Mechanisms of endothelial cell swelling from lactacidosis studied in vitro

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Behmanesh, S., and O. Kempski. Mechanisms of endothelial cell swelling from lactacidosis studied in vitro. Am J Physiol Heart Circ Physiol 279: H1512–H1517, 2000.—One of the early sequelae of ischemia is an increase of circulating lactic acid that occurs in response to anaerobic metabolism. The purpose of the present study was to investigate whether lactic acidosis can induce endothelial swelling in vitro under closely controlled extracellular conditions. Cell volume of suspended cultured bovine aortic endothelial cells was measured by use of an advanced Coulter technique employing the “pulse area analysis” signal-processing technique (CASY1). The isosmotic reduction of pH from 7.4 to 6.8 had no effect on cell volume. Lowering of pH to 6.6, 6.4, or 6.0, however, led to significant, pH-dependent increases of cell volume. Swelling was more pronounced in bicarbonate-buffered media than in HEPES buffer. Specific inhibition of Na\(^+/\)H\(^-\) exchange by ethylisopropylamiloride completely prevented swelling in HEPES-buffered media. Pretreatment with ouabain to partially depolarize the cells did not affect the degree of acidosis-induced swelling. In bicarbonate-buffered media, the inhibition of transmembrane HCO\(_3\)\(^-\) transport by DIDS reduced swelling to a level comparable with that seen in the absence of bicarbonate ions. Lactacidosis-induced endothelial swelling, therefore, is a result of intracellular pH regulatory mechanisms, namely, Na\(^+/\)H\(^-\) exchange and bicarbonate-transporting carriers.

Lactacidosis; bovine aortic endothelial cells; pH regulation

CAPILLARY DIAMETERS under pathophysiological conditions may be reduced below those of the traversing undeformed red and white blood cells (16) and, therefore, may jeopardize cell passage through the microcirculation as well as nutritive blood flow. The major cause of the reduction of capillary patency is endothelial swelling, as observed in hemorrhagic shock (14, 16) or systemic blood acidosis, but not at low flow conditions per se (15). Swelling in these experiments could be prevented by amiloride analogs, as an indication that a Na\(^+/\)H\(^-\) exchanger (NHE) participates in the swelling process. NHEs have been widely studied as membrane mechanisms involved in the regulation of intracellular pH (pHi) and are present in endothelial cells (4, 10). Five NHE isoforms (NHE1–NHE5) represent the major acid-extruding transporters in the absence of bicarbonate (HCO\(_3\)). In vivo, however, HCO\(_3\) is usually available, and HCO\(_3\)-dependent systems contribute to pH\(_i\) regulation; among these, the Na\(^+\)-dependent Cl\(^-\)/HCO\(_3\) exchanger (NCBE) has been found activated in acidosis in bovine aortic endothelium (4). In addition, Na\(^+\)-HCO\(_3\) cotransport is activated in corneal endothelium (1, 2, 10). Na\(^+\)-independent Cl\(^-\)/HCO\(_3\) exchange (CBE) is known to maintain pH\(_i\) after alkalinization. All three systems can be inhibited by DIDS.

There are very few in vitro studies concerned with cell-volume homeostasis of endothelial cells. Those available mostly deal with mechanisms involved in cell-volume regulation after exposure to osmotic stress (e.g., 11, 19, 23). In other cell types such as glia, swelling mechanisms during extracellular acidosis have been studied in detail (9, 12, 20, 22, 24). The purpose of the current in vitro study was, therefore, to evaluate the postulated swelling effect of extracellular lactacidosis on endothelial cell volume under closely controlled extracellular conditions and to characterize the transport systems involved.

