Hydrogen Peroxide Decreases pH_i in Human Aortic Endothelial Cells by Inhibiting Na+/H+ Exchange

Qinghua Hu, Yong Xia, Stefano Corda, Jay L. Zweier, Roy C. Ziegelstein

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_i, the effect of hydrogen peroxide on endothelial pH_i was examined. Hydrogen peroxide (100 μmol/L for 10 minutes) decreased pH_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+/H⁺ exchange with 5-(N-ethyl-N-isopropylamiloride (10 μmol/L), by removal of extracellular Na⁺, or by glycolytic inhibition with iodoacetic acid blocked the subsequent effect of hydrogen peroxide on pH_i. A 2-minute exposure to 100 μmol/L H₂O₂ 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_i in human endothelial cells by inhibiting Na⁺/H⁺ exchange. This appears to be mediated by activation of the DNA repair enzyme poly(ADP-ribose) polymerase and subsequent depletion of intracellular ATP. Since a decrease in pH_i in this range may alter the activity of NO synthase or affect the synthesis of vasodilator prostaglandins, the effect of hydrogen peroxide on the endothelial Na⁺/H⁺ exchanger may be important in the pathogenesis of postischemic endothelial dysfunction. (Circ Res. 1998;83:644-651.)

Key Words: pH_i ■ endothelium ■ Na⁺/H⁺ 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 (H₂O₂) and the superoxide anion (O₂⁻). The endothelium in vivo may be exposed to H₂O₂ as a consequence of the dismutation of O₂⁻ 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 H₂O₂ 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.

H₂O₂ decreases pH_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⁺/H⁺ exchanger activity. The related compound tert-butyl hydroperoxide has been shown to decrease Na⁺/H⁺ antiport activity in bovine pulmonary artery endothelial cells. Oxidants like H₂O₂ decrease cellular energy stores and induce DNA strand breaks in vascular endothelial cells. Within several minutes of exposure to micromolar concentrations of H₂O₂, 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⁺ to DNA. PARS activation rapidly decreases cellular NAD⁺ and, subsequently, ATP levels. The rapid intracellular ATP depletion induced by oxidants like H₂O₂ may result from PARS activation, since it can be prevented by PARS inhibitors in type II pneumocytes. We hypothesized that H₂O₂ decreases endothelial pH_i by inhibiting the ATP-dependent Na⁺/H⁺ exchanger through a pathway involving PARS activation. The present study reports that H₂O₂ 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 mg/L 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% CO₂. To examine the effects of H₂O₂ on HAEC pH_i, HAECs were plated at an approximate...
concentration of $1 \times 10^7$/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$_i$

HAEpH$_i$ was measured by using the fluorescent pH indicator carboxy SNARF 1 (c-SNARF 1) as previously reported. 10 HAEp monolayers on glass coverslips were incubated with culture medium containing 5 mmol/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, NaHPO$_4$ 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 wavelength 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). Autofluorescence from unloaded HAEp cells was <2% of c-SNARF–loaded HAEp and was subtracted automatically from c-SNARF fluorescence recordings.

A pH calibration was performed as previously described 10 from c-SNARF 1–loaded endothelial monolayers exposed to solutions of varying pH values containing 140 mmol/L KCl, 20 mmol/L MgCl$_2$ (Sigma), 1 mmol/L valinomycin (Calbiochem), and 1 mmol/L carboxyl cyanide p-trifluoromethoxyphenylhydrazone (Sigma) at room temperature.

### Measurement of H$^+$ Production

The amount of H$^+$ production in response to H$_2$O$_2$ was determined as previously described. 10, 31 HAEp 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$_2$O$_2$ (final concentration, 100 mmol/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 intrinsic intracellular buffer capacity ($\beta$, mmol/L per pH unit) was calculated as previously described 10 using the NH$_4$Cl acid-loading method. Briefly, the acid load was estimated as [NH$_3$]$_o$ after exposure to 20 mmol/L NH$_4$Cl, assuming that all NH$_4^+$ exits the cell as NH$_3$ after washout, yielding H$^+$ in the process. The last measured pH value in the presence of NH$_4$Cl (pH$_{me}$) was used to calculate [NH$_3$], with pH$_{me}$ considered the nadir pH value after NH$_4$Cl washout, assuming a pK of 9.0 and that [NH$_3$] = [NH$_4^+$] at a pH of 7.40. The change in pH ($\Delta$pH) was determined from the difference of the pH$_{me}$ and the pH$_{me}$ and $\beta$ was calculated as follows:

$$\beta = \Delta[pH] = [NH_3] \times 10^{\Delta pK}$$

### Experimental Protocol

HAEp monolayers were perfused (flow, 1.8 mL/min) with H$_2$O$_2$ (1 mmol/L to 1 mmol/L) made from a 3% stock solution (Sigma) in HBS. In some experiments, HAEp monolayers were acid-loaded by the NH$_4$Cl prepulse method 10 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 NH$_4$PO$_4$ was replaced by 1.2 mmol/L KH$_2$PO$_4$ (Sigma); the pH was adjusted to 7.40 at room temperature with KOH. An EIPA concentration of 10 mmol/L was used since this concentration has been shown to inhibit endothelial Na$^+$/H$^+$ exchange activity. 10 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 HAEp were measured by HPLC as previously described. 10 After HAEp cells in culture dishes were exposed to H$_2$O$_2$ (or other reagents described below), the reaction was stopped by the addition of 3 mol/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, HAEp 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 recirculating 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$_2$O$_2$ on HAEp pH$_i$

