Insulin inhibits Na\(^+\)/H\(^+\) exchange in vascular smooth muscle and endothelial cells in situ: involvement of H\(_2\)O\(_2\) and tyrosine phosphatase SHP-2

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Boedtkjer E, Aalkjaer C. Insulin inhibits Na\(^+\)/H\(^+\) exchange in vascular smooth muscle and endothelial cells in situ: involvement of H\(_2\)O\(_2\) and tyrosine phosphatase SHP-2. Am J Physiol Heart Circ Physiol 296: H247–H255, 2009. First published November 26, 2008; doi:10.1152/ajpheart.00725.2008—Insulin signals through several intracellular pathways. Here, we tested the hypothesis that insulin modulates Na\(^+\)/H\(^+\) exchange (NHE) activity in vascular cells through H\(_2\)O\(_2\)-mediated inhibition of tyrosine phosphatase Src homology 2 domain containing tyrosine phosphatase 2 (SHP-2). We measured intracellular pH (pHi) in isolated mouse mesenteric arteries using fluorescence confocal and wide-field microscopy. In the absence of CO\(_2\)/HCO\(_3\)\(^-\) solution, removal of both Na\(^+\) produced endothelial acidification (ΔpHi = 0.71 ± 0.12) inhibited by cariporide. Cariporide reduced endothelial steady-state pH\(_i\) (ΔpHi = 0.28 ± 0.08). Insulin and H\(_2\)O\(_2\) acidified endothelial cells 0.2–0.3 pH units and reduced the acidification upon Na\(^+\) removal by ~65%. Cariporide abolished the effect of insulin and H\(_2\)O\(_2\). In vascular smooth muscle cells, H\(_2\)O\(_2\) produced intracellular acidification (ΔpHi = −0.48 ± 0.06) as did high concentrations of insulin (ΔpHi = −0.03 ± 0.01). NHE activity after an NH\(_4\)\(^+\) prepulse was ~80% attenuated by H\(_2\)O\(_2\) and ~40% by high insulin concentrations. H\(_2\)O\(_2\) had no effect on Na\(^+\)/HCO\(_3\)-cotransporter activity. NHE1 (sle9a1) was the only plasma membrane NHE isoform detected in mouse mesenteric arteries by RT-PCR analyses. In both cell types, polyethylene glycol catalase abolished the effect of insulin on pHi. Exposure to insulin increased the intracellular concentration of reactive oxygen species estimated with the fluorophore 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein. The SHP-2 selective inhibitor NSC-87877 and protein tyrosine phosphatase (PTP) inhibitor IV reduced steady-state pH\(_i\) up to 0.3 pH units and inhibited NHE activity 60–80%; when applied in combination with insulin or H\(_2\)O\(_2\), no further effect was obtained. We conclude that NHE contributes to pH\(_i\) regulation in arterial endothelial and smooth muscle cells in situ and is inhibited by insulin and H\(_2\)O\(_2\). We propose that insulin signaling involves H\(_2\)O\(_2\) and inhibition of PTP SHP-2.

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INTRACELLULAR pH (pHi) in cells of the vascular wall has prominent effects on many aspects of vascular biology. In vascular smooth muscle cells (VSMCs), acute acidification results in contraction (2), whereas more prolonged acidification has been suggested to reduce the Ca\(^{2+}\) sensitivity of the contractile machinery (9). In endothelial cells (ECs), pH\(_i\) may affect the activity of nitric oxide synthase (16) and endothelin-converting enzyme (4) involved in the synthesis of vasoactive factors. Furthermore, the pH\(_i\) regulating Na\(^+\)/H\(^+\) exchangers (NHEs) are purportedly involved in the development of endothelial dysfunction resulting from hypoxia (8, 32) and may influence the process of apoptosis (14). NHEs are functionally present in VSMCs (9) and cultured ECs (15) and by extruding protons in exchange for Na\(^+\) defend cells against intracellular acidification. These transporters also play a role for regulation of cell volume, proliferation, and migration (15, 27).