MATERIALS AND METHODS

Cell culture. Bovine aortic endothelial (BAE) cells (Stanford University) (5) were maintained as monolayers in petri dishes with the use of DMEM containing 25 mmol/l HCO\(_3\), 10% bovine fetal calf serum (FCS), 10 IU/ml penicillin G, and 50 \(\mu\)g/ml streptomycin. The cells were cultured at 37°C in humidified room air containing 5% CO\(_2\). Subcultivation was performed 2–3 times/wk by washing with physiological saline and trypsinization (0.05% trypsin and 0.02% EDTA). They were subsequently suspended in medium containing FCS for inactivation of trypsin. After centrifugation, the cells were again washed in physiological saline to remove FCS. For experiments, cells were used once they had reached a confluent stage, usually 5–6 days after subcultivation. To investigate endothelial cell swelling, suspended cells were then transferred into an incubation chamber, which allowed for close control of the extracellular environment (12, 13, 24). The chamber was equipped with two apertures for measurement of pH and temperature by respective electrodes. A gas-permeable silicon rubber tubing within the chamber served as a membrane oxygenator, providing cells with a...
mixture of O₂, CO₂, and N₂ for experiments with HCO₃⁻ buffering or with O₂ and N₂ in HEPES-buffered experiments. A magnetic stirrer prevented cell sedimentation.

To confirm results obtained with BAE cells, endothelial cells from human umbilical cord were used. They were prepared by use of modifications of established methods (8). Briefly, the vessel was mounted on a proximal cannula to clear the clotted blood from the lumen, using medium 199. The endothelial cells from primary culture were grown in medium 199, which contained 25 mmol/l HCO₃⁻, 10% FCS, 100 IU/ml penicillin G, 50 μg/ml streptomycin, and 1% endothelial cell growth factor.

Measurement of cell volume. Cell volume was determined by CASY1 technology (Coulter technique employing the “pulse area analysis” signal-processing technique; Schärfe System, Reutlingen, Germany) (24). It combines an established measurement technique, the “resistance measuring principle,” with the pulse area analysis signal-processing technique. For measurement, the suspended cells are introduced into the measuring cell through a capillary of predefined geometry at a constant-stream velocity. During the measurement, a current is supplied to the capillary via two platinum electrodes. The capillary filled with electrolytes has a defined electrical resistance. While passing the capillary, the cells displace electrolyte solution in proportion to cell volume. Because intact cells have isolation properties, resistance along the capillary rises. The measuring signal is scanned by CASY1 at a frequency of 1 MHz. CASY1 captures the amplitude and width of the pulse and determines the integral of the measuring signal (pulse area analysis). This procedure allows for measurements with a dynamic range of >1.320,000 in volume. To store this wide volume range with high resolution, a multichannel analyzer with 512,000 volume-linear channels is used. Each of 512,000 registers contains the number of cells that have produced the corresponding pulse area value while passing through the measuring pore. From this volume-linear original size distribution, a diameter-linear size distribution with a resolution level of 1,024 channels is computed. All subsequent measuring parameters are determined on the basis of this size distribution.

Experimental groups. Experiments were performed after a 20-min control period utilized for baseline measurements of cell volume, cell viability, and medium osmolality at an extracellular pH (pHₑ) of 7.4. Data from three cell-volume measurements obtained during this control phase (15, 10, and 5 min before the experimental phase) were averaged, and all measurements, including the three baseline samples, were expressed as a percentage of the reference value thus obtained. This permitted the highlighting of volume changes constancy during the control phase as well as during experimental conditions. Unstable cell volume or an impaired cell viability (trypan blue exclusion) during the control phase led to discharge of the cells. In a control group, the pHₑ of the medium was maintained at 7.4 for 1 h (n = 3). In four experimental groups, pHₑ was lowered from an initial 7.4 (control phase) to either 6.8, 6.6, 6.4, or 6.0 by addition of isotonic lactic acid (350 mmol/l) to the HCO₃⁻-buffered cell suspension (5–8 experiments/group). The required volumes of lactic acid were read from a titration curve determined beforehand. CO₂ loss from buffering during acidosis was compensated for by increasing the PₐCO₂ in the membrane oxygenator under control of medium pH and PₐCO₂ (ABL Radiometer, Copenhagen, Germany). Cell volume and viability were monitored for 25 min during lactacidosis. Osmolarity was assessed under control conditions as well as after induction of lactacidosis (Osmomat 030, Gonotec). Respective experiments were repeated in HEPES-buffered medium (40 mmol/l) in the virtual absence of HCO₃⁻ (n = 5–6/group). To verify the data from experiments with BAE cells, the study was also performed with human umbilical cord endothelial cells (HUVEC) at pH 6.4 and 6.0 (n = 6/group) in HCO₃⁻-buffered media.

