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Extracellular Acidification Induces Human Neutrophil Activation[1]

Analía S. Trevani,[2]*[†] Graciela Andonegui,* Mirta Giordano,* Daniel H. López,* Romina Gamberale,* Fernando Minucci,* and Jorge R. Geffner*[†]

In the current work, we evaluated the effect of extracellular acidification on neutrophil physiology. Neutrophils suspended in bicarbonate-buffered RPMI 1640 medium adjusted to acidic pH values (pH 6.5–7.0) underwent: 1) a rapid transient increase in intracellular free calcium concentration levels; 2) an increase in the forward light scattering properties; and 3) the up-regulation of surface expression of CD18. By contrast, extracellular acidosis was unable to induce neither the production of H_2O_2 nor the release of myeloperoxidase. Acidic extracellular pH also modulated the functional profile of neutrophils in response to conventional agonists such as FMLP, precipiting immune complexes, and opsonized zymosan. It was found that not only calcium mobilization, shape change response, and up-regulation of CD18 expression but also production of H_2O_2 and release of myeloperoxidase were markedly enhanced in neutrophils stimulated in acidic pH medium. Moreover, extracellular acidosis significantly delayed neutrophil apoptosis and concomitantly extended neutrophil functional lifespan. Extracellular acidification induced an immediate and abrupt fall in the intracellular pH, which persisted over the 240-s analyzed. A similar abrupt drop in the intracellular pH was detected in cells suspended in bicarbonate-supplemented PBS but not in those suspended in bicarbonate-free PBS. A role for intracellular acidification in neutrophil activation is suggested by the fact that only neutrophils suspended in bicarbonate-buffered media (i.e., RPMI 1640 and bicarbonate-supplemented PBS) underwent significant shape changes in response to extracellular acidification. Together, our results support the notion that extracellular acidosis may intensify acute inflammatory responses by inducing neutrophil activation as well as by delaying spontaneous apoptosis and extending neutrophil functional lifespan.   *The Journal of Immunology,* 1999, 162: 4849–4857.

eutrophils play an important role in host defense against infectious agents and are also involved in the pathogenesis of a plethora of inflammatory conditions (1, 2). Previous studies have demonstrated that certain neutrophil responses can be affected by changes in the extracellular concentration of hydrogen ions (3–6). However, the effect of extracellular acidosis on neutrophil physiology has not been clearly established.

Extracellular acidosis is a condition commonly associated to a variety of physiological and pathological situations. It has been reported that urine pH values in distal tubules and bladder can fall below 5.5 under physiological conditions (7). It has also been shown that the mean crevicular pH varies from moderately basic (7.7) to mildly acidic (6.5) (8). Other studies have demonstrated that severe tissue ischemia induces extracellular acidosis (9, 10). Measurements of pH achieved in drainage fluids as well as in situ in different tissues have demonstrated that interstitial acidification is a common feature associated with inflammatory processes,

where extracellular pH (pH_o)[3] values as low as 6.1 have been documented (11–15). On the other hand, exhaustive researches have shown that tumor microenvironments are usually more acidic than the normal ones, with values of pH ranging from 5.8 to 7.6, both in human and rodent malignant tissues (16, 17).

Considering the widespread distribution of acidic microenvironments, it is surprising that to date the effect of interstitial acidosis on neutrophil physiology remains to be resolved. Early studies have shown that acidic pH_o per se increases neutrophil locomotion (3, 6, 18). Many studies have also examined the effect of pH_o on the activation of the respiratory burst (5, 19–21). These works have shown that O_2^- production induced either by opsonized zymosan (Zy), FMLP, or PMA is substantially inhibited at acidic pH_o (5, 19–21). By contrast, studies performed some years ago in our laboratory have shown that acidic pH_o markedly enhances oxygen-dependent cytotoxic responses elicited by conventional agonist such as precipiting immune complexes (pIC), Zy, FMLP, and Con A (22). These apparent contrasting findings may be explained considering that the production of reactive oxygen species other than O_2^- could be increased under extracellular acidic conditions and that these species were responsible for the cytotoxic effect.

The current study was undertaken to determine the effect of extracellular acidification on neutrophil functions. The results to be presented suggest that acidic pH_o is in itself able to induce neutrophil activation. Moreover, extracellular acidic conditions enhance neutrophil proinflammatory responses triggered by conventional agonists. Our findings are consistent with the hypothesis that

[3] Abbreviations used in this paper: pH_o, extracellular pH; pH_i, intracellular pH; $[Ca^{2+}]_i$, intracellular free calcium concentrations; pIC, precipiting immune complexes; Zy, opsonized zymosan; Fluo 3-AM, Fluo 3 acetoxymethyl ester; carboxy SNARF-1-AM, carboxy SemiNaphtoRhodaFluor acetoxymethyl ester; MFSC, mean forward scatter; MFI, mean fluorescence intensity; MPO, myeloperoxidase.

the abrupt decrease in the intracellular pH (pH_i) observed after extracellular acidification in neutrophils suspended in bicarbonate-buffered medium, which does not occur in those suspended in bicarbonate-free medium, plays a key role in the stimulation of neutrophil responses. Our observations also show that extracellular acidosis delays neutrophil apoptosis and extends its functional lifespan. Taken together, these results support the notion that acidic pH_o may intensify acute inflammatory responses by enhancing neutrophil functions.

Materials and Methods

Reagents and Abs

Reagents were obtained as follows: RPMI 1640 medium, dextran 266, FMLP, cytochrome c, superoxide dismutase from bovine erythrocytes (5000 U/mg of protein), amiloride, 4-aminoantipirine, horseradish peroxidase, phenol red, acridine orange, and ethidium bromide from Sigma (St. Louis, MO); Ficoll from Pharmacia Fine Chemicals (Uppsala, Sweeden); Hypaque from Winthrop Products (Buenos Aires, Argentine); carboxy-SNARF-1-AM and Fluo 3-AM from Molecular Probes (Eugene, OR); FCS from Life Technologies (Grand Island, NY), anti-CD18 mAb TS1/18 from American Type Culture Collection (Manassas, VA) and FITC-conjugated mouse anti-human myeloperoxidase (MPO) mAb MPO-7 from Dako Corporation (Carpinteria, CA). pIC were prepared at the equivalence zone with human IgG and rabbit IgG anti-human IgG, as we previously described (22). Zy was prepared by incubating 100 mg of zymosan with 10 ml of normal human serum for 30 min at 37°C.