The in vitro application of insulin and insulin-like substances has been shown to affect pH\(_i\) by modulating the activity of NHEs in a number of cell types, including adipocytes (5), erythrocytes (25, 28, 30), and hepatocytes (11). Although most studies suggest activation of NHEs (5, 28, 30), a few studies propose inhibition (11, 25). Insulin receptors are known to be expressed in VSMCs (33) and ECs (6) but whether insulin has an effect on NHE activity in these cells is unknown. The intracellular signaling pathways associated with insulin receptor activation are currently being elucidated, and, during the last three decades, it has become increasingly clear that the generation of reactive oxygen species (ROS) plays an important role for insulin signaling (18). In a number of cell types, including cultured VSMCs, H\(_2\)O\(_2\) has been shown to be present in increased amounts during insulin exposure, and exogenous H\(_2\)O\(_2\) has been shown to have insulin-like effects (18, 38). In addition to their putative role as second messengers, ROS are generated in increased amounts during inflammation (35) and hypoxia reoxygenation events (21) where endothelial pH\(_i\) regulation is also affected (17). Interestingly, H\(_2\)O\(_2\) has been reported to affect pH\(_i\) by inhibiting (20, 31) or activating (29) NHEs. The actions of H\(_2\)O\(_2\) are widespread, but one important action appears to be the inhibition of protein tyrosine phosphatases (PTPs) through stepwise oxidation of a critical cysteine residue at the catalytic site (18). PTPs make up a large family of enzymes involved in many cellular signaling pathways, and a recent study suggests that PTPs also affect the regulation of pHi (37). The Src homology 2 domain containing tyrosine phosphatase 2 (SHP-2) was shown to interact physically with NHE1, and overexpression of SHP-2 in BAF3 cells resulted in increased NHE activity (37). SHP-2 expression has previously been found to be abundant in VSMCs (3) and ECs (23).

In the past, studies on isolated arteries have provided important information about pH\(_i\) regulation in VSMCs (1, 9). Technical difficulties, however, have largely restricted the study of EC pH\(_i\) regulation to cultured cells. In the present study, we used a new approach employing fluorescence confocal and wide-field microscopy to evaluate the impact of insulin and H\(_2\)O\(_2\) on pH\(_i\) regulation in ECs and VSMCs of isolated mouse mesenteric arteries. More specifically, we tested the hypothesis that insulin modulates NHE activity in arterial VSMCs and ECs in situ through H\(_2\)O\(_2\)-mediated inhibition of the PTP SHP-2.
A better understanding of the biological effects of insulin and H$_2$O$_2$ is essential considering the involvement of metabolic dysfunction and oxidative stress in vascular disease.

**MATERIALS AND METHODS**

Male Naval Medical Research Institute mice (7–11 wk old; Taconic Europe) were killed by cervical dislocation. The whole mesenteric bed was placed in cold physiological salt solution (PSS), and mesenteric arteries were dissected free from surrounding connective tissue. All animal protocols were approved by the Institutional Animal Care and Use Committee in accordance with the licenses for the care and use of experimental animals issued by the Danish Ministry of Justice.

**Measurement of pHi in ECs in situ.** Isolated arteries were turned inside-out to expose the endothelial surface, mounted in a confocal wire-myograph (DMT) and kept at 37°C. Norepinephrine-precontracted inverted arteries maintained normal endothelium-dependent relaxation to 5 μmol/l acetylcholine (88 ± 5%; n = 3) compared with arteries in normal configuration (83 ± 5%; n = 9; P < 0.62). After being loaded with 5 μmol/l 2′,7′-bis-(carboxypropyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Invitrogen) in 0.02% dimethyl sulfoxide (DMSO) for 0.5 h, the arteries were investigated using the NO synthase inhibitor (12), and we tested the involvement of NO in the response to insulin (7). Nitric oxide (NO) is potentially also degraded by catalase to improve its cellular uptake, resulting in increased cellular catalase activity.

**Effect of insulin, H$_2$O$_2$, and SHP-2 on pHi.** We investigated the effect of insulin (Sigma) and H$_2$O$_2$, on steady-state pHi of ECs and VSMCs in the presence and absence of CO$_2$/HCO$_3$. For VSMCs, NH$_4$$_2$ prepulse experiments were performed in the absence of CO$_2$/HCO$_3$ to estimate the NHE activity. NH$_4$Cl (20 mmol/l) was washed out in a Na$^+$-free solution. When, subsequently, Na$^+$ was added to the bath solution, we recorded the Na$^+$-dependent and HCO$_3$-independent pHi recovery rate, which in the VSMCs is inhibited by 600 μmol/l amiloride (9). The Na$^+$-HCO$_3$ cotransport activity was estimated by similar NH$_4$$_2$ prepulse experiments performed in the presence of CO$_2$/HCO$_3$ and 600 μmol/l amiloride (9).

