Hydrogen Peroxide Decreases pH\textsubscript{i} in Human Aortic Endothelial Cells by Inhibiting Na\textsuperscript{+}/H\textsuperscript{+} Exchange

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Abstract—Postischemic endothelial dysfunction may occur as a result of the effects of endogenous oxidants like hydrogen peroxide. Since endothelium-dependent vasodilator function may be affected by pH, the effect of hydrogen peroxide on endothelial pH\textsubscript{i} was examined. Hydrogen peroxide (100 μmol/L for 10 minutes) decreased pH\textsubscript{i} from 7.24±0.01 to 7.02±0.02 and inhibited recovery from an ammonium chloride–induced intracellular acid load in carboxy SNARF 1 (c-SNARF 1)–loaded human aortic endothelial cells in bicarbonate-free solution. Prior inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchange with 5-(N-ethyl-N-isopropyl)amiloride (10 μmol/L), by removal of extracellular Na\textsuperscript{+}, or by glycolytic inhibition with iodoacetic acid blocked the subsequent effect of hydrogen peroxide on pH\textsubscript{i}. A 2-minute exposure to 100 μmol/L H\textsubscript{2}O\textsubscript{2} decreased intracellular ATP levels by ∼40%; this was prevented by 3-aminobenzamide and nicotinamide (1 mmol/L each), inhibitors of the DNA repair enzyme poly(ADP-ribose) polymerase. Both 3-aminobenzamide and nicotinamide significantly inhibited the hydrogen peroxide–induced intracellular acidification and the effect of hydrogen peroxide on recovery from an intracellular acid load. Hydrogen peroxide decreases pH\textsubscript{i} in human endothelial cells by inhibiting Na\textsuperscript{+}/H\textsuperscript{+} exchange. This appears to be mediated by activation of the DNA repair enzyme poly(ADP-ribose) polymerase. Both 3-aminobenzamide and nicotinamide significantly inhibited the hydrogen peroxide–induced intracellular acidification and the effect of hydrogen peroxide on recovery from an intracellular acid load. Hydrogen peroxide decreases pH\textsubscript{i} in human endothelial cells by inhibiting Na\textsuperscript{+}/H\textsuperscript{+} exchange. This appears to be mediated by activation of the DNA repair enzyme poly(ADP-ribose) polymerase.

Key Words: pH\textsubscript{i} ■ endothelium ■ Na\textsuperscript{+}/H\textsuperscript{+} exchange ■ free radical

An abnormal endothelium-dependent vasodilator response occurs as a consequence of ischemia and reperfusion. The endothelial dysfunction of ischemia/reperfusion (I/R) injury is at least in part mediated by oxidants and oxygen-derived free radicals, such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and the superoxide anion (O\textsubscript{2}−· ). The endothelium in vivo may be exposed to H\textsubscript{2}O\textsubscript{2} as a consequence of the dismutation of O\textsubscript{2}−· or from the products of polymorphonuclear leukocytes or monocytes that accumulate in the blood vessel wall as a consequence of I/R. Previous studies have shown that exogenous H\textsubscript{2}O\textsubscript{2} attenuates the endothelium-dependent vasodilator response of blood vessels in situ and of isolated coronary arteries in vitro, but the intracellular signaling pathways responsible for this effect are unknown.

H\textsubscript{2}O\textsubscript{2} decreases pH\textsubscript{i} in several cell types, including isolated rat cardiac myocytes, cultured rat cardiac myoblasts, and renal epithelial cells. In renal epithelial cells, this effect appears to be due, at least in part, to inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchanger activity. The related compound tert-butyl hydroperoxide has been shown to decrease Na\textsuperscript{+}/H\textsuperscript{+} antiport activity in bovine pulmonary artery endothelial cells. Oxidants like H\textsubscript{2}O\textsubscript{2} decrease cellular energy stores and induce DNA strand breaks in vascular endothelial cells. Within several minutes of exposure to micromolar concentrations of H\textsubscript{2}O\textsubscript{2}, activation of the nuclear DNA repair enzyme poly(ADP-ribose) polymerase (PARS, EC 2.4.2.30) occurs, which catalyzes the transfer of the ADP-ribosyl moiety of NAD\textsuperscript{+} to DNA. PARS activation rapidly decreases cellular NAD\textsuperscript{+} and, subsequently, ATP levels. The rapid intracellular ATP depletion induced by oxidants like H\textsubscript{2}O\textsubscript{2} may result from PARS activation, since it can be prevented by PARS inhibitors in type II pneumocytes. We hypothesized that H\textsubscript{2}O\textsubscript{2} decreases endothelial pH by inhibiting the ATP-dependent Na\textsuperscript{+}/H\textsuperscript{+} exchanger through a pathway involving PARS activation. The present study reports that H\textsubscript{2}O\textsubscript{2} stimulates intracellular acidification and ATP depletion in vascular endothelial cells and that this may be prevented by PARS inhibition.