Culture media

The standard medium employed throughout this study was bicarbonate-buffered RMPI 1640 supplemented with 1% FCS, adjusted to different pH values by the addition of distinct volumes of an isotonic HCl solution. Some assays were performed by employing bicarbonate-free PBS or isotonic PBS supplemented with 24 mM sodium bicarbonate (bicarbonate-supplemented PBS), both containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, and adjusted to the desired pH values by the addition of an isotonic HCl solution.

Preparation of human neutrophils

Blood samples were obtained from healthy donors by venipuncture of the forearm vein at the Instituto de Investigaciones Hematológicas Blood Bank. Neutrophils were isolated from heparinized human blood samples by dextran sedimentation and Ficoll-Hypaque gradient centrifugation, as described (23). Contaminating erythrocytes were removed by hypotonic lysis. Unless otherwise stated, after washing, the cell pellets (>96% of neutrophils on May Grunwald/Giemsa stained cytopreps) were resuspended in RPMI 1640 supplemented with 1% heat-inactivated FCS, previously adjusted to the desired pH values. In short incubations, cells were placed in a water bath at 37°C (15–30 min). In longer cultures (18 h), cells were maintained at 37°C in a humidified atmosphere either with 5% CO_2 (for cells suspended in medium at pH 7.4) or 7% CO_2 (for cells suspended in medium at lower pH values) to maintain the desired pH_o. In some experiments, after the hypotonic lysis step, cells were suspended either in bicarbonate-free PBS or in bicarbonate-supplemented PBS, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

Acidification of the culture medium

In most of the experiments performed throughout this work, extracellular acidification was achieved by suspending cell pellets in media previously adjusted to the desired pH values. However, in some experiments, acidification of cells suspended in media at pH_o 7.4 was accomplished by the addition of a precalculated volume of isotonic HCl solution (see Figs. 1, 8, and 9). By this experimental approach, after the addition of the HCl solution to neutrophil suspended in pH 7.4 medium, and until complete pH_o equilibration is attained, different cell fractions might be instantaneously exposed to pH_o values even lower than the desired pH_o. Hence, in these cases, control experiments were also performed in which cell pellets were suspended in media previously adjusted to the desired pH value. Similar results were obtained by both methods.

Flow cytometric measurements

Flow cytometric assays were performed in a FACScan argon laser flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA). Data were analyzed by employing CellQuest software (Becton Dickinson).

Calcium measurements

Changes in intracellular free calcium concentration ($[Ca^{2+}]_i$) were measured using Fluo 3-AM as previously described (24). Neutrophils, suspended at a concentration of 5×10^6 cells/ml in RPMI 1640 with 4 μM Fluo 3-AM, were incubated during 30 min at 30°C. Then, cells were washed three times with RPMI 1640 medium and resuspended at 5×10^6 cells/ml in RPMI 1640 supplemented with 5% FCS. Aliquots of 50 μl each were then added to 450 μl of 37°C RPMI 1640 medium containing 5% FCS (pH 7.4). The prewarmed sample was immediately loaded onto the flow cytometer and fluorescence was recorded during ~25 s. Then, the medium was acidified by the addition of a predetermined volume of isotonic solution of HCl to adjust its pH to the desired value, and the fluorescence was recorded during an additional 40 s. A gate based on forward and side scatter was used to exclude debris, whereas a time-based gate was used to divide the original data file and separate cells according to the time at which their fluorescence in FL1 was measured. Measurements of fluorescence in samples suspended at pH 7.4 were used to establish a marker at FL1 fluorescence channel number greater than that exhibited by at least 97% of these resting cells. This marker was then used to determine the relative percentage of activated cells. Cells that raised their $[Ca^{2+}]_i$ to higher levels than that shown by 97% of resting cells in response to the change in the pH_o were considered to be activated. Alternatively, some measurements were performed employing neutrophils loaded with Fluo 3-AM, washed, and suspended in bicarbonate-free PBS supplemented with 1 mM $CaCl_2$.

Neutrophil shape change

Cell pellets containing 2.5×10^6 neutrophils were suspended in 1 ml of RPMI 1640 medium with 1% FCS, previously adjusted to the desired pH value and incubated in a shaking water bath during 5 min at 37°C. In some cases, FMLP was employed as agonist. Cells were then centrifuged, suspended in PBS and fixed by the addition of an equal volume of 0.5% glutaraldehyde in PBS. On the other hand, in kinetic studies, neutrophil shape change was evaluated by flow cytometry simultaneously with pH_i changes after the addition of isotonic HCl solution to cells suspended in pH 7.4 medium. The shape change was measured as the shift in the forward light scatter parameter. Results were expressed as mean forward scatter (MFSC) values.

CD18 expression

Neutrophils (2×10^6) were suspended in 2 ml of RPMI 1640 medium containing 1% FCS, previously adjusted to the desired pH value. They were stimulated or not with FMLP and incubated in a shaking water bath at 37°C for the indicated time period. Then, cells were washed, fixed with 0.5% paraformaldehyde in PBS during 30 min at 4°C, and washed with PBS. Finally, cells were stained with anti-CD18 mAb and FITC-conjugated goat IgG anti-mouse IgG. Fluorescence was recorded by flow cytometry. Results were expressed as the mean fluorescence intensity (MFI) in arbitrary fluorescence units.