The ECs in our preparation released vesicles and lost their dye when exposed to concentrations of NH$_4$Cl higher than 5 mmol/l; hence, the NH$_4$$_2$ prepulse technique could not be reliably employed for measuring pHi recovery rates in ECs. Instead, we investigated the NHE activity by removing and subsequently adding bath Na$^+$ in the nominal absence of CO$_2$/HCO$_3$.

To test the involvement of H$_2$O$_2$ in the response to insulin, we used the H$_2$O$_2$ scavenger polyethylene glycol catalase (PEG-catalase; 250 U/ml; Sigma). The conjugation of catalase with PEG has previously been shown to decrease its sensitivity to proteolytic degradation and improve its cellular uptake, resulting in increased cellular catalase activity (7). Nitric oxide (NO) is potentially also degraded by catalase (12), and we tested the involvement of NO in the response to insulin using the NO synthase inhibitor N-nitro-L-arginine methyl ester (LNAME; 100 μmol/l; Sigma). Amiloride (600 μmol/l; Sigma) was used as an unspecific inhibitor of NHE, whereas cariporide (1 μmol/l; Aventis Pharma) was used to selectively inhibit NHE1 (24). To inhibit SHP-2, we used NSC-87877 (Calbiochem) at a concentration (30 μmol/l) that has previously been shown to inhibit Erk activation dependent on SHP-2 in Hek293 cells (13). NSC-87877 absorbs parts of the fluorescence spectrum. When pHi was clamped using the high-K$^+$ nigericin technique, NSC-87877 had only a minor effect on the fluorescence ratio (−2%), and we corrected the measurements accordingly. PTP inhibitor IV was used as an alternative inhibitor of SHP-2 at a concentration (2 μmol/l) previously shown to be effective (23).

**Buffering capacity and flux calculations.** We calculated the intracellular buffering capacity of ECs and VSMCs from the acidification seen upon washout of NH$_4$Cl as previously described (9). In experiments on ECs, the concentration of NH$_4$Cl was reduced to 5 mmol/l. In VSMCs, the average buffering capacity in the absence of CO$_2$/HCO$_3$ was 49 mmol/l at pHi 6.96 similar to previous findings in mouse smooth muscle cells (9, 10). Insulin, H$_2$O$_2$, PEG-catalase, NSC-87877, and PTP inhibitor IV had no significant effect on intracellular buffering capacity, allowing for direct comparison of pHi recovery rates. The rate of pHi recovery in VSMCs was calculated at multiple points over the relevant pHi range and presented as a function of the corresponding pHi values. In ECs, we estimated an intrinsic buffering capacity of 11 mmol/l at pHi 7.0. Because of low time resolution of these measurements, we did not calculate pHi recovery rates at multiple pHi values. Instead, the average net H$^+$ flux was calculated as the product of the intracellular buffering capacity and the pHi recovery rate at a single pHi value.

**Measurement of intracellular ROS concentrations.** The intracellular concentration of ROS was estimated using the fluorophore 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H$_2$DCF-DA; Invitrogen). Inverted arteries were loaded with 5 μmol/l CM-H$_2$DCF-DA in 0.02% DMSO for 30 min at 37°C. Confocal microscopy with a ×40 objective (LD C-Apochromat; NA 1.10; Zeiss) was performed with excitation at 488 nm and emission light collected at wavelengths longer than 515 nm. To estimate the relative contributions from NO and ROS, we performed control experiments in the presence of PEG-catalase and l-NAME. To rule out any effect of light-induced fluorescence changes, we performed experiments where no insulin was added. After background subtraction, the measured emissions were normalized to the initial fluorescence level.

