Intracellular pH in Human Resistance Arteries in Essential Hypertension

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To investigate intracellular pH (pH,) in human resistance arteries in essential hypertension, vessels were obtained from small biopsies of skin and subcutaneous fat from 14 untreated patients, and the results were compared with those from 14 matched normotensive control volunteers. Segments of isolated resistance arteries were mounted in a myograph and loaded with the pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein. Fluorescence signals were monitored using a series of barrier filters and chromatic beam splitters. In this way both resting pH, and the changes in pH, observed during isometric contractions initiated by agonists could be recorded. Resting pH, was not different in vessels from hypertensive patients (hypertensive, 7.24±0.06 versus control, 7.25±0.04 pH units). The application of ethylisopropylamiloride (EIPA) and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) demonstrated that both Na+-H+ exchange and bicarbonate-dependent membrane mechanisms contributed to pH, homeostasis but that neither system was overactive in hypertension (pH, change with EIPA in vessels from hypertensive versus control subjects was —0.11±0.02 and 0.13±0.03 pH units, respectively, and pH, change with DIDS in vessels from hypertensive versus control subjects was —0.097±0.05 and —0.091±0.03 pH units, respectively). The application of norepinephrine or 125 mM K+ solution induced contraction in the arterial segments with an accompanying fall in pH,. With norepinephrine this fall was significantly attenuated in vessels from hypertensive patients. These results fail to provide evidence for raised pH, in resistance arteries in human essential hypertension, and contrary to previous reports in circulating blood cells, Na+-H+ exchange is not overactive in the vessels of such patients. (Hypertension 1991;17:780-786)

Abnormalities of cell membrane ion transport have been frequently observed in cells from hypertensive humans and in animal models of hypertension.1 Furthermore, there has recently been considerable interest in the membrane regulation of intracellular pH (pH,) in hypertension because it has been suggested that pH, has an important function in the regulation of intracellular events.1-3 There are a number of reports on circulating cells from hypertensive humans concerning the activity of the Na+-H+ exchanger, a ubiquitous transporter in vertebrate cells that regulates pH, and that appears to be operative in platelets,4,6 erythrocytes,5 and leukocytes.7 However, the data are inconsistent with regard to resting pH, in hypertensive humans, with an increase,8 decrease,9 or no difference9 being observed. In hypertension, all studies that demonstrate abnormalities in surrogate cells assume that the disturbance is shared by vascular smooth muscle cells (VSMC). In such vascular tissues in hypertensive humans, increased Na+-H+ exchange has yet to be demonstrated. If such an abnormality is observed, this may result in cell alkalization; alternatively, Na+-H+ exchange may be increased as a consequence of increased metabolic acid production. It has been proposed that there is a link between a disturbance in cell calcium metabolism and increased Na+-H+ exchange in hypertension.10,11

In addition to Na+-H+ exchange, the bicarbonate ion at physiological concentrations has been shown to be of fundamental importance in pH, homeostasis in VSMC.12-14 Therefore with regard to pH, measurements, it is essential that experiments be performed in a CO2/HCO3- containing buffer.15 In this context, there are published studies in the lymphocyte and in resistance vessels from the spontaneously hypertensive rat where pH, has been examined in bicarbonate-containing buffers.3,11,16,17 However, such studies have not been reported in arteries from human hypertensive patients, and as a result it was decided...
to examine pH$_{i}$ in intact segments of resistance arteries from untreated essential hypertensive patients and matched normotensive controls. Because contraction is the primary function of VSMC, the technique we used enabled pH$_{i}$ and isometric contraction to be simultaneously recorded.\textsuperscript{18}

The present studies were performed to examine resting pH$_{i}$ in hypertensive resistance arteries and matched controls. The involvement of Na$^{+}$-H$^{+}$ exchange and bicarbonate transport under physiological conditions was determined by blocking these systems with ethylisopropylamiloride (EIPA) or 4,4'$'$-diisothiocyanatostilbene-2,2'$'$-disulfonic acid (DIDS) and observing the effect on pH$_{i}$. The pH$_{i}$ response to the contractile stimuli, namely, norepinephrine and high potassium buffer, were also observed.

**Methods**

**Subjects**

Fourteen patients with essential hypertension were recruited from the outpatient clinic of the Department of Medicine, Leicester Royal Infirmary, Leicester, UK. No subject had ever received any antihypertensive medication, and all patients were thoroughly screened for secondary causes of hypertension. All patients had blood pressures greater than 140/95 mm Hg when measured on at least three occasions using a Hawksley random-zero sphygmomanometer. The results obtained from this group of patients were compared with those from a group of normotensive subjects matched for age, sex, and body weight who were recruited by the use of an advertisement placed in a local newspaper. All participants were informed of the nature of the experiment and gave their consent in accordance with the regulations of the local ethical committee.

