. Although previous studies showed that H_2O_2 stimulates $O_2^{\bullet-}$ production from xanthine oxidase, mitochondria, and uncoupled endothelial NOS in endothelial cells,[21,23] our data do not support H_2O_2-induced $O_2^{\bullet-}$ production from these enzyme classes in SMCs. With this in mind, we interrogated the ability of Nox1 siRNA to inhibit H_2O_2-stimulated $O_2^{\bullet-}$. Nox1 is the identifying anchoring subunit of Nox1 oxidase, which co-associates with p22^{phox} in SMC caveolae to comprise the catalytic cytochrome portion of the enzyme system. Incidentally, cytosolic subunits p47^{phox} and NOXA1 are also implicated as essential for the active Nox1 oxidase complex in rASMC.[1] In preliminary studies, we tested the ability of three distinct oligonucleotides targeting Nox1 for their ability to specifically suppress Nox1 mRNA. Two of the three siRNA were effective, with the greatest inhibition (∼70%) rendered by oligonucleotide 2 (oligo 2, *Figure S3*, Supplementary material online). Nox1 siRNA oligo 2 ablated the four-fold increase in $O_2^{\bullet-}$ in response to H_2O_2 (in the presence of control siRNA), suggesting for the first time Nox1 as the major effector of H_2O_2 in SMCs (*Figure 2B*). Our data are supported by previous data showing that antisense oligonucleotides to p22^{phox} attenuated H_2O_2-stimulated ROS production in rASMC.[19] In that paper, however, the minimum effective concentration of H_2O_2 was 100 μM and knockdown of p22^{phox} per se was not sufficient to delineate the role of specific Nox isoforms, since p22^{phox} is critical for the activity of isoforms 1-4.

Our data show that suppressing Aqp1 using siRNA attenuated H_2O_2 entry into the cell as well as H_2O_2-induced $O_2^{\bullet-}$ production in rASMC. These data reveal for the first time a role for Aqp1 in paracrine vascular ROS signalling and challenge the widely accepted notion that H_2O_2 gains entry into vascular cells solely by virtue of its permeability across membranes. To date, only three studies addressed the role of aquaporins in mediating H_2O_2 transport across cellular membranes, and none was in cardiovascular cells.[5,6,24] Moreover, those previous studies demonstrate that Aqp3 and Aqp8 contribute to H_2O_2 transport yet none provide evidence for a role of Aqp1 in H_2O_2 transport. Those findings distinct from our current findings are likely explained by tissue and species differences in Aqp1 expression and regulation. Indeed, qPCR experiments (data not shown) demonstrated that only Aqp1, and not Aqp3 nor Aqp8, is expressed in rAMSC. This is consistent with previous findings by Herrera *et al.*[25] These differences notwithstanding, the data could foster a paradigm shift in our understanding of the means by which H_2O_2 enters cells and mediates its paracrine effects. At a more basic level, the data appear to provide the first evidence for a role of aquaporins in H_2O_2 transport across parenchymal cells.

Western blot analysis in the present study showed the activation of Ask1 in response to H_2O_2 treatment, and siRNA transfection revealed a role for Nox1 in this process. Although Ask1 is activated by a number of stress stimuli including ROS in multiple cell types, its role as a downstream target of Nox enzymes was until now unknown. Ask1 was shown to be activated by high micromolar concentrations of H_2O_2 in HeLa, HEK293 cells and mouse embryonic fibroblasts.[8,13,26] Despite this evidence, only one study in neurons suggests a link between Ask1 activation and Nox enzymes.[27] Indeed, the current data support a direct link between Nox1 and Ask1 and demonstrate that activation of Ask1 is downstream of Nox1-derived $O_2^{\bullet-}$ production in H_2O_2-treated rASMC. Nonetheless, direct activation of Ask1 by exogenous H_2O_2 cannot be ruled out. What is clear, however, is that knockdown of Nox1 was sufficient to abolish both endogenous and overexpressed Ask1 activation in response to H_2O_2 (*Figure 4* and Supplementary material online, *Figure S7*). Interestingly, knockdown of Aqp1 in cells overexpressing Ask1 was not enough to block Ask1 phosphorylation in response to H_2O_2 (*Figure S9*, Supplementary material online). We attribute this negative result to be due to a technical limitation of an overexpressed Ask1 on the one hand and a less than optimal antibody for endogenous phospho-Ask1 detection on the other hand.