**RT-PCR analyses.** The expression of NHEs in mouse mesenteric arteries was investigated at the mRNA level using RT-PCR analysis. We designed primers against the NHE isoforms reported to be restricted to intracellular organelles (NHE6, NHE7, and NHE9) or to be pseudogenes (26, 27). The primer sequences used were as follows: NHE1: forward (F) 5′-GAG CAG AGA CC-3′ and reverse (R) 5′-TGG CAA GTG -3′; NHE2: F 5′-CAC ACA CAA CAT CCG GGT CA-3′, R 5′-AGA AGG CCA GGA TGA AGC AG-3′; NHE3: F 5′-TGG CAG GAG CAG AGC AG-3′, R 5′-CCA CAG GGT TAA TGG CCA GG-3′; NHE4: F 5′-AGA AGG CCA GGA TGA AGC AG-3′, R 5′-TGG CAG AAG AGC AGA GTG AG-3′; NHE5: F 5′-GGA AGG CAA CAT CTC CCA TAA G-3′, R 5′-GGG GGA TGA CGA CAA AGG-3′; NHE8: F 5′-TGC TAC GGG GGA TAC TTG TCA AG-3′, R 5′-TGG TGG TCA TCA TCA TCA TCA TCA TCA GAG CTT GG-3′. Primer synthesis was performed by MWG Biotech (Germany). Kidney or brain samples were used as positive controls. RNA isolation was performed using an RNeasy minikit (Qiagen). After DNase treatment, total RNA was reverse transcribed using random decamers and SuperScript III (Invitrogen). In RT-controls, the reverse transcriptase was omitted. In subsequent PCR reactions, the following cycles were run: 1 cycle at 94°C for 1 min; 35 cycles at 94°C for 30 s, 58°C for 45 s, and 72°C for 1 min; 1 cycle at 72°C for 5 min. Gel electrophoresis of PCR products was performed on 2% agarose gels. DNA was visualized with ethidium bromide and photographed using ultraviolet illumination.

**Solutions.** The composition of the HCO$_3$-containing PSS was as follows (in mmol/l): 139 Na$^+$, 5.5 K$^+$, 1.6 Ca$^{2+}$, 1.2 Mg$^{2+}$, 122 Cl$^-$, 25 HCO$_3$-, 1.2 SO$_4^{2-}$, 1.2 H$_2$PO$_4^{−}$, 10 HEPES, 5.5 glucose, and 0.026

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EDTA. In Na⁺-free solutions, Na⁺ was substituted with an equimolar amount of N-methyl-d-glucammonium, except for NaHCO₃, which was replaced with choline-HCO₃. In the HCO₃⁻-free PSS, HCO₃⁻ was replaced with an equimolar amount of Cl⁻. HCO₃⁻-containing solutions were bubbled with 5% CO₂ in air, whereas HCO₃⁻-free solutions were bubbled with air; pH was adjusted to 7.40 at 37°C.

Statistics and experimental design. Data are expressed as means ± SE. Paired two-tailed Student’s t-test was used for comparing two interventions on the same arteries, whereas more than two interventions were compared by one-way ANOVA for repeated measures followed by a Dunnett or Bonferroni posttest. Two-way ANOVA for repeated measures followed by a Bonferroni posttest was used to compare successive interventions between groups. The pHi recovery rates were fitted as a function of pHi with a one-phase exponential decay function, and the resulting parameters were compared. P < 0.05 was considered statistically significant; n equals the number of mice (one artery from each animal). Statistical analyses were performed using GraphPad Prism 4.02 software.

RESULTS

Typically, both the EC and the VSMC layer were loaded with BCPCF. Light emitted from the two cell layers could, however, be distinctly separated and endothelial pHi recorded (Fig. 1).

NHE regulates endothelial pHᵢ. The removal of bath Na⁺ in the absence of CO₂/HCO₃ resulted in strong intracellular acidification (ΔpHi = −0.71 ± 0.12; P < 0.01; Fig. 1B) consistent with the presence of an Na⁺-dependent and HCO₃⁻-independent acid extrusion mechanism. An original trace is shown in Supplemental Fig. S1 (Supplemental data for this article can be found on the American Journal of Physiology: Heart and Circulatory Physiology website.). Addition of 600 μmol/l amiloride in the absence of CO₂/HCO₃ produced a significant, yet smaller, decrease in pHᵢ (ΔpHi = −0.27 ± 0.05; P < 0.01; Fig. 1C). When bath Na⁺ was removed in the presence of amiloride, no acidification was seen (ΔpHi = 0.00 ± 0.05; P = 1.00; Fig. 1C). Taken together, these results strongly suggest that amiloride-sensitive NHE is involved in pHᵢ regulation in arterial ECs in situ. The difference between the acidification seen after removal of bath Na⁺ and after addition of amiloride is likely explained by a reversal of NHE after extracellular Na⁺ removal as opposed to inhibition of the transport after addition of amiloride.