Materials and Methods

Culture of Human Aortic Endothelial Cells

Human aortic endothelial cells (HAECs) were obtained as proliferating quaternary cultures (Clonetics) and were grown to confluence at passages 5 to 9 in endothelial cell growth medium supplemented with 2% FBS, 10 μg/L human recombinant epidermal growth factor, 1 ng/mL hydrocortisone, 50 μg/mL gentamicin, 50 ng/mL amphotericin B, and 12 μg/mL bovine brain extract (Clonetics) in a 37°C humidified atmosphere of 95% air/5% CO\textsubscript{2}. To examine the effects of H\textsubscript{2}O\textsubscript{2} on HAEC pH\textsubscript{i}, HAECs were plated at an approximate
concentration of \(1 \times 10^6\) cells/mL and grown to \(70\%\) confluence on 25-mm-diameter circular glass coverslips (VWR Scientific) precoated with 2% gelatin solution (Sigma Chemical Co). The glass coverslips were washed 3 times with PBS (Quality Biological, Inc) before cell seeding.

**Measurement of \(pH_i\)**

HAEC \(pH\) was measured by using the fluorescent \(pH\) indicator carboxy SNARF 1 (c-SNARF 1) as previously reported.30 HAEC monolayers on glass coverslips were incubated with culture medium containing 5 \(\mu\)mol/L of the acetoxymethyl ester form of c-SNARF 1 (Molecular Probes) and maintained at room temperature in a 95% air/5% CO\(_2\) incubator for 30 minutes. Monolayers were washed for 30 minutes with indicator-free HEPES-buffered saline (HBS) of the following composition (in mmol/L): NaCl 137, KCl 4.9, CaCl\(_2\) 1.5, MgSO\(_4\) 1.2, NaH\(_2\)PO\(_4\) 1.2, D-glucose 15, and HEPES 20 (pH adjusted to 7.40 at room temperature with NaOH). The glass coverslips were then transferred to a perfusion chamber and mounted on the stage of a modified Nikon Diaphot inverted epifluorescence microscope. c-SNARF 1 fluorescence was excited at 530±5 nm with the use of a Xenon short-arc lamp (UXL-75 XE, Ushio Inc), and bandpass interference filters (Omega Optical) selected fluorescence wave-length bands of 590±5 and 640±5 nm, corresponding to the \(H^+\)-bound and \(H^+\)-free forms of c-SNARF, respectively. The ratio of the emission fluorescence was monitored by using a spectrofluorometer (PTI, Deltascan). Auto-fluorescence from unloaded HAECs was \(<2%\) of c-SNARF–loaded HAECs and was subtracted automatically from c-SNARF fluorescence recordings.

A \(pH\) calibration was performed as previously described30 from c-SNARF 1–loaded endothelial monolayers exposed to solutions of varying \(pH\) values containing 140 mmol/L KCl, 20 \(\mu\)mol/L nigericin (Sigma), 1 \(\mu\)mol/L valinomycin (Calbiochem), and 1 \(\mu\)mol/L carbonyl cyanide p-(trifluoromethoxy)phénylhydrazone (Sigma) at room temperature.

**Measurement of \(H^+\) Production**

The amount of \(H^+\) production in response to \(H_2O_2\) was determined as previously described.13,10 HAEC monolayers were trypsinized, harvested, and centrifuged at 1000 rpm for 8 to 10 minutes at room temperature. The pellet from three 100-mm tissue culture dishes was then washed with 0.5 mmol/L HEPES-buffered saline (pH 7.40). The cell suspension (final volume, 4 mL) was then stirred by a magnetic stirrer during continuous \(pH\) measurement at room temperature using a \(pH\) meter (Beckman Instruments, Inc) and covered to prevent equilibration with air. The rate of \(H^+\) production after the addition of \(H_2O_2\) (final concentration, 100 \(\mu\)mol/L) was calculated from the amount of \(OH^-\) required per unit time to maintain the \(pH\) of the cell suspension at a constant value of 7.40±0.01 using 0.1 mmol/L NaOH.

**Measurement of Intracellular Buffer Capacity**

The intracellular buffering capacity (\(\beta\), mmol/L per \(pH\) unit) was calculated as previously described15 using the Na\(_2\)Cl acid-loading method. Briefly, the acid load was estimated as \([NH_4]^+\) after exposure to 20 mmol/L Na\(_2\)Cl, assuming that all Na\(_2\) exits the cell as Na\(_2\) after washout, yielding \(H^+\) in the process. The last measured \(pH\) value in the presence of Na\(_2\)Cl (\(pH_{post}\)) was used to calculate \([NH_4]^+\), with \(pH_{rest}\) considered the nadir \(pH\) value after Na\(_2\)Cl washout, assuming a \(pK\) of 9.0 and that \([NH_4]^+\) equals \([NH_3]\) at a \(pH\) of 7.40. The change in \(pH\) (\(\Delta pH\)) was determined from the difference of the \(pH_{rest}\) and the \(pH_{post}\) and \(\beta\) was calculated as follows:

\[
\beta = \frac{[(NH_4)^+] \Delta pH}{[(NH_4)^+] \times 10^{-(pH_{rest}-pH_{post})}} = 0.49 \times 10^{-(pH_{rest}-pH_{post})} + 0.04\times 10^{-(pH_{rest}-pH_{post})}
\]