Hydrogen peroxide generation

Hydrogen peroxide production was measured by a modification of the oxidation of phenol red assay previously described (25). This assay was chosen because it is not influenced by pH variations of the reaction mixture (our unpublished observations). A buffered phenol red solution containing 116 mM NaCl, 24 mM $NaHCO_3$, 10 mM potassium phosphate buffer, pH 7.0, 5.5 mM dextrose, 0.28 mM phenol red, was mixed with bicarbonate-buffered RPMI 1640 medium containing 1% FCS at a 2:1 ratio, and, immediately before starting the assay, 8.5 U/ml of horseradish peroxidase was added. In the course of the experiments, pellets containing 5×10^6 neutrophils were suspended in 1 ml of the reaction mixture previously adjusted to the desired pH values. Then, 1 mM NaN_3 was added to block H_2O_2 consumption by MPO and catalase, and the production of H_2O_2 was triggered either by FMLP, Zy, or pIC. After a 120-min incubation at 37°C, cells were centrifuged and 900 μl of the supernatants were collected and added to tubes containing 100 μl of 1 mM NaOH. Absorbance at 610 nm was determined in a Hewlett Packard 8452A spectrophotometer. Results, calculated by interpolating the sample absorbance on a linear regression curve obtained from known concentrations of H_2O_2, were expressed as nmol H_2O_2 produced per 5×10^6 neutrophils per 120 min.

MPO release

The release of MPO to the extracellular medium was evaluated by using 4-aminoantipyrine as chromogen, as previously described (26). Briefly, pellets containing 2.5×10^6 neutrophils were resuspended in 1 ml RPMI 1640 medium containing 1% FCS previously adjusted to the desired pH

value. Then, cells were stimulated with pIC or Zy and incubated in a steady water bath during 30 min at 37°C. After this period, cells were centrifuged and the supernatants were recovered and placed in an incubator with 5% CO_2 to equilibrate their pH at 7.4. In a 3-ml cuvette, 1.4 ml of 2.5 mM 4-aminoantipyrine in 170 mM phenol was mixed with 1.5 ml of freshly prepared 1.7 mM H_2O_2 and incubated 4 min at 25°C. An aliquot of 100 μl of the supernatant was added and the increase in A510 measured for 5 min with continuous stirring at 25°C in a Hewlett Packard (Waldbronn, Germany) model 8452A spectrophotometer. The amount of MPO released was calculated by interpolating the sample activities on a linear regression curve obtained from known concentrations of horseradish peroxidase. MPO attached to neutrophil surface after stimulation under acidic and control pH_o conditions was evaluated by flow cytometry by employing a FITC-conjugated mouse anti-human MPO mAb.

Quantitation of cellular apoptosis and viability by fluorescence microscopy

Neutrophils (5×10^6) were resuspended in 2 ml RPMI 1640 medium containing 1% FCS, previously adjusted to the desired pH value, and cultured during 18 h at 37°C. Then, apoptosis was quantitated using the fluorescent DNA-binding dyes acridine orange (100 μg/ml), to determine the percentage of cells that had undergone apoptosis, and ethidium bromide (100 μg/ml), to differentiate between viable and nonviable cells, as previously described (27, 28). With this method, nonapoptotic cell nuclei show variations in fluorescent intensity that reflect the distribution of euchromatin and heterochromatin. By contrast, apoptotic nuclei exhibit highly condensed chromatin that is uniformly stained by acridin orange. To assess the percentage of cells showing morphological features of apoptosis, at least 200 cells were scored in each experiment.

Cytotoxic assay

Neutrophils were cultured during 18 h at 37°C in RPMI 1640 medium previously adjusted to pH 6.7 or 7.4. Then, cells were washed, suspended in pH 7.4 RPMI 1640 medium, and employed as effector cells in cytotoxic assays. The assays were performed as we previously described (29). Briefly, neutrophils (2×10^5) were stimulated by pIC, FMLP, and Con A in the presence of 2×10^5 ^{51}Cr-labeled chicken RBC. After incubation for 18 h at 37°C, the culture plate was centrifuged, the radioactivity of supernatants and pellets measured in a γ counter, and the percentage of lysis determined. Spontaneous release was always <6%.

Measurement of pH_i

Measurement of pH_i was performed using carboxy-SNARF-1-AM as previously described (30). Neutrophils (5×10^6/ml in PBS) were loaded with 10 μM carboxy-SNARF-1-AM during 30 min at 37°C, washed in PBS, and resuspended in the same buffer at 5×10^6 in 100 μl. Then, 10 μl of the cell suspension were added to 2 ml of the indicated medium at pH 7.4, and allowed to equilibrate at 37°C during 20 min in a steady water bath. Then, the time courses of changes in internal pH was evaluated before and after the addition of a precalculated volume of isotonic HCl solution to adjust the pH_o to 6.7. Assays were performed by flow cytometry, with excitation at 488 nm and emission analysis at FL2 and FL3. Ten thousand events were collected. The pH_i was estimated from the ratio of emission intensities at the two wavelengths, standardizing by comparison with the fluorescence intensity ratios of cells whose pH_i values were fixed by incubation with nigericin (10 μM) in high-potassium buffers, as previously described (31).

Statistical analysis

Data were analyzed using Wilcoxon nonparametric paired test, in which p values < 0.05 were considered statistically significant.