We investigated the expression of different NHE isoforms in isolated arteries using RT-PCR. As shown in Fig. 2A, only NHE1 was detected at the mRNA level. The importance of NHE1 was supported by the finding that 1 μmol/l cariporide reported to be selective for NHE1 (24) reduced steady-state pHi (ΔpHi = −0.27 ± 0.07; n = 8; P < 0.01) in the absence of CO₂/HCO₃. Furthermore, in the presence of 1 μmol/l cariporide, no acidification was seen upon removal of bath Na⁺ (P < 0.05 vs. control; Fig. 2B).

Insulin induces endothelial acidification by inhibition of NHE. Addition of insulin resulted in a concentration-dependent decrease in endothelial pHi in the presence as well as in the absence of CO₂/HCO₃ (Fig. 3A). Under both conditions, prior application of 600 μmol/l amiloride completely abolished the acidification, as illustrated in Fig. 4, A and B for recordings made in the absence of CO₂/HCO₃. Similarly, 1 μmol/l cariporide inhibited the effect of insulin on steady-state pHᵢ (Fig. 4, A and B). Furthermore, insulin concentration-dependently inhibited the magnitude of the acidification seen upon removal of bath Na⁺ in the absence of CO₂/HCO₃ (Fig. 3, B and D). Also, the rate of acidification and the rate of recovery were significantly slowed by the presence of insulin (Fig. 3, B and D). Taken together, these results strongly suggest that insulin induces intracellular acidification through inhibition of NHE.

H₂O₂ induces endothelial acidification by inhibition of NHE and is required for the effects of insulin on endothelial pHᵢ. Exogenous H₂O₂ (100 μmol/l) produced endothelial acidification (ΔpHi = −0.22 ± 0.03; n = 8; P < 0.001) when applied in the absence of CO₂/HCO₃. This acidification was inhibited by the prior addition of 600 μmol/l amiloride (ΔpHi = −0.01 ± 0.05; n = 4; P < 0.01) or 1 μmol/l cariporide (ΔpHi = −0.03 ± 0.04; n = 3; P < 0.01), suggesting an effect via NHE. In the presence of 100 μmol/l H₂O₂, the rate and extent of acidification after removal of bath Na⁺ and the rate of

![Fig. 1. Na⁺/H⁺ exchange (NHE) is functionally present in arterial endothelial cells (ECs) in situ. A: confocal image of 2',7'-bis-(carboxypropyl)-5(6)-carboxyfluorescein (BCPCF)-loaded ECs in the wall of an isolated artery. Scale bar: 20 μm. B: average response (n = 5) of ECs to removal and readdition of bath Na⁺ in the absence of CO₂/HCO₃. C: average response (n = 5) of ECs to addition of 600 μmol/l amiloride and subsequent removal and readdition of bath Na⁺ in the absence of CO₂/HCO₃.](http://ajpheart.physiology.org/content/296/2/210/figurecards/210_F1)
recovery after readdition of Na\(^+\) were significantly smaller than in the absence of H\(_2\)O\(_2\) (Fig. 3, C–E).

The similar effects of H\(_2\)O\(_2\) and insulin led us to hypothesize that insulin inhibits NHE through production of H\(_2\)O\(_2\). Indeed, the acidification to insulin was completely inhibited by 250 U/ml of the H\(_2\)O\(_2\) scavenger PEG-catalase (Fig. 4, A and B). Also, 80 nmol/l insulin had no effect on the rate and extent of intracellular acidification upon Na\(^+\) removal or the rate of pH\(_i\) recovery after Na\(^+\) readdition when PEG-catalase was present in the chamber (Fig. 4, C–E).