**Preparation**

Artery segments about 2 mm long were dissected from biopsies (about 0.5x0.5x1.5 cm) of skin and subcutaneous tissue taken under local anesthesia (3–5 ml 2% lidocaine hydrochloride) from the gluteal region.\textsuperscript{18}

**Solutions and Chemicals**

Vessels were removed and normally held unstimulated in physiological salt solution (PSS) with the following composition (mmol/l): NaCl 119, KCl 4.7, CaCl$_{2}$ 2.5, MgSO$_{4}$ 1.17, NaHCO$_{3}$ 25, KH$_{2}$PO$_{4}$ 1.18, ethylenediamine tetraacetic acid (EDTA) 0.026, and glucose 5.5. K-PSS was similar except for equimolar substitution of KG for NaCl. Both solutions had a pH of 7.45–7.50 when gassed with 5% CO$_{2}$ in O$_{2}$. Calibration experiments were carried out using K-HEPES buffer that had the following composition (mmol/l): KCl 140, MgCl$_{2}$ 1, CaCl$_{2}$ 1.6, EDTA 0.026, glucose 10, and HEPES 5.

Norepinephrine hydrochloride nigericin and DIDS were purchased from Sigma Chemical Co., Poole, Dorset, UK, and 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and BCECF-AM were obtained from Molecular Probes, Eugene, Oregon. EIPA was obtained from Dr. E.J. Cragoe Jr. and synthesized as previously described.\textsuperscript{19}

**Vessel Mounting, Morphology, and Normalization**

After removal and cleaning, the resistance vessels were mounted in a myograph.\textsuperscript{20} After mounting, the segments were maintained in PSS at 37°C for 1 hour and then set to an internal circumference at which they were held just under tension. By means of water immersion microscopy the smooth muscle layer thickness was measured at six sites and the lumen diameter at three sites.\textsuperscript{18} The resting tension–internal circumference relation was then determined\textsuperscript{18} and the vessels set to a normalized internal circumference, which was 90% of that which the vessel would have when relaxed and under a transmural pressure of 100 mm Hg.\textsuperscript{18} The vessels were then activated with K-PSS three times followed by norepinephrine once (5 µmol/l in K-PSS). Each solution was applied for 2 minutes before washing with PSS, and the vessels were allowed to relax fully between these activations. Criteria for rejection of results from experiments were arteries that were found to have an internal diameter greater than 300 µm or that were unable to contract against a pressure of 100 mm Hg.\textsuperscript{18} In the present study, none of the vessels were rejected from either group of subjects.

**Measurement of Intracellular pH**

Intracellular pH in intact vessels was measured as previously described.\textsuperscript{16,17} The myograph was placed on the stage of a standard laboratory 16 upright microscope (Carl Zeiss, Oberkochen Ltd, FRG) fitted with a photomultiplier tube and a xenon light source. The mounted vessel segment was visualized using a ×25 water immersion objective. The light source for fluorescence measurements was provided by a xenon lamp connected to a voltage stabilizer and attenuated 80% by a neutral density filter. The preparation was excited at two wavelengths (485 and 455 nm) each with a band width of 10 nm by using an assembly of excitation filters and chromatic beam splitters. Emissions from the vessels at 520–560 nm were collected through appropriate barrier filters. Light signals were recorded using a BBC computer linked to the photomultiplier tube, and induced isometric contractions were measured using a chart recorder (Grass Instruments, Quincy, Mass.).

**Background Fluorescence**

Before loading the artery with fluorescent dye, it was important to measure background natural fluorescence. Therefore, readings were taken when the vessels were excited at 485 and 455 nm for 2.5 seconds at each wavelength. Five readings were taken, each separated by a period of 1 minute, from which the mean background fluorescence was calculated at each excitation wavelength.
Dye Loading

The pH-sensitive fluorescent dye used was BCECF. The methyl ester BCECF-AM (5 μmol/l) was added to the myograph and left to load for 60 minutes, at which time the excess was washed out. A fluorescence ratio was determined every 60 seconds: this was achieved by dividing the emission with excitation at 485 nm minus background at 485 nm by emission with excitation at 455 nm minus background at 455 nm. As with the background measurements, excitation was for 2.5 seconds at each wavelength.