Results

Calcium mobilization in response to extracellular acidification

It is well established that the $[Ca^{2+}]_i$ is increased upon neutrophil stimulation with different agonists such as FMLP, LPS, and pIC (32). In the present study, Fluo 3-AM was used to determine whether extracellular acidification was able to induce calcium mobilization. With this aim, different volumes of an isotonic HCl solution were added to the sample tubes at pH 7.4 to adjust the pH_o to the desired values. Fig. 1, which shows the dot plot data from a single donor, illustrates the pattern of $[Ca^{2+}]_i$ changes after extracellular acidification. A fraction of neutrophils exhibited a rapid transient increase in $[Ca^{2+}]_i$, followed by a gradual decrease to the
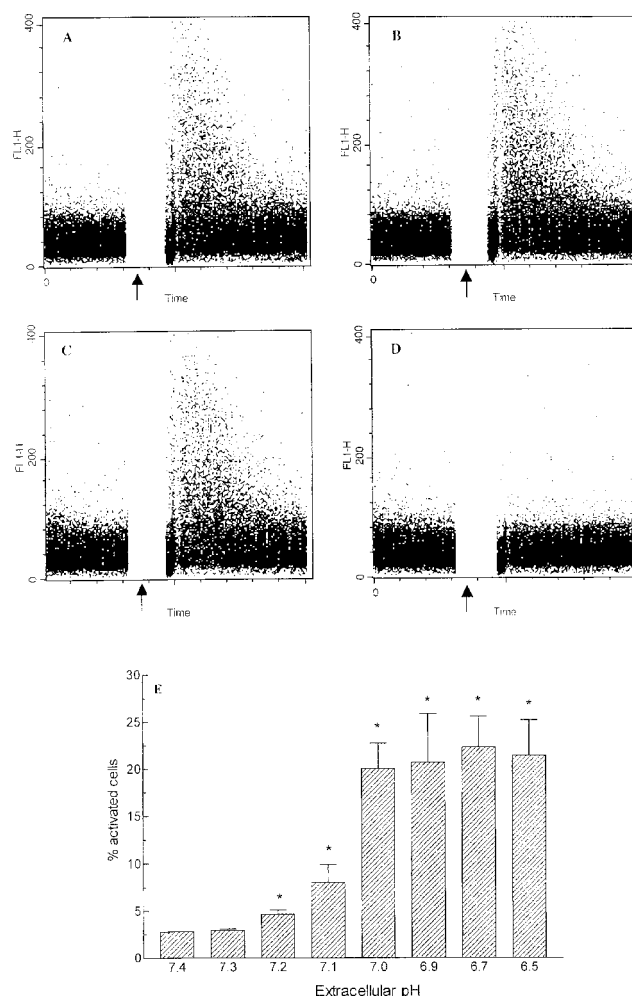

FIGURE 1. Calcium mobilization induced by extracellular acidification. Dot plots showing Fluo 3-AM fluorescence vs time from a single representative experiment ($n = 7$). Neutrophils were suspended in RPMI 1640 medium (pH 7.4). Arrows indicate the addition of an isotonic HCl solution to each sample tube to adjust the pH_o to 6.5 (A), 6.7 (B), and 7.0 (C). D, Time-dependent dot plot representing Fluo 3-AM fluorescence of control neutrophils in which the HCl solution was replaced by RPMI 1640 to maintain the pH_o at 7.4 throughout the time recorded. E, Data depicts the percentage of the neutrophil population that underwent an increase in $[Ca^{2+}]_i$ above the $[Ca^{2+}]_i$ exhibited by 97% of resting cells, which were considered as activated cells. *, Statistical significance ($p < 0.05$) compared with neutrophils at pH_o 7.4.

resting $[Ca^{2+}]_i$ levels, ~40 s after the addition of the HCl solution (Fig. 1, A–C). Similar results were observed when extracellular acidification was achieved by an alternative procedure, i.e., by suspending neutrophils in culture medium previously adjusted to pH 6.7 (not shown). As expected, no differences in fluorescence emission were observed when pH_o was maintained at pH 7.4 throughout the experiment (Fig. 1D).

As Fig. 1E shows, similar cellular activation levels (expressed as the percentage of the neutrophil population that underwent an increase in $[Ca^{2+}]_i$ above the threshold we defined as baseline) were documented at pH_o values comprised in the range 6.5–7.0. A gradation of responses was observed in the intervening pH_o range between 7.0 and 7.2, while no activation was observed at pH_o 7.3 (Fig. 1E). It is important to point out that the activation observed at acidic pH_o is presumably underestimated because previous works have shown that the affinity of Fluo 3-AM for calcium

FIGURE 2. Neutrophil shape change in response to extracellular acidification. Forward light scatter histograms of neutrophils suspended for 5 min at 37°C in RPMI 1640 medium at pH_o 7.4 (shaded histogram), pH_o 7.0 (black dotted line), pH_o 6.7 (black line), and pH_o 6.5 (gray line). Overlaid histograms of a representative experiment from seven done. The inset depicts MFSC values of neutrophils suspended for 5 min at 37°C in RPMI 1640 medium adjusted to pH_o 7.4 or pH_o 6.7. These results are expressed as MFSC $\pm$ SEM values from 17 experiments. *, Statistical significance ($p < 0.01$) compared with neutrophils at pH_o 7.4.
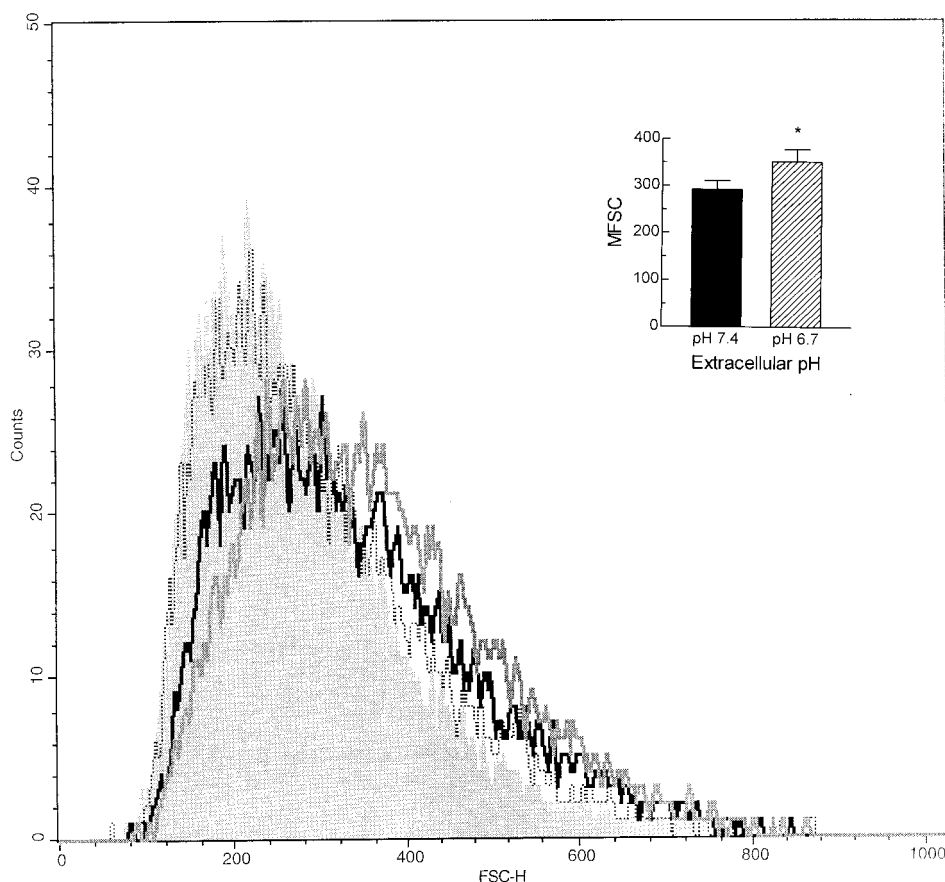
markedly declines as pH decreases from 7.4 to 5.5, with a twofold increase in the K_d of Fluo 3-AM for calcium from pH 7.4 to 6.5 (33, 34).