Catalase has been proposed to degrade NO in addition to H\(_2\)O\(_2\) (12). We therefore investigated whether NO is involved in the insulin-induced changes in NHE activity. In four experiments, the change of pH\(_i\) upon Na\(^+\) removal was 0.65 ± 0.17 under control conditions, 0.19 ± 0.12 in the presence of 80 nmol/l insulin, and −0.14 ± 0.15 in the combined presence

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**Fig. 2.** NHE1 is the only plasma membrane NHE isoform detected by RT-PCR in isolated mouse mesenteric arteries, and the NHE1 selective inhibitor cariporide inhibits endothelial acidification upon Na\(^+\) removal. A: agarose gels showing the results of RT-PCR analyses. + and −, RT+ and RT−, respectively. B: average traces (n = 4) showing the response of ECs in situ to Na\(^+\) removal in the presence and absence of 1 μmol/l cariporide. Experiments were performed in the absence of CO\(_2\)/HCO\(_3^−\).

**Fig. 3.** Insulin and H\(_2\)O\(_2\) induce intracellular acidification and inhibit NHE in arterial ECs in situ. A: average response of ECs to increasing concentrations of insulin. Measurements were performed in the presence (n = 5) and in the absence (n = 9) of CO\(_2\)/HCO\(_3^−\). B: average response (n = 6) of ECs to removal and readdition of bath Na\(^+\) in the presence of increasing concentrations of insulin. Experiments were performed in the absence of CO\(_2\)/HCO\(_3^−\). C: average response (n = 6) of ECs to removal and readdition of bath Na\(^+\) in the presence and absence of 100 μmol/l H\(_2\)O\(_2\). Experiments were performed in the absence of CO\(_2\)/HCO\(_3^−\). D: average net H\(^+\) flux (n = 6) upon Na\(^+\) removal and Na\(^+\) readdition. E: magnitude of acidification (n = 6) recorded 6 min after Na\(^+\) removal. *P < 0.05 and **P < 0.01 vs. control. The treated groups were compared with their paired controls with a one-way ANOVA followed by a Dunnett posttest or with a paired Student’s t-test.
of 80 nmol/l insulin and 100 μmol/l L-NAME. Thus the effect of insulin on EC pH was unaffected by prior inhibition of NO synthesis (P = 0.54).

H₂O₂ and insulin acidify VSMCs. In VSMCs, 80 nmol/l insulin had no effect on steady-state pH in the absence of CO₂/HCO₃⁻ (ΔpHᵢ = 0.00 ± 0.00; n = 4; P = 0.61). Application of 800 nmol/l insulin produced significant acidification (ΔpHᵢ = −0.03 ± 0.01; n = 7; P < 0.01; Fig. 5A).

Addition of 100 μmol/l H₂O₂ resulted in intracellular acidification of VSMCs (Fig. 5B). In the presence of CO₂/HCO₃⁻, exposure to H₂O₂ for 15 min decreased pH by 0.19 ± 0.03 pH units (n = 6), and this effect was completely reversible upon washout (Fig. 5B). In the nominal absence of CO₂/HCO₃⁻, even larger acidification (ΔpHᵢ = −0.43 ± 0.07; n = 5; P < 0.001) was produced (Fig. 5B).

H₂O₂ and insulin inhibit NHE in VSMCs. To determine the effect of H₂O₂ and insulin on NHE activity in VSMCs, we performed NH₄⁺ prepulse experiments in the presence and absence of these agents. The experiments were performed in the absence of CO₂/HCO₃⁻ to rule out any contribution from HCO₃⁻-dependent transporters. An original trace is shown in Supplemental Fig. S2. Washout of NH₄Cl produced intracellular acidification, and the subsequent pHᵢ recovery was studied.

Insulin (80 nmol/l) inhibited NHE activity in VSMCs (P < 0.05; Fig. 6B) while a considerably larger effect was seen with 800 nmol/l insulin (P < 0.001; Fig. 6, A and B). The inhibitory effect of 800 nmol/l insulin on NHE activity was completely abolished in the presence of 250 U/ml PEG-catalase (P < 0.001 vs. 800 nmol/l insulin; Fig. 6B).