When HAEp monolayers were exposed to H$_2$O$_2$ for 10 minutes, a concentration-dependent intracellular acidification was observed. The threshold of this effect was $\approx$10 mmol/L (pH$_i$ decrease of 0.15±0.02 at a concentration of 10 mmol/L, $n=7$, $P<0.05$ versus control, Figure 1A). The decrease in HAEp pH$_i$ was typically observed within $\approx$2 minutes of exposure (Figure 1B) and was partially reversible on washout of H$_2$O$_2$ at concentrations of 1 to 100 mmol/L. At a concentration of 100 mmol/L H$_2$O$_2$, pH$_i$ decreased from 7.24±0.01 to 7.02±0.02 ($n=12$) and then was partially reversible (pH$_i$=7.16±0.01 at 15 minutes) in 5 of the 6 monolayers in which pH$_i$ was examined during H$_2$O$_2$ washout. At H$_2$O$_2$ concentrations of 250 mmol/L to 1 mmol/L, the intracellular acidification was irreversible on washout of H$_2$O$_2$ ($n=7$ for each concentration). Since 100 mmol/L H$_2$O$_2$ was the maximal concentration for which a reversible effect on pH$_i$ was observed, this concentration was used in subsequent experiments.

### Effect of Na$^+$/H$^+$ Exchange Inhibition on the H$_2$O$_2$-Induced Intracellular Acidification

To determine whether the effect of H$_2$O$_2$ on pH$_i$ was related to Na$^+$/H$^+$ exchange inhibition, experiments were performed in which the Na$^+$/H$^+$ exchanger was inhibited before H$_2$O$_2$ exposure either pharmacologically (by pretreatment with...
Effects of H₂O₂ on HAEC pHi. A, Averaged data show-
ing the relationship between H₂O₂ concentration (1 μmol/L to
1 mmol/L) and the decrease in pHi from baseline (ΔpHi). H₂O₂
initiated a concentration-dependent decrease in pHi, which
became significant at a concentration of 10 μmol/L (P<0.05 vs
control; n=12 monolayers for 100 μmol/L concentration and
n=7 for all others). B, Representative c-SNARF 1 fluores-
cence from an HAEC monolayer exposed to 100 μmol/L H₂O₂ in
HEPES buffer with 1.5 mmol/L Ca²⁺. An irreversible decrease in pHi that occurred within ~2 minutes of exposure
was observed (n=12).

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 pHi. When HAEC
monolayers were pretreated with 10 μmol/L EIPA, the effect
of 100 μmol/L H₂O₂ on pHi was significantly inhibited
(ΔpHi=0.04±0.01 pH unit at 10 minutes, n=5, P<0.05 vs 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 ~20-
minute period from 7.23±0.01 to 6.98±0.02 (n=6). After
this decrease in pH1, the subsequent exposure to 100 μmol/L
H₂O₂ in Na⁺-free buffer (n=4) did not significantly affect pHi
(ΔpHi=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 iodoacetic 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 pHi 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 (ΔpHi=0.02±0.01, P=NS versus con-
trol). 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 pHi 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
(ΔpHi=0.13±0.00, P=NS versus 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 recovery 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).
Recovery from the intracellular acid load was almost completely inhibited when NH₄Cl washout occurred in the presence of 10 μmol/L EIPA (Figure 3B), with an increase in pH of 0.02±0.00 pH unit at 10 minutes (n=6, P<0.05 versus no EIPA). Recovery from the NH₄Cl prepulse-induced acid load was also inhibited when washout occurred in the presence 100 μmol/L H₂O₂ (Figure 3C, increase in pH of 0.02±0.00 pH unit at 10 minutes, n=6, P<0.05 versus no H₂O₂). To determine whether H₂O₂ had a similar effect under conditions of greater acid loading, which would be expected to result in more significant activation of the Na⁺/H⁺ exchanger, HAECs were exposed sequentially to 2 separate 50 mmol/L NH₄Cl pulses, the first in HBS and the second in HBS with 100 μmol/L H₂O₂. Under these conditions, pH decreased to a nadir of 6.78±0.01 after washout of the first prepulse and 6.79±0.08 after washout of the second prepulse. Recovery from the 50 mmol/L NH₄Cl prepulse-induced acid load was inhibited by 100 μmol/L H₂O₂ (0.46±0.07 versus 0.18±0.06 pH unit, both at 10 minutes, n=3, P<0.02). Thus, the effects of this concentration of H₂O₂ on pH, and on recovery from an intracellular acid load may be explained by Na⁺/H⁺ exchange inhibition.