Stimulation of neutrophil shape change by acidic pH_o

After appropriate stimulation, neutrophils undergo transient shape changes, which can be detected by analyzing variations in their light scattering properties by flow cytometry (35). To ascertain whether acidic pH_o was also able to stimulate cellular shape changes, neutrophils were cultured during 5 min at 37°C in media adjusted to different pH values, then they were fixed and analyzed by flow cytometry. Neutrophils suspended in medium adjusted to pH 6.7 showed higher forward light scattering values than cells suspended at pH_o 7.4 (inset of Fig. 2). Moreover, a hydrogen ion concentration-dependent increase in the forward light scatter parameter was observed, with maximal shape changes at lower pH values (Fig. 2).

Regulation of CD18 expression by acidic pH_o

To determine whether acidic pH_o was able to induce the up-regulation of neutrophil adhesion-promoting receptors for endothelial cells, the expression of the β_2 integrin CD18 was evaluated. Kinetic assays indicated that CD18 expression at pH_o 6.7 peaked at about 5 min and started to decline after 15 min of incubation (Fig. 3). As expected, the expression of CD18 at pH 7.4 was not significantly modified over the period of time evaluated (Fig. 3).

Acidic pH_o is unable to trigger H_2O_2 production and MPO release

The ability of acidic pH_o to induce H_2O_2 production and MPO release (a measurement of neutrophil degranulation) was also de-

termined. Neither H_2O_2 generation nor MPO release could be detected either under acidic or control pH_o conditions.

Enhancement by acidic pH_o of neutrophil responses induced by conventional agonists

Additional experiments indicated that extracellular acidic conditions were also able to enhance neutrophil responses triggered by agonists. Data in Table I show that acidic pH_o markedly increased
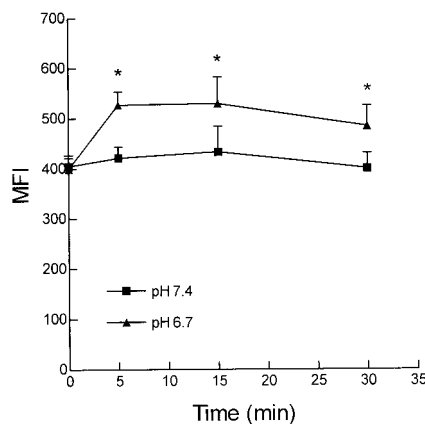
FIGURE 3. Time course of expression of CD18 after extracellular acidification. Neutrophils were cultured in RPMI 1640 medium adjusted to pH_o 7.4 or 6.7 for the periods of time indicated. Then, they were fixed, stained, and the expression of CD18 revealed by flow cytometry. Data points are displayed as means $\pm$ SEM values from 8–12 experiments. *, Statistical significance ($p < 0.01$) compared with neutrophils at pH_o 7.4.

Table I. *Effect of extracellular acidic conditions on neutrophil responses induced by FMLP[a]*

Neutrophil Response	Control		FMLP (100 nM)		FMLP (10 nM)		FMLP (1 nM)		FMLP (0.1 nM)	
	pH 7.4	pH 6.7	pH 7.4	pH 6.7	pH 7.4	pH 6.7	pH 7.4	pH 6.7	pH 7.4	pH 6.7
Calcium mobilization (% activated cells ± SEM)	<3	17 ± 5*	ND	ND	ND	ND	49 ± 8	56 ± 8*	10 ± 3	38 ± 6*
Shape change (MFSC ± SEM)	259 ± 28	320 ± 22*	391 ± 17	415 ± 26	405 ± 35	442 ± 38*	378 ± 71	414 ± 67*	325 ± 68	335 ± 48
Expression of CD18 (MFI ± SEM)	376 ± 22	490 ± 34*	528 ± 50	606 ± 46	488 ± 40	570 ± 36*	458 ± 26	584 ± 38*	ND	ND

[a] Responses were evaluated as indicated in Figs. 1–3.*, Statistical significance ($p < 0.05$) compared with neutrophils stimulated in pH 7.4 medium.

calcium mobilization triggered by 0.1 nM FMLP, while moderately augmented the response induced by 1 nM FMLP. Acidic conditions also increased the shape change response and the upregulation of the expression of CD18 to every FMLP concentration tested (Table I). However, these differences were found statistically significant only for 10 and 1 nM FMLP concentrations.

The effect exerted by acidic pH_o on H_2O_2 production induced by conventional agonists was also evaluated. As shown in Fig. 4, neutrophils stimulated either with FMLP, pIC, or Zy during 120 min under extracellular acidic conditions generated markedly higher amounts of H_2O_2 than those stimulated in pH 7.4 medium. Additional experiments indicated that the release of MPO elicited by either pIC or Zy was also significantly higher when neutrophils were stimulated at acidic pH_o (Fig. 5A). Because MPO is a highly charged enzyme that once released binds with high avidity to cell membranes (2), the increased MPO activity detected in the supernatants of neutrophils incubated in acidic pH medium could be due to a diminished binding of MPO to the neutrophil membrane. To rule out this possibility, the MPO attached to the cell surface after stimulation under acidic and control pH_o conditions was evaluated by flow cytometry. No differences in the fluorescence emission signal were observed (Fig. 5B).