In VSMCs, H₂O₂ concentration-dependently inhibited the Na⁺-dependent and HCO₃⁻-independent pHᵢ recovery after an NH₄⁺ prepulse with an IC₅₀ of ~40 μmol/l (Fig. 6, C and D).
Taken together, these results strengthen the hypothesis that insulin-mediated effects on NHE activity are mediated via H$_2$O$_2$ in cells of the vascular wall.

**H$_2$O$_2$ does not affect Na$^+$/HCO$_3$-cotransport activity in VSMCs.** Under physiological conditions, acid extrusion from VSMCs takes place partly via NHE and partly via Na$^+$/HCO$_3$-cotransport (9). Na$^+$/HCO$_3$-cotransport activity was calculated from the Na$^+$/HCO$_3$-dependent pHi recovery after an intracellular acid load in the presence of CO$_2$/HCO$_3$ and 600 μmol/l amiloride (Fig. 7A). Below pHi 6.8, the NHEs were the dominant acid extruders, but in the pH range >6.8, acid extrusion via Na$^+$/HCO$_3$ cotransport was comparable to or greater than acid-extrusion via NHE (Figs. 6D and 7B). Furthermore, H$_2$O$_2$ had no effect on the Na$^+$/HCO$_3$ cotransport activity ($P=0.36$; Fig. 7, A and B). The maintained Na$^+$/HCO$_3$ cotransport activity likely explains the attenuated steady-state pHi response to H$_2$O$_2$ in the presence of CO$_2$/HCO$_3$ (Fig. 5B).

**ROS are present in increased amounts during insulin exposure.** We assessed the intracellular ROS concentration using the fluorophore CM-H$_2$DCF. In ECs in situ, we found an increase in CM-H$_2$DCF fluorescence during exposure to 80 nmol/l insulin (Fig. 8, A and C). This increase was not observed when no insulin was added ($P=0.01$) and was completely abolished by the prior application of 250 U/ml PEG-catalase ($P=0.01$; Fig. 8, A and C). CM-H$_2$DCF dis-
plays some cross-reactivity with NO; however, the CM-H$_2$DCF response to insulin was unaffected by preincubation with 100 $\mu$mol/l L-NAME ($P = 0.75$; Fig. 8, A and C).

In VSMCs, the increase in ROS concentration upon exposure to 80 nmol/l insulin was less pronounced, but a significant increase in CM-H$_2$DCF fluorescence was observed compared with experiments where no insulin was added ($P < 0.05$) and experiments performed in the presence of 250 $\mu$mol/l PEG-catalase ($P < 0.05$; Fig. 8, B and C). Again, 100 $\mu$mol/l L-NAME had no effect on the increase in CM-H$_2$DCF fluorescence ($P = 0.67$; Fig. 8, B and C).

Inhibition of SHP-2 reduces NHE activity and steady-state pHi in VSMCs. The tyrosine phosphatase SHP-2 has recently been shown to interact physically with NHE1, and overexpression of SHP-2 in BAF3 cells increased NHE activity (37). Additionally, SHP-2 isolated from cultured ECs and other sources has been shown to be inhibited by H$_2$O$_2$ with an IC$_{50}$ around 75 $\mu$mol/l (34, 39) similar to the concentration range inhibiting NHE activity in the present study. We therefore hypothesized that H$_2$O$_2$ reduces NHE activity through inhibition of SHP-2 and investigated the effect of the SHP-2 selective inhibitors NSC-87877 and PTP inhibitor IV. In VSMCs, 30 $\mu$mol/l NSC-87877 and 2 $\mu$mol/l PTP inhibitor IV reduced NHE activity significantly ($P < 0.001$; Fig. 9A). The effect of the inhibitors was completely abolished in the absence of H$_2$O$_2$ ($P = 0.67$; Fig. 9B).

NSC-87877 ($\Delta$pHi = $-0.16 \pm 0.06$; $n = 6$; $P < 0.05$) and PTP inhibitor IV ($\Delta$pHi = $-0.29 \pm 0.07$; $n = 4$; $P < 0.05$) both reduced steady-state pH$_i$ of VSMCs.