**Effect of H₂O₂ on H⁺ Production and on Intracellular Buffer Capacity**

The rate of H⁺ production was assessed as net cellular H⁺ efflux from HAEC suspensions at baseline and after addition of H₂O₂. Under control conditions, HAECs produce H⁺ at a rate of 0.24±0.03 μmol/h per 10⁶ cells (n=6). There was a nonsignificant trend for the rate of acid production to decrease after the addition of 100 μmol/L H₂O₂ (0.21±0.02 μmol/h per 10⁶ cells, n=6, P=0.06 versus control). There was no significant change in the rate of acid production in time control experiments in which H₂O₂ was not added.

The effect of H₂O₂ on 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⁺ exchanger, 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 versus 16.61±1.15 nmol/10⁶ 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/10⁶ 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/10⁶ 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 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).
induced intracellular acid load, and decreases intracellular
by H2O2 was prevented by NIC pretreatment (n
20 mmol/L NH4Cl under control conditions (first exposure) and

c-SNARF 1 fluorescence from an HAEC monolayer exposed to

of recovery from an intracellular acid load. A, Representative

A431 carcinoma cells, 20 alveolar type II cells, 24 renal epithe-

Discussion

The present study demonstrates that H2O2 decreases pH, 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 μmol/L, H2O2 decreases HAEC pH by 0.22 pH unit, inhibits pH recovery from an NH4Cl prepulse-induced intracellular acid load, and decreases intracellular

ATP levels by ~40%. Although we cannot exclude the possibility that the effect of H2O2 on endothelial pH is in part due to an increase in acid production resulting from ATP hydrolysis, no effect on H+ efflux was observed when HAECs were exposed to 100 μmol/L H2O2 in the present study, and H2O2 did not increase H+ production in other cell types.10 The effects of H2O2 on pH, and cellular ATP are prevented by pretreatment with the PARS inhibitors 3-AB and NIC, which themselves have no effect on pH, on pH recovery from an acid load, on intracellular buffer capacity, or on ATP levels. The H2O2-induced acidification is pre-

The present study shows that H2O2 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 H2O2 does not result in complete inhibition of the exchanger, since pH recovery from an acid load was significantly, although not completely, inhibited by 100 μmol/L H2O2 when 50 mmol/L NH4Cl was used to decrease HAEC pH to a degree that would be expected to maximally activate the Na+/H+ exchanger. H2O2 has been shown to rapidly induce time- and concentration-dependent intracellular ATP depletion in several cell types10,44 including vascular endothelial cells,11 although the mechanism of this effect has not been determined.

Plasmalemmal Na+/H+ exchangers are present in all mam-

malian cells and are involved in pH regulation in many cell

types, including endothelial cells.15,36,43 The Na+/H+ exch-

H2O2 has been shown to rapidly induce time- and con-

centration-dependent intracellular ATP depletion in several

cell types12,16–18 without affecting cell viability.12,17 In the
present study, H2O2 decreased HAEC pH within minutes of oxidant exposure and produced a similarly rapid depletion of intracellular ATP. After only a 2-minute exposure, 100 μmol/L H2O2 decreased intracellular ATP concentration by ~40%. This is similar to previously reported observations in alveolar type II cells, in which a 2-minute exposure to 500 μmol/L H2O2 reduced ATP levels by ~60%.16

Changes in the concentration of intracellular ATP induced by H2O2 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,21 rat ventricular cells,22 sheep Purkinje fibers,26 A431 carcinoma cells,20 alveolar type II cells,24 renal epithe-

lium cells,23 and fibroblasts transfected with the growth factor–
sensitive isofrom of the exchanger, NHE-1.27 Indeed, ATP deple-
tion 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-1–transfected fibroblasts without affecting NHE-1 phosphorylation.27
inhibition by 2-deoxyglucose inhibits the Na\(^+\)/H\(^+\) exchanger, whereas the oxidative inhibitor cyanide does not, despite similar degrees of intracellular ATP depletion (~70%) produced by each.\(^1\) 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.\(^2\)

In the present work, the effects of 100 \(\mu\)mol/L \(\text{H}_2\text{O}_2\) on pH 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.\(^3\) This appears to be due to direct oxidation of sulfhydryl groups on the active site of GAPDH.\(^4\) At lower concentrations (≤100 \(\mu\)mol/L), GAPDH may be partially inhibited,\(^5\) 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\(^+\).\(^6\) 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.\(^7\) 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.,\(^8\) 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\(^9\) containing \(\text{H}_2\text{O}_2\) (100 \(\mu\)mol/L) and 10 \(\mu\)mol/L Fe\(^{3+}\)-nitriloacetate 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.\(^10\)\(^14\) 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 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 ~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 pathophysiologic 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.

Acknowledgments
This study was supported in part by National Heart, Lung, and Blood Institute grants HL-03102 and HL-52315.

References
Hydrogen Peroxide Decreases pH\textsubscript{i} in Human Aortic Endothelial Cells by Inhibiting Na\textsuperscript{+}/H\textsuperscript{+} Exchange
Qinghua Hu, Yong Xia, Stefano Corda, Jay L. Zweier and Roy C. Ziegelstein

Circ Res. 1998;83:644-651
doi: 10.1161/01.RES.83.6.644

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/83/6/644

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/