In the second part of the study, we focused on the mechanisms responsible for swelling using inhibitors of transport systems involved in pHₑ control: 5-(N-ethyl-N-isopropyl) amiloride (EIPA) hydrochloride was applied in a concentration of 5 μmol/l to inhibit Na⁺/H⁺ exchange in the virtual absence of HCO₃⁻ (HEPES buffered) in experiments where pHₑ was reduced to 6.0. A concentration of 1 mmol/l DIDS was used in the presence of HCO₃⁻ (25 mmol/l, 5% CO₂) to inhibit HCO₃⁻ transporters such as Na⁺/HCO₃⁻ co-transport or Cl⁻/HCO₃⁻ exchange, again during acidosis at pHₑ = 6.0. Ouabain (1 mM) was used to inhibit Na⁺/K⁺ exchange (1) during 45 min of baseline conditions at pHₑ = 7.4 and 2) during acidosis (pHₑ = 6.0) after 10 min of pretreatment with ouabain to partially depolarize cells. In addition, experiments with acidosis at pHₑ = 6.0 were performed in Na⁺-free HEPES-buffered medium, where NaCl was replaced by choline chloride and HCO₃⁻ by HEPES.

Statistics. Data are expressed as means ± SE. As a parametric test, a one-way repeated-measures analysis of variance (ANOVA) was used, and, as a nonparametric test, a repeated-measures ANOVA on ranks according to Friedman was used. Experimental groups were compared by ANOVA (Sigmastat, Jandel Scientific). Differences were considered significant if P < 0.05.

RESULTS

Under control conditions, at pHₑ = 7.4 and 37°C, volume and viability of the BAE cells remained stable for up to 70 min; longer periods were not studied. The average BAE cell volume was 1,232.3 ± 38.1 μm³ for up to 70 min; longer periods were not studied. The volume and viability of the BAE cells remained stable during acidosis. Osmolarity was assessed under control conditions as well as after induc-
viability decreased fast, which may have prevented the detection of further cell-volume increases.

Endothelial cells suspended in HEPES-buffered medium had a mean cell volume of 1,200.4 ± 12.6 μm³, i.e., cells were only negligibly smaller than in HCO₃⁻-buffered medium. In HEPES-buffered medium, lactic acidosis again induced significant increases of cell volume if pHₑ was reduced to or below 6.6 (Fig. 2). During the whole observation period, swelling had approximately the same time course as in HCO₃⁻-buffered media, although the degree of swelling was significantly attenuated.

Experiments with HUVECs were in agreement with above observations (Fig. 3). Again, swelling occurred at pHₑ ≤ 6.6 (data not shown).

Inhibition of acidosis-induced endothelial cell swelling. In the absence of HCO₃⁻, the increase of cell volume at pHₑ = 6.0 could have been completely prevented if the cells had been pretreated with EIPA, the specific inhibitor of Na⁺/H⁺ exchange (Fig. 4). Under baseline conditions, the presence of EIPA had no effect on cell volume.
As indicated by Fig. 5, addition of DIDS, an inhibitor of Cl⁻/HCO₃⁻ countertransport and Na⁺-HCO₃⁻ cosubstrate, did not fully block cell swelling in HCO₃⁻-buffered media. Interestingly, in the presence of DIDS, the remaining volume response to lactacidosis was very similar to that observed in HEPES-buffered medium without DIDS pretreatment (Fig. 5).