Modulation of neutrophil survival by extracellular acidic conditions

Different stimuli that induce neutrophil activation also extend its lifespan through the inhibition of apoptosis (36). Therefore, we investigated whether acidic pH_o, besides triggering neutrophil activation, was also able to modulate neutrophil survival. Apoptotic rates of cells cultured during 18 h at 37°C in media adjusted to different pH values were evaluated by fluorescence microscopy. Extracellular acidosis induced a significant inhibition of neutrophil apoptosis without inducing cellular necrosis. Cell integrity was always >94% as judged by the exclusion of both trypan blue and ethidium bromide dyes (Fig. 6).

Extension of neutrophil functional lifespan by acidic pH_o

Considering the ability of acidic pH_o to delay neutrophil apoptosis, we then evaluated whether the prevention of apoptosis was associated with an extension of neutrophil lifespan. To test this possibility, neutrophils were cultured during 18 h at 37°C in media adjusted to either pH 6.7 or 7.4. Then, they were washed, resuspended in pH 7.4 medium, and employed as effector cells in cytotoxic assays. Results in Fig. 7 indicate that neutrophils cultured under acidic conditions exhibited a markedly higher cytotoxic potential than those cultured at pH 7.4.

### Neutrophil cytoplasmic acidification in response to extracellular acidification

In an attempt to determine the underlying mechanisms of neutrophil stimulation by acidic pH_o, we evaluated the time course of changes in the pH_i upon the addition of HCl solution to the extracellular medium, employing carboxy-SNARF-1-AM-loaded neutrophils. Results of seven experiments indicated that pH_i of resting neutrophils suspended in RPMI 1640 medium adjusted to pH 7.4 averaged 7.15 ± 0.04. The addition of HCl-isotonic solution to adjust the pH_o to 6.7 elicited a reproducible pattern of changes in the pH_i (Fig. 8A). As early as 15 s after extracellular acidification (the earliest time point analyzed), an abrupt fall in the pH_i of 0.46 ± 0.06 U was observed ($n = 7$), which persisted over the course of 240 s recorded (Fig. 8A). A similar drop in the pH_i was observed when extracellular acidification was achieved by an alternative procedure, i.e., by suspending neutrophils in culture medium previously adjusted to pH 6.7 (not shown). As expected, the pH_i of neutrophils maintained in medium adjusted to pH 7.4 remained unaffected over the time analyzed ($n = 7$) (Fig. 8A).

The above described experiments were conducted with neutrophils suspended in RPMI 1640 medium, which contains physiological concentrations of sodium bicarbonate, to provide culture conditions as close as possible to those found in vivo (37). We then reasoned that the abrupt drop in the pH_i observed after extracellular acidification may be due to the reaction of the H^+ supplied by the acid load with the bicarbonate present in the culture medium ($HCO_3^- + H^+ \rightleftarrows CO_2 + H_2O$), resulting in the production of CO_2. This compound would rapidly diffuse into the cell, causing an overproduction of intracellular protons ($CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$) (37). Considering this hypothesis, we investigated the role
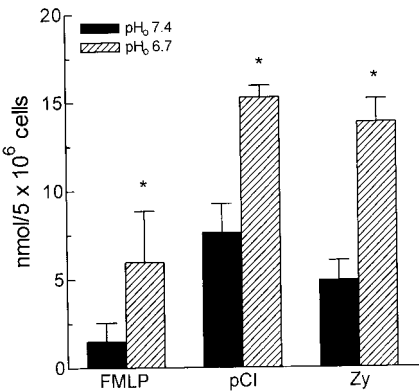
FIGURE 4. Hydrogen peroxide production in response to extracellular acidification. Neutrophils pellets (5×10^6) were suspended in 1 ml of phenol red reaction mixture adjusted to pH_o 7.4 or 6.7 and stimulated either by FMLP (1 μM), pIC (20 μg/ml), or Zy (20 μg/ml) for 120 min at 37°C. Then, they were centrifuged and 900 μl of the supernatants were collected and transferred to tubes containing 100 μl of 1 mM NaOH. Absorbance at 610 nm was determined by spectrophotometry. *, Statistical significance ($p < 0.01$) compared with neutrophils stimulated in pH 7.4 medium.

FIGURE 5. Modulation of MPO secretion by acidic pH_o. *A*, MPO release elicited by pIC (20 $\mu g/ml$) or Zy (50 $\mu g/ml$) in neutrophils cultured in RPMI 1640 medium adjusted to pH_o 7.4 or 6.7 for 30 min at 37°C. Results are expressed as the arithmetic mean ± SEM of five to six experiments. *, Statistical significance ($p < 0.01$) compared with the amount of MPO released from neutrophils incubated in medium at pH_o 7.4. *B*, MPO attached to neutrophil surface after stimulation with pIC under control (shaded histogram) and acidic (dotted line) pH_o conditions.
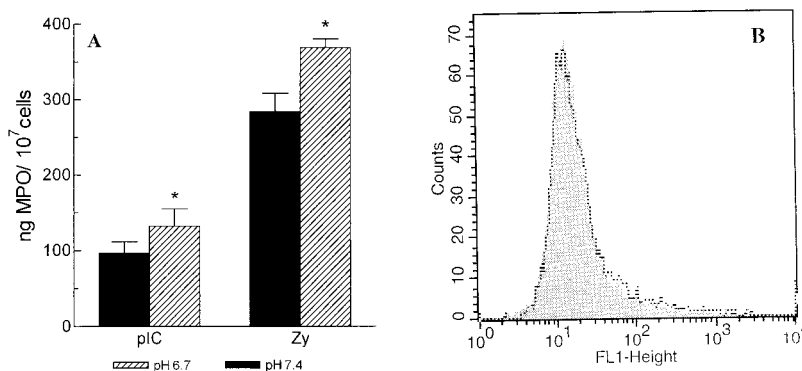
of sodium bicarbonate in the intracellular acidification induced by external acidification. Experiments were conducted employing neutrophils suspended either in bicarbonate-free PBS or isotonic bicarbonate-supplemented PBS. The addition of isotonic-HCl solution to neutrophils suspended in bicarbonate-free PBS slightly reduced the pH_i over the 240 s analyzed (0.11 ± 0.06 U, $n = 5$) (Fig. 8*B*). By contrast, the addition of HCl to neutrophils suspended in bicarbonate-supplemented PBS induced an abrupt fall in the pH_i (0.38 ± 0.05 U, $n = 4$) (Fig. 8*C*), similar to that observed upon extracellular acidification of neutrophils suspended in RPMI 1640 medium. As expected, the pH_i of neutrophils maintained at pH 7.4, either in bicarbonate-free or bicarbonate-supplemented PBS, remained unaffected ($n = 5$) (Fig. 8, *B* and *C*).