**Experimental Protocol**

HAEC monolayers were perfused (flow, 1.8 mL/min) with \(H_2O_2\) (1 mmol/L to 1 mmol/L) made from a 3% stock solution (Sigma) in HBS. In some experiments, HAECs were acid-loaded by the Na\(_2\)Cl prepulse method15 by replacing 20 mmol/L NaCl of the standard HBS with 20 mmol/L NH\(_4\)Cl (J.T. Baker Chemical Co). To examine the role of the Na\(^+/H^+\) exchanger, some monolayers were exposed to 5-(N-ethyl-N-isopropyl)amiloride (EIPA, Molecular Probes) or to a Na\(^+\)-free HBS in which NaCl was replaced by 138.2 mmol/L choline chloride (Sigma) and NaH\(_2\)PO\(_4\) was replaced by 1.2 mmol/L K\(_2\)HPO\(_4\) (Sigma); the \(pH\) was adjusted to 7.40 at room temperature with KOH. An EIPA concentration of 10 \(\mu\)mol/L was used since this concentration has been shown to inhibit endothelial Na\(^+/H^+\) exchange activity.13 Cells were exposed to EIPA for 30 to 60 minutes or to Na\(^+\)-free HBS for 20 to 30 minutes before the remainder of the experimental protocol to allow \(pH\) to achieve a stable baseline after inhibition of Na\(^+/H^+\) exchange.

**Measurement of Intracellular ATP Concentrations**

Intracellular ATP concentrations in HAECs were measured by HPLC as previously described.31 After HAECs in culture dishes were exposed to \(H_2O_2\) (or other reagents described below), the reaction was stopped by the addition of 3 mmol/L ice-cold HClO\(_4\). The cells were then harvested, centrifuged at 16 000g, and frozen at –20°C. For measurements of intracellular ATP concentrations, cells were thawed and vortex-mixed. After ultrasonication (Dower 10-15v, Heat Systems, Inc) 3 times on ice, HAECs were centrifuged at 16 000g for 3 minutes at 4°C, and the supernatant was neutralized by 1:3 (vol:vol) mixing with Freon/triethylamine (4:1). The mixture was centrifuged at 16 000g for 3 minutes at 4°C, and the upper aqueous layer was recovered for HPLC analysis. Reverse-phase HPLC was performed using a Waters Bondapak C18 column and a Waters HPLC system (Waters Associates) with a model 484 UV detector, 2 model 510 reciprocating pumps, and Maxima software. The intracellular ATP concentration was expressed as nanomoles per 10\(^6\) cells.

**Data Analysis and Statistics**

Data are reported as mean±SE. Statistical comparisons were made using the Student \(t\) test for paired and unpaired groups. ANOVAs were performed when multiple comparisons were involved. A difference was considered significant at \(P<0.05\).

**Results**

**Effects of \(H_2O_2\) on HAEC \(pH_i\)**

When HAEC monolayers were exposed to \(H_2O_2\) for 10 minutes, a concentration-dependent intracellular acidification was observed. The threshold of this effect was \(\approx 10\) \(\mu\)mol/L (\(pH\) decrease of 0.15±0.02 at a concentration of 10 \(\mu\)mol/L, \(n=7\), \(P<0.05\) versus control, Figure 1A). The decrease in HAEC \(pH\) was typically observed within \(\approx 2\) minutes of exposure (Figure 1B) and was partially reversible on washout of \(H_2O_2\) at concentrations of 1 to 100 \(\mu\)mol/L. At a concentration of 100 \(\mu\)mol/L \(H_2O_2\), \(pH\) decreased from 7.24±0.01 to 7.02±0.02 (\(n=12\)) and then was partially reversible (\(pH\)=7.16±0.01 at 15 minutes) in 5 of the 6 monolayers in which \(pH\) was examined during \(H_2O_2\) washout. At \(H_2O_2\) concentrations of 250 \(\mu\)mol/L to 1 \(mmol/L\), the intracellular acidification was irreversible on washout of \(H_2O_2\) (\(n=7\) for each concentration). Since 100 \(\mu\)mol/L \(H_2O_2\) was the maximal concentration for which a reversible effect on \(pH\) was observed, this concentration was used in subsequent experiments.

**Effect of Na\(^+/H^+\) Exchange Inhibition on the \(H_2O_2\)-Induced Intracellular Acidification**

To determine whether the effect of \(H_2O_2\) on \(pH\) was related to Na\(^+/H^+\) exchange inhibition, experiments were performed in which the Na\(^+/H^+\) exchanger was inhibited before \(H_2O_2\) exposure either pharmacologically (by pretreatment with...
EIPA), by removal of extracellular Na⁺ and replacement with equimolar choline, or by metabolic inhibition. Prior inhibition of the Na⁺/H⁺ exchanger by any of these 3 methods blocked the subsequent effect of H₂O₂ on pH. When HAEC monolayers were pretreated with 10 μmol/L EIPA, the effect of 100 μmol/L H₂O₂ on pH, was significantly inhibited (ΔpH = 0.04 ± 0.01 pH unit at 10 minutes, n = 5, P < 0.05 versus 100 μmol/L H₂O₂ without EIPA and P = NS versus time control). When HAEC monolayers were exposed to Na⁺-free buffer (Figure 2A), pH decreased over an approximate period from 7.23 ± 0.01 to 6.98 ± 0.02 (n = 6). After this decrease in pH, the subsequent exposure to 100 μmol/L H₂O₂ in Na⁺-free buffer (n = 4) did not significantly affect pH (ΔpH = 0.03 ± 0.01 pH unit, P = NS versus control and P < 0.05 versus 100 μmol/L H₂O₂ in Na⁺-containing HBS).