Protocol

The effects of inducing contraction on resting pH were investigated using two different stimuli, namely K-PSS and norepinephrine (5 μmol/l). Fluorescence signals were measured five times at the start and the mean of each set of readings was used to determine resting pH. Before any stimulus was added, pH was measured three times to define a baseline; K-PSS and norepinephrine were added for 5 minutes, and changes in pH were monitored at minute intervals.

The effect on resting pH of blockade of Na⁺-H⁺ exchange was then examined using EIPA, with fluorescence measurements being made every minute for 10 minutes. Resting pH was also investigated in the presence of DIDS (200 μmol/l), an anion transport inhibitor that blocks bicarbonate-dependent pH regulation. A representative trace of the ratio recordings during the protocol is shown in Figure 1.

Calibration

Calibration of the intensity ratio was achieved using nigericin. At the end of the experiments, three solutions of 140 mmol/l K-HEPES were set to different pH values in the range 6.6–7.6 (which is the range in which the fluorescence ratio is linear with pH), and 10 μmol/l nigericin was added to each. These solutions were warmed to 37°C, and the exact pH of each was measured using a Corning microcombination pH electrode (Fisons, Loughborough, UK). The first solution was applied to the myograph for 8 minutes, and the subsequent solutions were added for 5 minutes each. Fluorescence ratios were recorded throughout the procedure. Nigericin is a K⁺-H⁺ ionophore that will equilibrate pH and extracellular pH (pHe) in high potassium buffers. The mean of the last three readings with each solution was recorded, at which time the fluorescence signals had reached a plateau, indicating the pHe and pH had reached equilibrium. Using this technique, a linear regression line could be calculated and the other intensity ratios evaluated to give true pH readings. In all experiments the correlation coefficient was 0.98 or greater indicating an effectively linear curve.

Results are expressed as mean±SEM. Data from hypertensive patients and control subjects were analyzed with the Mann-Whitney U test, and changes in pH with time within and between groups were analyzed with analysis of variance.

Results

Resistance arteries were studied from 14 patients (nine men) with essential hypertension. These were matched with 14 normotensive control subjects (nine men). There were no significant differences in mean age, height, or body weight, although the control subjects were slightly older (Table 1). The hypertensive patients had significantly higher blood pressures in both lying and standing positions (Table 1).

Morphology

Fourteen arteries were studied from each group. Mean media thickness was increased in vessels from hypertensive patients, although this did not attain statistical significance when compared with control arteries (14.9±0.72 versus 12.8±0.75 μm, p=0.059).
Similarly, media volume (medial cross-sectional area) was not significantly different between vessels from hypertensive patients compared with control subjects (9,656±734 versus 8,887±759 μm², p=0.48). Mean lumen diameter was not different between the two groups of vessels (190±9.0 versus 206±10.3 μm, p=0.25). Mean media/lumen ratio was significantly raised in arteries from hypertensive patients (7.92±0.4 versus 6.4±0.47%, p=0.022). The 16.4% increase in media thickness was less than we have previously recorded, although the current group of patients had lower blood pressures compared with the previously studied group. The other parameters are in agreement with previous work.

Resting Intracellular pH

The resting pH in resistance arteries from hypertensive patients was not different from that observed in vessels from control subjects (7.24±0.06 versus 7.25±0.04 pH units, n=14; Figure 2). There was no correlation between age and pH, or blood pressure and pH, in arteries from patient or control groups or when the data were pooled (results not shown).

Experiments in Potassium–Physiological Salt Solution

After exposure to K-PSS for 5 minutes, there was a significant acidification observed in arteries from both hypertensive patients and normotensive control subjects (Figure 3). The change in pH, was not significantly different between the two groups of arteries (Figure 3).

Experiments With Norepinephrine

Activation of vessels with 5 μmol/l norepinephrine again led to a significant acidification (Figure 4) that was significantly attenuated in vessels from hypertensive patients (p<0.01; Figure 4).

Contractility Studies

Activation with norepinephrine (5 μmol/l) resulted in the production of media stress (force per unit media area), which did not differ in vessels from patients compared with those from control subjects (117±11.4 versus 109±11.0 mN/mm², NS). Similarly, K-PSS did not induce a difference in media stress production (hypertensive, 91±10.3 versus control, 94±12.3 mN/mm², NS).

Experiments With Ethylisopropylamiloride

EIPA (60 μmol/l) applied for 10 minutes caused a fall in pH, in both groups of arteries (Figure 5). The change in pH, was not different between vessels from patients compared with those from control subjects (−0.11±0.02 versus −0.13±0.03 pH units, NS; Figure 5).