These findings suggest that Na⁺ and HCO₃⁻ transport across the cell membrane are the main factors responsible for acidosis-induced endothelial swelling. Further evidence supporting this view came from experiments in Na⁺-free medium (Fig. 6). Na⁺ and HCO₃⁻ were replaced by choline chloride and HEPES under strict maintenance of isotonicity. This by itself was found to cause a progressive, gradual decrease of cell volume, probably because of outward leakage of intracellular Na⁺ down the concentration gradient. As shown in Fig. 6, virtually no swelling was found at lactacidosis of pHe = 6.0 in the absence of Na⁺.

In an effort to affect membrane potential and intracellular Na⁺ concentrations, cells were pretreated with ouabain. As shown in Fig. 7, the degree of swelling in HEPES-buffered media was unaffected by ouabain. Swelling kinetics, however, appeared slowed down because the swelling maximum was reached after 10 min only. Ouabain did not affect cell volume during baseline conditions (pHe = 7.4; Fig. 7, inset).

DISCUSSION

The data accumulated herein indeed confirm former in vivo observations that extracellular acidosis may promote endothelial swelling (15). To understand the mechanisms of endothelial swelling, we have to assume the following sequence of events.

Extracellular acidosis leads to an influx of acid equivalents into the cell as described for astrocytes (12, 17, 18, 20, 22). The degree of acidosis studied here is within a range expected to occur in the parenchyma of heart (21), muscle (25), or brain (reviewed in Ref. 12) during ischemia. In HCO₃⁻/CO₂-buffered media, a fast acid entry is likely. It is caused by CO₂ influx, which diffuses easily through the cell membrane, forming carbonic acid and subsequently H⁺ and HCO₃⁻ in the cytosol. The electrochemical gradient for H⁺ favors the development of cytosolic acidosis already at physiological pH levels. Hence, a normal pH can only be maintained by active pH regulation involving ion transporters, ion channels, and metabolic processes.

Under CO₂/HCO₃⁻-free conditions, the situation is more complex. One might expect enhanced intracellular acidosis compared with baseline conditions, because the cells are HCO₃⁻ depleted. However, because
CO₂ diffusion over the cell membrane is thought to be the major mechanism of intracellular acidification, omission of CO₂/HCO₃ may rather result in an attenuated intracellular acidosis, as seen in glial cells (20).

The observed swelling is assumed to result from pHᵢ regulatory systems such as NHE: the uptake of Na⁺ in exchange for H⁺, which comes from intracellular buffers and is therefore thought to be osmotically inactive (3), increases the osmotic load of the cell and is followed by influx of water. Inhibition of NHE either by EIPA (Fig. 4) or the absence of Na⁺ (Fig. 6) hence prevents swelling in HCO₃-free media. The use of ouabain to partially depolarize the cells results in a delay of slowdown of the swelling response (Fig. 7). This presumably is related to a gradual rise in intracellular Na⁺, which suppresses Na⁺ entry through the NHE.

In the presence of HCO₃, other pHᵢ regulatory transport systems are activated in extracellular acidosis in addition to NHE, i.e., Na⁺-HCO₃ cotransport or NCBE: an involvement of HCO₃-dependent transport processes is suggested by the larger volume increases seen in HCO₃-containing compared with HEPES-buffered media (Figs. 1 and 2) and by the reduction of swelling in DIDS-treated cells to the level seen in the absence of HCO₃ (Fig. 5). HCO₃-dependent transporters so far have not been discussed with respect to endothelial swelling, although their participation in the regulation of endothelial pHᵢ is known (1, 2, 4, 10); at physiological pHᵢ, HCO₃-dependent transport systems contribute more to pHᵢ homeostasis than NHE. In most cell types, the contribution of NHE to pHᵢ homeostasis increases with decreasing pHᵢ (4, 24).