In some experiments, the effect of Na⁺/H⁺ exchange inhibition by ATP depletion on the H₂O₂-induced acidification was examined by exposing cells for ~20 minutes to the glycolytic inhibitor iodoacetate acid (IAA), a nonspecific alkylating agent that irreversibly and noncompetitively inhibits GAPDH. This concentration of IAA was used because a previous study showed that 10 minutes after exposure to 100 μmol/L IAA, endothelial pH decreased to a similar extent as did pH, after exposure to 100 μmol/L H₂O₂ in the present study. As shown in Figure 2B, 100 μmol/L IAA decreased pH from 7.24 ± 0.01 to 6.96 ± 0.03 and inhibited the effect of a subsequent H₂O₂ exposure on pH (ΔpH = 0.02 ± 0.01, n = 5, P = NS versus control). This was not a nonspecific effect of IAA, since the subsequent exposure to 4.7 mmol/L propionic acid (PA) initiated a further decrease in pH of 0.11 ± 0.02 pH unit, which was no different from that observed when HAEC monolayers were exposed to 4.7 mmol/L PA without IAA pretreatment (ΔpH = 0.13 ± 0.00, n = 4, P = NS versus pH change induced by PA after IAA).

**Effect of H₂O₂ on Recovery From an NH₄Cl Prepulse-Induced Intracellular Acid Load**

The NH₄Cl prepulse method of intracellular acid loading was used to examine the effects of H₂O₂ on Na⁺/H⁺ exchanger activity, since pH recovery from an acid load in bicarbonate-free buffer is due to H⁺ extrusion via the Na⁺/H⁺ exchanger and is blocked by pharmacological inhibition of the exchanger. As shown in Figure 3, when HAECs were exposed to NH₄Cl in HBS, pH increased from 7.23 ± 0.01 to 7.83 ± 0.02 (n = 9) because of the rapid diffusion of NH₄⁺ into cells and its combination with H⁺. During exposure to NH₄Cl, pH gradually began to recover because of the slower diffusion of NH₄⁺ into cells, which subsequently dissociates into NH₃ and H⁺. When HAEC monolayers were then returned to NH₄Cl-free HBS, pH decreased to 7.03 ± 0.02 because of the rapid diffusion of NH₃ from cells, leaving an excess of intracellular H⁺. Recovery then proceeded slowly over 15 to 20 minutes, with an increase in pH of 0.15 ± 0.02 pH unit at 10 minutes. After pH recovered to baseline, a second NH₄Cl prepulse stimulated a similar effect on pH, (Figure 3A) with an increase in pH of 0.12 ± 0.01 pH unit at 10 minutes (n = 9).
Figure 1B). As shown in Figure 4, 100 μmol/L H2O2 decreased ATP levels by 40% (9.95 ± 0.76 nmol/10^6 cells, n = 4 for each, P < 0.05). This effect was partially reversible at this concentration of H2O2 after a 15-minute washout (13.10 ± 0.85 nmol/10^6 cells, n = 4, P = NS versus control). A higher concentration of H2O2 (1 mmol/L) decreased ATP levels by 54% (7.65 ± 0.21 nmol/10^6 cells, n = 4, P = NS versus 100 μmol/L H2O2), but the effect on intracellular ATP levels at this concentration was irreversible (P = NS versus no washout). Cell viability, as measured by Trypan blue exclusion, was not significantly affected by either concentration of H2O2.

Since H2O2-induced ATP depletion may occur as a result of DNA strand breaks, with depletion of cellular energy stores occurring as a result of the subsequent activation of the repair enzyme PARS, the effect of the PARS inhibitors 3-aminobenzamide (3-AB) and nicotinamide (NIC) on H2O2-induced ATP depletion was examined. When HAEC monolayers were pretreated for 15 minutes with either 3-AB or NIC (each 1 mmol/L) before exposure to 100 μmol/L H2O2,

**Effect of H2O2 on Intracellular Buffer Capacity**

Intracellular buffer capacity was examined using the NH4Cl acid-loading method, since this represents an important mechanism to maintain pH homeostasis. In HBS, the calculation of buffer capacity β from the first NH4Cl exposure was 26.66 ± 1.71 mmol/L per pH unit (n = 21), and that from the second NH4Cl exposure was 28.07 ± 2.70 mmol/L per pH unit (n = 9, P = NS). Addition of 100 μmol/L H2O2 during washout of a second NH4Cl prepulse did not affect the β value (31.50 ± 3.19 mmol/L per pH unit, n = 6, P = NS versus second control NH4Cl exposure).