On a functional level, the current study establishes a role for Aqp1, Nox1 and Ask1 in H_2O_2-induced hypertrophy of rASMC. Using protein to DNA ratio measurements, FACS analysis, average cell area quantification, and a three-pronged approach utilizing Aqp1 or Nox1 siRNA transfection and adenoviral transduction of DN or Tg Ask1, we conclude that H_2O_2 enters the cells via Aqp1 and induces Nox1 activation, which in turn activates Ask1 leading to SMC hypertrophy. Importantly, by the use of DN Ask1, we have demonstrated the essential role of Ask1 in this pathway. H_2O_2 has been implicated in the development of SMC hypertrophy and pro-hypertrophic signalling pathways;[2,28] however, the precise mechanisms have been elusive. Based on the current findings implicating Ask1 in rASMC hypertrophy, and recent findings from our laboratory implicating medial SMC p38 MAPK activation in response to adventitia-derived H_2O_2,[4] it is reasonable to speculate a signalling cascade involving the p38 MAPK pathway. Indeed, the activation of p38 MAPK downstream of Nox-derived ROS has been demonstrated.[7] However, based on the literature, links between Ask1 and p38 MAPK are expected to be complex and currently outside the scope of this study.

Induction of rASMC proliferation by H_2O_2 was ruled out by assessing radiolabelled thymidine incorporation; that is, H_2O_2 did not stimulate thymidine incorporation of rASMCs (*Figure 5E*). Of note is the observation of a significant inhibition of thymidine incorporation by Nox1 siRNA in vehicle-treated cells, which is consistent with previous findings.[29] Furthermore, there was no effect on cell count or appearance of cell death in rASMC treated with H_2O_2 in our studies, consistent with no effect on apoptosis of the concentrations used in this study. This is in line with the literature showing that apoptosis is typically observed at millimolar concentrations of H_2O_2.[19,26,30]

In summary, our findings challenge existing dogma on the biological action of H_2O_2 in the vasculature in a number of ways. Our results demonstrate that circulating or interstitial pathophysiological concentrations of H_2O_2 may induce paracrine $O_2^{\bullet-}$ production in rASMC. The findings suggest a novel mechanism by which H_2O_2 enters SMCs via Aqp1 and induces Nox1-derived $O_2^{\bullet-}$ production and SMC hypertrophy. This, combined with the fact that Nox1 siRNA completely blocked the $O_2^{\bullet-}$ signal, Ask1 phosphorylation and SMC hypertrophy and the fact that DN Ask1 blocked SMC hypertrophy, is consistent with a role for Ask1 activation in this pathway. Given the growing evidence that H_2O_2 acts on vascular cells as both a paracrine and autocrine signalling mediator, our findings have important implications for hypertension- and inflammatory disease-related SMC growth. From a therapeutic standpoint, the data provide key insights into the development of drugs to prevent medial thickening via new multiple targets.

Supplementary material

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References

1. Al Ghouleh I, Khoo NK, Knaus UG, Griendling KK, Touyz RM, Thannickal VJ *et al.* Oxidases and peroxidases in cardiovascular and lung disease: new concepts in reactive oxygen species signaling. *Free Radic Biol Med* 2011;**51**:1271–1288.

2. Csanyi G, Taylor WR, Pagano PJ. NOX and inflammation in the vascular adventitia. *Free Radic Biol Med* 2009;**47**:1254–1266.

3. Liu J, Ormsby A, Oja-Tebbe N, Pagano PJ. Gene transfer of NAD(P)H oxidase inhibitor to the vascular adventitia attenuates medial smooth muscle hypertrophy. *CircRes* 2004;**95**:587–594.

4. Cascino T, Csanyi G, Al Ghouleh I, Montezano AC, Touyz RM, Haurani MJ *et al.* Adventitia-derived hydrogen peroxide impairs relaxation of the rat carotid artery via smooth muscle cell p38 mitogen-activated protein kinase. *Antioxid Redox Signal* 2011;**15**:1507–1515.

5. Miller EW, Dickinson BC, Chang CJ. Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. *Proc Natl Acad Sci USA* 2010; **107**:15681–15686.

6. Bienert GP, Moller AL, Kristiansen KA, Schulz A, Moller IM, Schjoerring JK *et al.* Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *J Biol Chem* 2007;**282**:1183–1192.

7. Ushio-Fukai M, Alexander RW, Akers M, Griendling KK. p38 mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy. *J Biol Chem* 1998;**273**:15022–15029.

8. Tobiume K, Matsuzawa A, Takahashi T, Nishitoh H, Morita K, Takeda K *et al.* ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO Rep* 2001;**2**:222–228.

9. Csanyi G, Cifuentes-Pagano E, Al Ghouleh I, Ranayhossaini DJ, Egana L, Lopes LR *et al.* Nox2 B-loop peptide, Nox2ds, specifically inhibits the NADPH oxidase Nox2. *Free Radic Biol Med* 2011;**51**:1116–1125.