Role of intracellular acidification in neutrophil activation induced by extracellular acidification

To define the biochemical basis of neutrophil activation in response to extracellular acidification, the time relationship between the pH_i and the light scattering responses elicited by the addition of isotonic HCl solution to the extracellular medium was examined simultaneously in cells suspended either in RPMI 1640 medium, bicarbonate-free or bicarbonate-supplemented PBS, both containing 1 mM CaCl (Fig. 8). It was found a close correlation between both parameters. Thus, the abrupt drop in the pH_i observed in neutrophils suspended either in RPMI 1640 medium or bicarbonate-supplemented PBS after extracellular acidification was accom-

panied by cellular shape changes (Fig. 8, *A* and *D*, *C* and *F*). Conversely, the slight reduction in the pH_i of neutrophils suspended in bicarbonate-free PBS was associated with a modest alteration in the light scattering properties (Fig. 8, *B* and *E*). Finally, the effect of extracellular acidification on calcium mobilization was evaluated in neutrophils suspended in bicarbonate-free PBS. As shown in Fig. 9, no changes in $[Ca^{2+}]_i$ levels were observed at any pH_o values analyzed.

Discussion

The results presented here indicate that neutrophils exposed to extracellular acidic conditions undergo: 1) a transient increase in $[Ca^{2+}]_i$ over the resting levels; 2) a shape change response; 3) the up-regulation of the expression of the β_2 integrin CD18; 4) a delay in the spontaneous apoptotic rate; and 5) an extension in their functional lifespan.

Taking into account that acidic pH_o is a common feature of several inflammatory sites where neutrophils are recruited from circulation, it is surprising that its impact on the development of inflammatory processes remains uncertain. Early studies showed that acidic pH_o stimulates per se neutrophil locomotion (18). Nahas et al. demonstrated that the rate of neutrophil locomotion as a
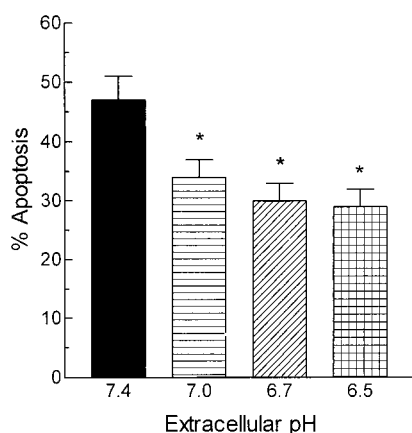

FIGURE 6. Acidic pH_o delays neutrophil apoptosis. Percentage of apoptotic neutrophils observed after 18 h of culture in RPMI 1640 medium adjusted to different pH_o. Apoptosis was evaluated by fluorescence microscopy. Results are expressed as the arithmetic mean ± SEM of 21 experiments. *, Statistical significance ($p < 0.01$) compared with neutrophils cultured in medium at pH_o 7.4.
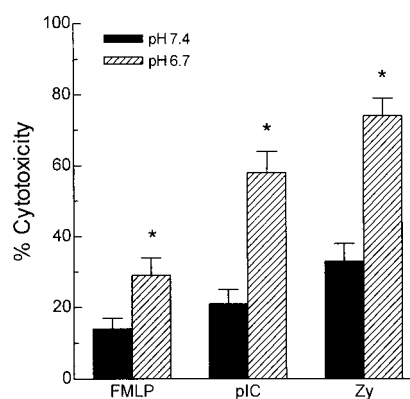
FIGURE 7. Acidic pH_o extends neutrophil functional lifespan. Neutrophils were cultured in RPMI 1640 medium adjusted to pH_o 7.4 or 6.7 for 18 h at 37°C. Then, cells were washed and resuspended in RPMI 1640 medium (pH 7.4) and employed as effector cells in cytotoxic assays. Neutrophils were stimulated with pIC (10 $\mu g/ml$), Zy (50 $\mu g/ml$), and FMLP (10^{-7} M) in the presence of ^{51}Cr-labeled chicken RBC and further cultured for 18 h at 37°C. Then, the percentage of cytotoxicity was determined as described under *Materials and Methods*. Results are expressed as the arithmetic mean ± SEM of seven experiments. *, Statistical significance ($p < 0.01$) compared with cytotoxicity mediated by neutrophils cultured in medium at pH_o 7.4.
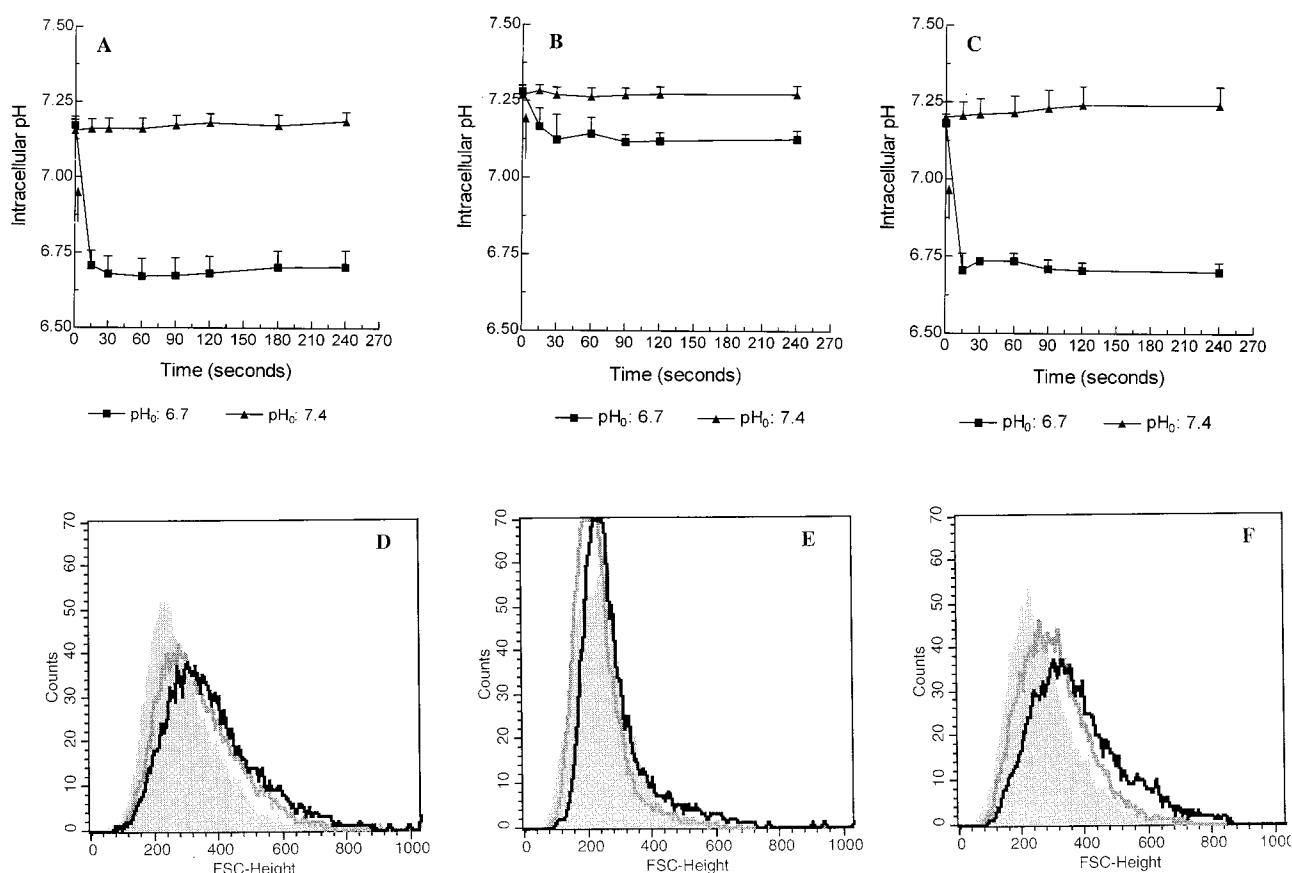
FIGURE 8. Time course of changes in neutrophil pH_i and forward light scatter properties after extracellular acidification. Both parameters were evaluated simultaneously. Upper and lower data show the pH_i and the forward light scatter properties of neutrophils incubated in RPMI 1640 medium (*A* and *D*), bicarbonate-free PBS (*B* and *E*), and bicarbonate-supplemented PBS (*C* and *F*), respectively. At the time indicated by the arrow, an isotonic HCl solution was added to each sample tube to adjust the pH_o to 6.7. Intracellular pH data are expressed as the arithmetic mean ± SEM of four to seven experiments. Lower histograms are representative of one of the experiments considered in the upper curves. Histograms depict forward scatter values of neutrophils, before and after extracellular acidification: pH_o 7.4, shaded histograms; pH_o 6.7 at $t = 15$ s, gray line; and pH_o 6.7 at $t = 240$ s, black line.