**Effect of H2O2 on Intracellular ATP Concentration With and Without PARS Inhibition**

Since ATP depletion reduces the activity of the Na+/H+ exchanger21,27 and H2O2 decreases cellular ATP in several cell types, the effect of H2O2 on intracellular ATP concentration in HAECs was examined. In these experiments, the effect of a 2-minute exposure to H2O2 was studied, since the effect of H2O2 on pH begins within this time period (see Figure 1B). As shown in Figure 4, 100 μmol/L H2O2 decreased intracellular ATP levels by 40% (9.95 ± 0.76 nmol/10^6 cells, n = 4 for each, P < 0.05). This effect was partially reversible at this concentration of H2O2 after a 15-minute washout (13.10 ± 0.85 nmol/10^6 cells, n = 4, P = NS versus control). A higher concentration of H2O2 (1 mmol/L) decreased ATP levels by 54% (7.65 ± 0.21 nmol/10^6 cells, n = 4, P = NS versus 100 μmol/L H2O2), but the effect on intracellular ATP levels at this concentration was irreversible (P = NS versus no washout). Cell viability, as measured by Trypan blue exclusion, was not significantly affected by either concentration of H2O2.

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ATP depletion was largely prevented. This concentration of 3-AB and NIC was used on the basis of previous studies showing that these PARS inhibitors prevented oxidant stress–induced DNA strand breaks and ATP depletion in several cell types, including endothelial cells.13,19,40–42 In HAEC monolayers pretreated with 3-AB, ATP levels were ∼93% of control after exposure to 100 μmol/L H₂O₂ (n=5, P=NS versus control). ATP levels were similarly preserved by NIC pretreatment (∼94% of control after 100 μmol/L H₂O₂, n=4, P=NS versus control). Neither 3-AB nor NIC alone affected ATP levels (∼93% and ∼95%, respectively; P=NS versus control, n=4 for each). In contrast to the effect of PARS inhibition before exposure to 100 μmol/L H₂O₂, 3-AB pretreatment did not prevent the effect of 1 mmol/L H₂O₂ on ATP levels (∼47% depletion, n=4, P<0.05 versus control, P=NS versus 1 mmol/L H₂O₂ without 3-AB pretreatment).

To determine whether prevention of ATP depletion by PARS inhibitors occurred by a nonspecific effect rather than one related to damage and repair of DNA, the effect of 3-AB pretreatment was examined before ATP depletion by a mechanism that is not known to involve PARS activation. HAECs were exposed to the glycolytic inhibitor IAA for 2 minutes. Exposure to 30, 40, and 100 μmol/L IAA decreased ATP levels by ∼36%, ∼45%, and ∼60%, respectively (n=4, P<0.05 versus control, P=NS versus 100 μmol/L H₂O₂ for each), but this was not affected by 3-AB pretreatment for either IAA concentration examined (∼44% depletion for 40 μmol/L IAA and ∼54% depletion for 100 μmol/L IAA, n=4, P=NS versus no 3-AB, not shown). IAA did not affect cell viability at these concentrations, as assessed by Trypan blue exclusion.

Effect of PARS Inhibitors on H₂O₂-Induced Intracellular Acidification and Inhibition of Recovery From an Intracellular Acid Load

Neither 3-AB nor NIC pretreatment (1 mmol/L for 15 minutes) affected HAEC pHᵢ (ΔpHᵢ=0.02±0.01 pH unit for 3-AB and 0.02±0.02 pH unit for NIC, n=6 for both, P=NS versus control). When HAEC monolayers were exposed to 100 μmol/L H₂O₂ after 3-AB or NIC pretreatment, the effect of H₂O₂ on pHᵢ was significantly inhibited (ΔpHᵢ=0.05±0.01 and 0.03±0.01 pH unit for 3-AB and NIC, respectively; n=6 for both, P<0.05 versus 100 μmol/L H₂O₂ without pretreatment, Figure 5). The ability of PARS inhibitors to attenuate the H₂O₂-induced acidification was not observed when HAEC monolayers were acidified by exposure to IAA. After 3-AB pretreatment, the subsequent exposure to 100 μmol/L IAA decreased pHᵢ by 0.24±0.01 pH unit (P=NS versus IAA without 3-AB pretreatment).

The effect of PARS inhibition on the recovery from an NH₄Cl prepulse-induced acid load was examined with and without H₂O₂ present in the washout period (Figure 6). When HAEC monolayers were exposed to NH₄Cl in the presence of either 3-AB (n=7) or NIC (n=6), pHᵢ recovery on washout (first exposure in Figure 6A and 6B) was no different from that in the absence of these inhibitors (0.13±0.01 pH unit at 10 minutes for both 3-AB and NIC, P=NS versus control). As summarized in Figure 6C, both 3-AB and NIC blocked the inhibition of pHᵢ recovery induced by 100 μmol/L H₂O₂ during the second NH₄Cl washout period (0.09±0.01 pH unit 10 minutes after washout of NH₄Cl for 3-AB pretreatment and 0.09±0.01 pH unit for NIC pretreatment, P<0.05 versus 100 μmol/L H₂O₂ and P=NS versus control for both). Neither 3-AB nor NIC pretreatment affected the calculated β value (34.15±4.24 mmol/L per pH unit for 3-AB and 33.08±2.73 mmol/L per pH unit for NIC, n=6 and P=NS versus control for each).