Experiments With DIDS

Exposure to the anion exchange inhibitor DIDS also resulted in acidification in both groups of vessels

**FIGURE 2.** Plotting of individual intracellular pH (pHi) values from resistance vessels from 14 hypertensive patients (○) and 14 matched normotensive control subjects (●). NS, not significant.

**FIGURE 3.** Line graph showing change in intracellular pH (pHi) induced over 5 minutes by 125 mmol/l potassium physiological salt solution in subcutaneous arteries from hypertensive (○) and control (●) subjects. Results show mean±SEM at minute intervals. NS, not significant (analysis of variance, hypertensive versus control subjects).

**FIGURE 4.** Line graph showing change in intracellular pH (pHi) induced over 5 minutes by norepinephrine (5 μmol/l) in subcutaneous arteries from hypertensive (○) and control (●) subjects. Results show mean±SEM at minute intervals. *p<0.01 (analysis of variance, hypertensive versus control subjects).
FIGURE 5. Line graph showing mean±SEM change in resting intracellular pH (pHi) over 10-minute period in presence of ethylisopropylamiloride (60 µmol/l) in subcutaneous arteries from hypertensive (○) and control (●) subjects. Results show mean±SEM at minute intervals. NS, not significant (analysis of variance, hypertensive versus control subjects).

Discussion

The present study is the first to investigate pHi in human resistance arteries in essential hypertension. Using the pH-sensitive dye BCECF, we measured pHi and found no difference in resting pHi in arteries from hypertensive patients compared with those from matched normotensive controls. Although segments of resistance arteries comprise several cell types, smooth muscle cells make up nearly 90% of the tissue at this level. Therefore, when using BCECF, the obtained signal will be essentially from smooth muscle. However, it might be suggested that, because an intact resistance vessel is a composite of cell types, a difference in VSMC pHi could be missed in vessels from patients with essential hypertension. This is unlikely because a significant difference in pHi was observed in spontaneously hypertensive rat mesenteric resistance vessels using the same technique,16 which prompted the current investigation. Blockade of the Na+-H+ exchange with EIPA resulted in an acidification that was of the same magnitude in both groups, suggesting that Na+-H+ exchange is involved in maintaining resting cell pHi and that the reliance of the cells on this system is identical in arteries from both the hypertensive and the control groups. The anion exchange inhibitor DIDS also produced a fall in pHi. This disulfonic stilbene derivative will inhibit bicarbonate-dependent acid extrusion, possibly via a Na+-dependent HCO3−-Cl− exchanger14 or an inwardly directed Na+-HCO3− cotransporter, as recently demonstrated in a smooth muscle–like cell line.23 Also, this stilbene derivative will inhibit Cl−-HCO3− exchange, which extrudes alcali; evidence of this transport system in vascular smooth muscle has been demonstrated.21,24 The acidification observed indicates that in the resting state, bicarbonate-dependent acid extrusion is the dominant mechanism. Because the fall in pHi was identical in hypertensive patients and control subjects, the data do not suggest that there is any disturbance in DIDS-sensitive pHi regulation in resistance arteries in established essential hypertension.

Stimulation of the vessels with K-PSS and norepinephrine produced a sustained acidification over a 5-minute period similar to that reported in rat mesenteric vessels.16 Because depolarization with K-PSS reverses the inwardly directed H+ ion electrochemical gradient, it seems plausible that contraction results in an increase in metabolic acid production and pHi falls in consequence. In addition, K-PSS contains 25 mmol/l Na+ that may inhibit the capacity of Na+-dependent pH regulators to extrude acid. The acidification observed on activation with K-PSS was not different between the hypertensive and control groups. Conversely, the acidification observed when the vessels were stimulated with 5 µmol/l norepinephrine (which elicits a maximum contractile response to this agonist in human vessels18) was significantly reduced in vessels from the hypertensive group. It is uncertain how this is brought about; many aspects of cell metabolism might be responsible, among which would be the possibility of enhanced Na+-H+ exchange in resistance vessels from hypertensive patients during stimulation with norepinephrine. At this time the exact mechanism has not been elucidated.