Inhibition of SHP-2 reduces NHE activity in ECs. Similar to the effects in VSMCs, 30 $\mu$mol/l NSC-87877 and 2 $\mu$mol/l PTP inhibitor IV produced significant inhibition of NHE activity in ECs in situ. The extent and rate of acidification upon removal of bath Na$^+$ and the recovery upon readdition of Na$^+$ were reduced in the presence of the SHP-2 inhibitors (Fig. 9, C–F). In the presence of the SHP-2 inhibitors, insulin had no effect on NHE activity (Fig. 9, C–F).

Taken together, the SHP-2 inhibitor results support the hypothesis that insulin and H$_2$O$_2$ affect NHE activity via SHP-2 inhibition.

**DISCUSSION**

In the present study, we investigated the role and regulation of NHE by insulin and H$_2$O$_2$ in VSMCs and ECs of isolated mouse mesenteric arteries. Using confocal fluorescence microscopy, light emitted from ECs and VSMCs was separated, and pH$_i$ regulation was studied.

In ECs, insulin concentrations close to physiological levels and within the plasma concentration range of type 2 diabetes (11, 22, 36) produced intracellular acidification and inhibition of NHE. In VSMCs, higher concentrations of insulin were needed, and smaller effects on pH$_i$ and NHE activity were evident. The pH$_i$ effects of insulin were mimicked by H$_2$O$_2$ and inhibited by the H$_2$O$_2$ scavenger PEG-catalase. Furthermore, the concentration of ROS evaluated as CM-H$_2$DCF fluorescence was significantly increased during insulin exposure. Our results support previous suggestions that H$_2$O$_2$ serves as a second messenger for insulin (18) and expand our understanding of this phenomenon to pH$_i$ regulation and vascular cells.

A greater increase in CM-H$_2$DCF fluorescence was recorded in ECs compared with VSMCs after exposure to insulin. This apparent difference in ROS generation likely explains the higher concentrations of insulin needed to affect NHE activity in VSMCs compared with ECs. This view is further supported by the strong response of VSMCs to exogenous H$_2$O$_2$.

Intriguingly, ROS have been detected in increased amounts during hypoxia-reoxygenation events (21) when increased Na$^+$ influx through NHEs purportedly contributes to intracellular Ca$^{2+}$ overload and cellular damage (19). Inhibition of NHEs under these conditions seems favorable (8, 32), and, consequently, H$_2$O$_2$-mediated inhibition of NHE could play an important role as a cellular defense mechanism.

In previous studies, H$_2$O$_2$ has been shown to activate (29) or inhibit (20) NHEs, but the link between increased amounts of H$_2$O$_2$ and changes in NHE activity is not well defined. H$_2$O$_2$ has been suggested to inhibit NHEs in cultured ECs through the induction of DNA damage causing a reduced cellular ATP concentration (20), whereas activation of NHEs in cardiac myocytes has been suggested to result from increased activity of the mitogen-activated protein kinase (29). Here, we inves-
tigated a possible pathway via SHP-2, a PTP that is highly expressed in the vasculature (3, 23). SHP-2 was recently reported to interact physically with NHE1 and cause increased NHE activity when overexpressed in BAF3 cells (37). The concentrations of H2O2 used in the present study have previously been shown to inhibit the activity of purified SHP-2 in vitro (34, 39). We show that the SHP-2 selective inhibitors NSC-87877 and PTP inhibitor IV reduce NHE activity in vascular cells. When applied in combination with insulin or H2O2, no further effect was obtained, suggesting that these substances work through the same cellular pathway.

Our results provide evidence for a novel signaling pathway linking insulin and H2O2 exposure to decreased NHE activity through a redox-induced change in intracellular phosphatase activity.

We have previously shown that Na+/HCO3− cotransport mediated by NBCn1 (slc4a7) plays an important role for regulation of pHi in VSMCs (9, 10). In the current study, we find no effect of H2O2 on the Na+/HCO3− cotransport activity.

Consequently, during conditions of oxidative stress when the NHE activity is inhibited, the relative quantitative importance of Na+/HCO3− cotransport is increased.

In conclusion, we propose that insulin-stimulated H2O2 production results in decreased NHE activity in VSMCs and ECs through inhibition of SHP-2. These functional interactions are intriguing considering the effect of pHi on cellular and vascular functions and the involvement of metabolic disturbances and oxidative stress in the development of human disease and vascular dysfunction.

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