[Figure 4: Effect of H₂O₂ on intracellular ATP. Averaged data show the effect of a 2-minute exposure to H₂O₂ on intracellular ATP levels in HAECs. H₂O₂ produced a significant (P<0.05 vs control, n=4) decrease in intracellular ATP levels, which was partially reversible on washout and was prevented by the PARS inhibitors 3-AB (1 mmol/L, n=5) and NIC (1 mmol/L, n=4) (P<0.05 vs 100 μmol/L H₂O₂). At a higher concentration of H₂O₂ (1 mmol/L), ATP depletion was irreversible (n=4).

[Figure 5: Effect of inhibition of PARS on H₂O₂-induced ΔpHᵢ. A, Representative c-SNARF 1 fluorescence from an HAEC monolayer exposed to 100 μmol/L H₂O₂ for 15 minutes after pretreatment with the PARS inhibitor 3-AB (1 mmol/L present throughout). 3-AB inhibited the subsequent effect of H₂O₂ on pHᵢ (n=6, P<0.05 vs 100 μmol/L H₂O₂ without 3-AB). B, Averaged data showing the effect of 100 μmol/L H₂O₂ on pHᵢ with and without pretreatment with 3-AB or NIC. Both 3-AB and NIC (each 1 mmol/L) inhibited the subsequent effect of H₂O₂ on pHᵢ (n=6, P<0.05 vs H₂O₂ alone for each).]
ATP levels by \(\approx 40\%\). Although we cannot exclude the possibility that the effect of \(\text{H}_2\text{O}_2\) on endothelial \(\text{pH}_i\) is in part due to an increase in acid production resulting from ATP hydrolysis, no effect on \(\text{H}^+\) efflux was observed when HAECs were exposed to 100 \(\mu\text{mol/L}\) \(\text{H}_2\text{O}_2\) in the present study, and \(\text{H}_2\text{O}_2\) did not increase \(\text{H}^+\) production in other cell types.\(^{10}\) The effects of \(\text{H}_2\text{O}_2\) on \(\text{pH}_i\) and cellular ATP are prevented by pretreatment with the PARS inhibitors 3-AB and NIC, which themselves have no effect on \(\text{pH}_i\), on \(\text{pH}_i\) recovery from an acid load, on intracellular buffer capacity, or on ATP levels. The \(\text{H}_2\text{O}_2\)-induced acidification is prevented after inhibition of the Na+/H+ exchanger by EIPA, by removal of extracellular Na+, or by glycolytic inhibition with IAA.

Plasmalemmal Na+/H+ exchangers are present in all mammalian cells and are involved in \(\text{pH}_i\) regulation in many cell types, including endothelial cells.\(^{15,36,43}\) The Na+/H+ exchanger extrudes \(\text{H}^+\) from the cell in electroneutral exchange for Na+ in a 1:1 stoichiometry.\(^{28}\) Inhibition of the Na+/H+ exchanger by oxidant stress has been reported previously in several cell types,\(^{10,44}\) including vascular endothelial cells,\(^{11}\) although the mechanism of this effect has not been determined.

The present study shows that \(\text{H}_2\text{O}_2\) induces rapid inhibition of Na+/H+ exchange in human endothelial cells by a process associated with PARS activation and depletion of intracellular ATP. It is likely that this concentration of \(\text{H}_2\text{O}_2\) does not result in complete inhibition of the exchanger, since \(\text{pH}_i\) recovery from an acid load was significantly, although not completely, inhibited by 100 \(\mu\text{mol/L}\) \(\text{H}_2\text{O}_2\); when 50 \(\mu\text{mol/L}\) \(\text{NH}_4\text{Cl}\) was used to decrease HAEC \(\text{pH}_i\) to a degree that would be expected to maximally activate the Na+/H+ exchanger, \(\text{H}_2\text{O}_2\) has been shown to rapidly induce time- and concentration-dependent intracellular ATP depletion in several cell types\(^{12,16–18}\) without affecting cell viability.\(^{12,17}\) In the present study, \(\text{H}_2\text{O}_2\) decreased HAEC \(\text{pH}_i\) within minutes of oxidant exposure and produced a similarly rapid depletion of intracellular ATP. After only a 2-minute exposure, 100 \(\mu\text{mol/L}\) \(\text{H}_2\text{O}_2\) decreased intracellular ATP concentration by \(\approx 40\%\). This is similar to previously reported observations in alveolar type II cells, in which a 2-minute exposure to 500 \(\mu\text{mol/L}\) \(\text{H}_2\text{O}_2\) reduced ATP levels by \(\approx 60\%\).\(^{16}\)