In contrast to the results found in leukocytes and erythrocytes,7,8 we found no difference in resting intracellular pH in arteries from hypertensive patients compared with those from the control subjects. Moreover, the acidification observed on blockade of the Na+-H+ exchanger was identical in both groups,
which is at variance with the possibility of increased Na\(^+\)-H\(^+\) activity secondary to increased metabolic acid production.\(^6\) However, there was a reduced acidification in vessels from the hypertensive group in the presence of norepinephrine; whether this finding is a fundamental difference or a consequence of the blood pressure rise is uncertain at this time. However, after activation with norepinephrine media stress was the same in both groups; therefore, this finding does not appear to bear any direct relation to vascular contraction.

The findings in the presence of EIPA do not rule out the possibility of subtle differences in Na\(^+\)-H\(^+\) exchange activity in resistance vessels from hypertensive patients compared with those from normotensive control subjects. A powerful inhibitor such as EIPA might abrogate such differences. However, if Na\(^+\)-H\(^+\) exchange were increased in the resting cell to offset increased metabolic acid production in hypertension, then blockade of this transporter would result in a greater acidification than in resistance vessels from control subjects. However, this was not observed. To examine Na\(^+\)-H\(^+\) exchange or DIDS-sensitive acid extrusion in physiological conditions (i.e., at resting pH\(_i\)), the transporter is blocked and the effect on pH\(_i\) recorded. Other methods of assessing these exchangers require the measurement of the initial rate of recovery from an acid load. However, when this technique is used transport activity is assessed at pH\(_i\) values well below resting pH\(_i\). The same argument applies to kinetic studies. The present investigation was not intended to investigate the kinetics of Na\(^+\)-H\(^+\) exchange or bicarbonate transport in resistance vessels in hypertension. It was felt that it was important to examine the dependence of resting pH\(_i\) on these transporters that may be of pathophysiological importance.

The arteries in which these measurements were made are small enough to be intimately involved in the control of peripheral vascular resistance. Furthermore, the use of an intact segment permitted simultaneous recordings of contraction to be made. Therefore, the findings in terms of pH\(_i\) cannot be ascribed to "phenotype modulation,"\(^20\) which is so often the case with work using cells held in culture media.\(^27\) It might be argued that in vivo differences in arteries from hypertensive patients compared with those from control subjects are lost as a result of removing the tissues from the body and studying them in vitro. This is unlikely for a number of reasons. First, we have shown previously that there is an increased pH\(_i\) in resistance arteries from spontaneously hypertensive rats due to enhanced Na\(^+\)-H\(^+\) exchange using the same technique,\(^16\) and recently these findings have been confirmed in vivo using nuclear magnetic resonance.\(^28\) Second, resting pH\(_i\) in surrogate tissues such as blood cells studied in similar conditions ex vivo reported differences in hypertension although at variance with each other.\(^7\) Third, we have recently reported significant differences in the effects of DIDS and EIPA on resting pH\(_i\) in resistance arteries from rats with experimentally induced hypertension at various time points from the inception of hypertension.\(^29\) Finally, by using ex vivo methods, increased Na\(^+\)-H\(^+\) exchange has been observed in isolated renal brush border membrane vesicles from rats treated with thyroid hormone or subjected to an acidosis.\(^30\)

It has been hypothesized that increased Na\(^+\)-H\(^+\) exchange in vascular smooth muscle in essential hypertension will increase vascular tone as a consequence of cell alkalinization.\(^31\) In addition, agonist-induced alkalinization has been proposed to be of importance in sustained vascular contraction.\(^32,33\) Studies using isolated vascular tissue argue against these possibilities. First, studies using mesenteric arteries,\(^34\) rabbit aorta,\(^35\) and rabbit ear arteries\(^36\) have shown that alkalinization dilates and acidification enhances contraction; this may be due to competition between protons and calcium ions for common intracellular buffer sites.\(^37,38\) Using skinned vascular tissue, a lower pH shifts the calcium dose-response curve to the left.\(^39\) Second, with regard to the pH\(_i\) change in response to pressor stimuli, no alkalinization has been observed in hog carotid arteries,\(^40\) rat mesenteric arteries,\(^12,16\) or rat femoral arteries,\(^41\) a finding that we also report here for the first time in human subcutaneous resistance arteries.

We found no difference between resting pH\(_i\) in resistance arteries from hypertensive humans compared with those from age- and sex-matched control subjects. Furthermore, there is no evidence of an increase or decrease in reliance of basal pH\(_i\) on the EIPA- or DIDS-sensitive pH regulators, suggesting that in the resting state, proton metabolism is normal, although a difference was observed on activation with norepinephrine. In conclusion, we found no evidence of an elevation in basal pH\(_i\), enhanced Na\(^+\)-H\(^+\) exchange, or functional changes in subcutaneous resistance vessels from hypertensive humans.

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