10. Song GJ, Barrick S, Leslie KL, Sicari B, Fiaschi-Taesch NM, Bisello A. EBP50 inhibits the anti-mitogenic action of the parathyroid hormone type 1 receptor in vascular smooth muscle cells. *J Mol Cell Cardiol* 2010;**49**:1012–1021.

11. Ellmark SH, Dusting GJ, Fui MN, Guzzo-Pernell N, Drummond GR. The contribution of Nox4 to NADPH oxidase activity in mouse vascular smooth muscle. *Cardiovasc Res* 2005;**65**:495–504.

12. Matsukawa J, Matsuzawa A, Takeda K, Ichijo H. The ASK1-MAP kinase cascades in mammalian stress response. *J Biochem* 2004;**136**:261–265.

13. Tobiume K, Saitoh M, Ichijo H. Activation of apoptosis signal-regulating kinase 1 by the stress-induced activating phosphorylation of pre-formed oligomer. *J Cell Physiol* 2002;**191**:95–104.

14. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 1994;**74**:1141–1148.

15. Miller FJ Jr, Chu X, Stanic B, Tian X, Sharma RV, Davisson RL *et al.* A differential role for endocytosis in receptor-mediated activation of Nox1. *Antioxid Redox Signal* 2010; **12**:583–593.

16. Lacy F, O'Connor DT, Schmid-Sch'nbein GW. Plasma hydrogen peroxide production in hypertensives and normotensive subjects at genetic risk of hypertension. *JHypertens* 1998;**16**:291–303.

17. Varma SD, Devamanoharan PS. Hydrogen peroxide in human blood. *Free Radic Res Commun* 1991;**14**:125–131.

18. Halliwell B, Clement MV, Long LH. Hydrogen peroxide in the human body. *FEBS Lett* 2000;**486**:10–13.

19. Li WG, Miller FJ Jr, Zhang HJ, Spitz DR, Oberley LW, Weintraub NL. H_2O_2-induced O_2 production by a non-phagocytic NAD(P)H oxidase causes oxidant injury. *J Biol Chem* 2001;**276**:29251–29256.

20. Liu X, Zweier JL. A real-time electrochemical technique for measurement of cellular hydrogen peroxide generation and consumption: evaluation in human polymorphonuclear leukocytes. *Free Radic Biol Med* 2001;**31**:894–901.

21. Thomas SR, Chen K, Keaney JF Jr. Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. *J Biol Chem* 2002; **277**:6017–6024.

22. Colston JT, de la Rosa SD, Strader JR, Anderson MA, Freeman GL. H_2O_2 activates Nox4 through PLA2-dependent arachidonic acid production in adult cardiac fibroblasts. *FEBS Lett* 2005;**579**:2533–2540.

23. Witting PK, Rayner BS, Wu BJ, Ellis NA, Stocker R. Hydrogen peroxide promotes endothelial dysfunction by stimulating multiple sources of superoxide anion radical production and decreasing nitric oxide bioavailability. *Cell Physiol Biochem* 2007;**20**: 255–268.

24. Dynowski M, Schaaf G, Loque D, Moran O, Ludewig U. Plant plasma membrane water channels conduct the signalling molecule H2O2. *Biochem J* 2008;**414**:53–61.

25. Herrera M, Garvin JL. Novel role of AQP-1 in NO-dependent vasorelaxation. *Am J Physiol Renal Physiol* 2007;**292**:F1443–F1451.

26. Nadeau PJ, Charette SJ, Landry J. REDOX reaction at ASK1-Cys250 is essential for activation of JNK and induction of apoptosis. *Mol Biol Cell* 2009;**20**:3628–3637.

27. Yamamoto E, Tamaki N, Nakamura T, Kataoka K, Tokutomi Y, Dong YF *et al.* Excess salt causes cerebral neuronal apoptosis and inflammation in stroke-prone hypertensive rats through angiotensin II-induced NADPH oxidase activation. *Stroke* 2008;**39**:3049–3056.

28. Liu J, Yang F, Yang XP, Jankowski M, Pagano PJ. NAD(P)H oxidase mediates angiotensin II-induced vascular macrophage infiltration and medial hypertrophy. *Arterioscler Thromb Vasc Biol* 2003;**23**:776–782.

29. Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D *et al.* Cell transformation by the superoxide-generating oxidase Mox1. *Nature* 1999;**401**:79–82.

30. Li J, Niu XL, Madamanchi NR. Leukocyte antigen-related protein tyrosine phosphatase negatively regulates hydrogen peroxide-induced vascular smooth muscle cell apoptosis. *J Biol Chem* 2008;**283**:34260–34272.