Changes in the concentration of intracellular ATP induced by \(\text{H}_2\text{O}_2\) would be expected to affect the activity of the Na+/H+ exchanger. Intracellular ATP depletion inhibits the exchanger in several cell types, including vascular smooth muscle cells,\(^{23}\) rat ventricular cells,\(^{27}\) sheep Purkinje fibers,\(^{26}\) A431 carcinoma cells,\(^{20}\) alveolar type II cells,\(^{24}\) renal epithelial cells,\(^{23}\) and fibroblasts transfected with the growth factor–sensitive isofrom of the exchanger, NHE-I.\(^{27}\) Indeed, ATP depletion results in decreased activity of Na+/H+ exchange by all 3 exchanger isoforms.\(^{25,26}\) Although it has been suggested that ATP depletion affects basal Na+/H+ exchanger activity by decreasing phosphorylation of the antipporter,\(^{22,45,46}\) this concept has been challenged, since ATP depletion rapidly inhibits the Na+/H+ exchanger in NHE-I–transfected fibroblasts without affecting NHE-I phosphorylation.\(^{27}\) The mechanism of ATP depletion may be important in determining the effect on exchanger activity. In Purkinje fibers, glycolytic

**Discussion**

The present study demonstrates that \(\text{H}_2\text{O}_2\) decreases \(\text{pH}_i\), depletes intracellular ATP, and inhibits the Na+/H+ exchanger in human endothelial cells by a mechanism that is prevented by inhibition of the DNA repair enzyme PARS. At a concentration of 100 \(\mu\text{mol/L}\) \(\text{H}_2\text{O}_2\), decreases HAEC \(\text{pH}_i\) by 0.22 \(\text{pH}\) unit, inhibits \(\text{pH}_i\) recovery from an \(\text{NH}_4\text{Cl}\) prepulse-induced intracellular acid load, and decreases intracellular

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**Figure 6.** Effect of inhibition of PARS on \(\text{H}_2\text{O}_2\)-induced inhibition of recovery from an intracellular acid load. A, Representative c-SNARF 1 fluorescence from an HAEC monolayer exposed to 20 \(\mu\text{mol/L} \text{NH}_4\text{Cl}\) under control conditions (first exposure) and in the presence of the 100 \(\mu\text{mol/L} \text{H}_2\text{O}_2\) during washout (second exposure). The PARS inhibitor 3-AB (1 \(\mu\text{mol/L}\)) was present throughout. Inhibition of \(\text{pH}_i\) recovery from an \(\text{NH}_4\text{Cl}\) prepulse-induced acid load by \(\text{H}_2\text{O}_2\) was prevented by 3-AB pretreatment (\(n=7\), \(P=\text{NS}\) vs control). B, Representative c-SNARF 1 fluorescence from an HAEC monolayer exposed to 20 \(\mu\text{mol/L} \text{NH}_4\text{Cl}\) under control conditions (first exposure) and in the presence of the 100 \(\mu\text{mol/L} \text{H}_2\text{O}_2\) during washout (second exposure). The PARS inhibitor NIC (1 \(\mu\text{mol/L}\)) was present throughout. Inhibition of \(\text{pH}_i\) recovery from an \(\text{NH}_4\text{Cl}\) prepulse-induced acid load by \(\text{H}_2\text{O}_2\) was prevented by NIC pretreatment (\(n=6\), \(P=\text{NS}\) vs control). C, Averaged data showing the effect of PARS inhibitors on \(\text{H}_2\text{O}_2\)-induced inhibition of recovery from an intracellular acid load. The average \(\text{pH}_i\) recovery 10 minutes after washout of 20 \(\mu\text{mol/L} \text{NH}_4\text{Cl}\) is shown on the y-axis. \(\text{H}_2\text{O}_2\) (100 \(\mu\text{mol/L}\)) significantly inhibited recovery from the intracellular acid load (\(P<0.05\) vs control, \(n=6\)). This effect was inhibited by pretreatment with either 3-AB (1 \(\mu\text{mol/L}\), \(n=7\)) or NIC (1 \(\mu\text{mol/L}\), \(n=6\)) (\(\#P<0.05\) vs 100 \(\mu\text{mol/L} \text{H}_2\text{O}_2\) alone and \(P=\text{NS}\) vs control for each).
inhibition by 2-deoxyglucose inhibits the Na\(^+/\)H\(^+\) exchanger, whereas the oxidative inhibitor cyanide does not, despite similar degrees of intracellular ATP depletion (\(\approx 70\%\)) produced by each.\(^{10}\) The Na\(^+/\)H\(^+\) exchanger has a relatively low affinity for ATP, with inactivation reported at ATP concentrations in the millimolar range, consistent with a 10-fold lower affinity for ATP than the Na\(^+/\)-K\(^+\) pump.\(^{47}\)