It remains to be determined whether NCBE or Na⁺-HCO₃ cotransport is responsible for HCO₃-dependent swelling, because both are present in endothelium (1, 2, 4, 10). NCBE, however, is less likely to be involved in the swelling response, because uptake of HCO₃ by this transporter and the ensuing buffering of protons generates CO₂, which can readily leave the cell. Because Cl⁻ is exported in exchange for HCO₃, this would imply rather a loss of osmotic activity and, hence, cell shrinkage. Na⁺-HCO₃ cotransport, on the other hand, imports Na⁺ as osmotically active particles together with HCO₃ and, therefore, better explains the observed swelling response. Na⁺-HCO₃ cotransport is electroneutral and can function only if membrane potential permits.

In glial cells, despite the activation of pHᵢ regulatory mechanisms after induction of extracellular acidosis, pHᵢ does not normalize (17, 18, 20). With ongoing pHᵢ regulation, one would assume cell swelling to continue until pHᵢ has normalized. Because swelling kinetics are similar in endothelial cells (Figs. 1–3) and glia (12, 22) (cells swell on acidification with cell volume, reaching a new steady state after a few minutes), it is quite likely that endothelium is likewise acidified in extracellular acidosis. This has to be verified in future experiments. Meller-gard et al. (17, 18) offered three possible explanations for the failing pHᵢ regulation at reduced extracellular pH in astroglia: 1) pHᵢ is not the regulated parameter, 2) the H⁺ extrusion capacity is reduced, and 3) H⁺ leak fluxes are too high. As an explanation, they favored a reduced acid-extrusion capacity by competitive inhibition of the NHE by extracellular protons.

Likewise unexplained so far is the observation that cell swelling ceases at a given plateau in glia (12, 20, 22) as well as in endothelium (Figs. 1 and 2), particularly if pHᵢ does not normalize (20). Under these conditions, pHᵢ regulatory mechanisms and swelling would be expected to continue ion transport until pHᵢ normalizes. A possible explanation might involve an opening of volume-regulated anion channels once cell volume increases (19), which would render cell membranes more permeable for CI⁻, HCO₃⁻, and lactate. Recently, Voets et al. (23) elegantly demonstrated that a decrease of intracellular ionic strength rather than an increase of cell volume triggers the opening of such channels, at least in conditions such as hypotonic endothelial swelling. All processes of the current project were studied in strict isotonicity, which, therefore, makes an involvement of volume-regulated anion channels less likely.

Another possible explanation would be the recently described control of NHE1 activity by an intracellular Na⁺ receptor (7). That receptor is thought to provide a general mechanism for regulating the intracellular Na⁺ concentration in epithelia, where its activation reduces NHE1 activity. In nonepithelial cells, NHE has also been reported to be inhibited by increased intracellular Na⁺ concentration (6). Inhibition of NHE would reduce Na⁺ influx and, hence, cell swelling and could explain the plateau phase observed at all levels of acidosis tested. The experiments employing ouabain to partially depolarize cells, however, only yielded a slowdown of the swelling response but did not affect the plateau phase. Inhibition of Na⁺-K⁺-ATPase by ouabain was presumably followed by a gradual increase of intracellular Na⁺ and should thereby inhibit NHE (and swelling) via activation of the intracellular Na⁺ receptor (7) earlier than in the absence of ouabain. This did not occur. Hence, further experiments including measurements of pHᵢ are required to verify details of the mechanisms of the swelling response in extracellular acidosis.

In conclusion, endothelial swelling in extracellular lactacidosis is a result of an activation of ion transport systems involved in pHᵢ regulation, in particular, NHE and Na⁺-HCO₃ cotransport. Under in vivo conditions, acidosis-induced endothelial swelling may hamper microcirculatory blood flow. Drugs that interfere with pHᵢ regulation can prevent the swelling response and, hence, may positively affect postischemic microcirculation.

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REFERENCES

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