In the present work, the effects of 100 \(\mu\)mol/L \(\text{H}_2\text{O}_2\) on pH\(_i\) and ATP levels were at least partially reversible after washout, but at a higher concentration (1 mmol/L), these effects were irreversible. At high concentrations (>100 \(\mu\)mol/L), \(\text{H}_2\text{O}_2\) inhibits ATP generation by affecting the glycolytic enzyme GAPDH.\(^{40,48}\) This appears to be due to direct oxidation of sulfhydryl groups on the active site of GAPDH.\(^{40}\) At lower concentrations (<100 \(\mu\)mol/L), GAPDH may be partially inhibited,\(^{8}\) but intracellular ATP depletion also occurs as a result of \(\text{H}_2\text{O}_2\)-induced DNA damage, subsequent activation of PARS, and consumption of NAD\(^+\).\(^{4}4\) Of note, in the present study, pretreatment with 3-AB or NIC prevented the effects of 100 \(\mu\)mol/L \(\text{H}_2\text{O}_2\) on pH\(_i\) and intracellular ATP levels, but PARS inhibition did not prevent these effects of \(\text{H}_2\text{O}_2\) at a concentration of 1 mmol/L and did not alter the effects of the GAPDH inhibitor IAA.\(^{17}\) These data are most consistent with the predominant effect of 100 \(\mu\)mol/L \(\text{H}_2\text{O}_2\) resulting from PARS activation in HAECs. Our results differ from those of Wu et al,\(^{9}\) who reported that 100 \(\mu\)mol/L \(\text{H}_2\text{O}_2\) decreased pH\(_i\) in freshly dissociated rat cardiac myocytes and rat cardiac myoblasts by inhibiting glycolysis. This may relate to differences in cell type and to cell type-specific differences in glycolytic activity and dependence on glycolysis for ATP production. The effects of 3-AB and NIC cannot be explained by free radical scavenging activity, since neither of these PARS inhibitors affected free radicals generated by use of a Fenton reaction system\(^{49}\) containing \(\text{H}_2\text{O}_2\) (100 \(\mu\)mol/L) and 10 \(\mu\)mol/L \(\text{Fe}^{3+}\)-nitrilotriacetate and assessed by electron paramagnetic resonance spectroscopy (data not shown).

Previous studies show that \(\text{H}_2\text{O}_2\) induces rapid and concentration-dependent DNA strand breaks in vascular endothelial cells.\(^{15,15\text{a}}\) DNA strand breaks activate the nuclear enzyme PARS, resulting in depletion of intracellular NAD\(^+\) and ATP levels. Pharmacological inhibition of PARS has been shown to prevent this effect in many cell types, including vascular endothelial cells.\(^{13}\) In murine macrophages, Schraufstatter et al\(^{15}\) showed that DNA single-strand breaks occur within seconds of exposure to micromolar concentrations of \(\text{H}_2\text{O}_2\). Only 26% of DNA remained double-stranded after 5 minutes of exposure to 100 \(\mu\)mol/L \(\text{H}_2\text{O}_2\). Within 1 minute, decreases in NAD and ATP levels were observed, and within several minutes, activation of PARS was noted.\(^{15}\)

The effect of \(\text{H}_2\text{O}_2\) on endothelial pH\(_i\) may play a role in the endothelial dysfunction that occurs as a consequence of ischemia and reperfusion. On reperfusion after a period of ischemia, the response to endothelium-dependent vasodilators is diminished, and previous work shows that oxidants play a role in this process.\(^{3}\) The \(\text{H}_2\text{O}_2\)-induced decrease in pH\(_i\) and Na\(^+/\)H\(^+\) exchange inhibition may affect agonist-stimulated vasodilation due to either NO or prostaglandins. Relatively small pH changes within a narrow physiological range affect the activity of the constitutive NO synthase in endothelial cells,\(^{50}\) with a decrease in pH\(_i\) inhibiting the enzyme. Pharmacological inhibition of endothelial Na\(^+/\)H\(^+\) exchange significantly blocked the production of NO >15 minutes after stimulation with bradykinin, suggesting a role for the exchanger or endothelial pH\(_i\) in determining the activity of agonist-induced endothelium-dependent vasodilator function. The decrease in pH\(_i\) from 7.24 to 7.02 (due to exposure to 100 \(\mu\)mol/L \(\text{H}_2\text{O}_2\) reported in the present study) would be expected to decrease the activity of constitutive NO synthase by \(\approx 35\%\) (I. Fleming, unpublished data, 1997). Inhibition of Na\(^+/\)H\(^+\) exchange activity may also reduce endothelial synthesis or release of vasodilatory prostaglandins, since the stimulation of the exchanger appears to mediate agonist-induced prostaglandin release.\(^{45}\) It is interesting that a recent study reports that inhibitors of PARS activity reduce myocardial infarct size in a rabbit model of I/R injury.\(^{51}\) Since 100 \(\mu\)mol/L \(\text{H}_2\text{O}_2\) has been shown to mimic some of the pathophysiological responses observed in I/R injury,\(^{52,53}\) and oxidants are associated with impaired endothelium-dependent relaxation,\(^{3}\) the effects of micromolar concentrations of \(\text{H}_2\text{O}_2\) on pH\(_i\) described in the present study may be important in the pathogenesis of postischemic endothelial dysfunction.

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References


Hydrogen Peroxide Decreases pH\textsubscript{i} in Human Aortic Endothelial Cells by Inhibiting Na\textsuperscript{+}/H\textsuperscript{+} Exchange

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