function of pH presents a bimodal distribution, with peak velocities at pH_o 6.5 and 7.4 (3), while Zigmond and Hargrove observed that human and rabbit neutrophils, placed in a pH gradient between 5.0 and 8.0, orient their locomotion toward the acidic pH_o (6). In a recent report, Serrano et al. demonstrated that neutrophil CD18 expression is markedly increased under hypercarbic acidic conditions (20% CO_2) (38). The authors also found that, despite acidosis which notably decreases ICAM-1 expression on human aortic endothelial cells, it significantly enhances neutrophil adhesion to these cells, promoted by the up-regulated expression of CD18 (38). Although performed by employing a different experimental setting, our findings indicating that acidic pH_o increases the expression of neutrophil CD18 are in agreement with Serrano's studies. Moreover, in experiments designed to determine whether pH_o affects neutrophil transendothelial migration, we observed an increase in spontaneous neutrophil locomotion through HUVEC monolayers under acidic conditions (A. Trevani, unpublished results). Taking into account that neutrophil shape change and its adhesive interaction with endothelial cells are essential steps in the process of granulocyte migration to sites of inflammation, our data suggest that extracellular acidic pH may promote neutrophil recruitment at inflammatory sites. Two mechanisms might account for the shape change observed at acidic pH_o: 1) cytoskeleton reorganization that involves changes in the ratio of polymeric (F) to monomeric (G) actin; and 2) cell swelling induced by ionic movement and water uptake, which depends, at least in part, on the

activation of the Na^+/H^+ antiport (39). Indeed, previous studies have demonstrated that the activation of this antiport, which occurs at low pH_i, is involved in the cell swelling necessary for efficient neutrophil migration (39). In this regard, we observed that neutrophil shape change promoted by acidic pH_o was substantially prevented by 1 mM amiloride, an inhibitor of the Na^+/H^+ antiport, supporting a role for the antiport in this response (A. Trevani, unpublished results). Additional experiments are required to determine whether the shape change response induced by extracellular acidification also involves changes in the ratio of polymeric (F) to monomeric (G) actin.

In the absence of appropriate stimuli, neutrophils rapidly undergo characteristic changes indicative of programmed cell death or apoptosis, including cell shrinkage, nuclear chromatin condensation, and DNA fragmentation into nucleosome-length fragments (40, 41). Apoptosis, which represents an alternative fate to necrosis, not only determines neutrophil uptake by macrophages, but also is associated with a loss of neutrophil functions, such as chemotaxis, phagocytosis, stimulated shape change, degranulation, and respiratory burst (41, 42). A variety of stimuli that induce neutrophil activation, such as C5a, FMLP, and LPS, have been described to prolong lifespan via inhibition of apoptosis (43–45). We here demonstrated that cellular activation induced by external acidification is able to delay spontaneous neutrophil apoptosis. Moreover, this delay in apoptotic rates is associated with an extension of neutrophil functional lifespan.
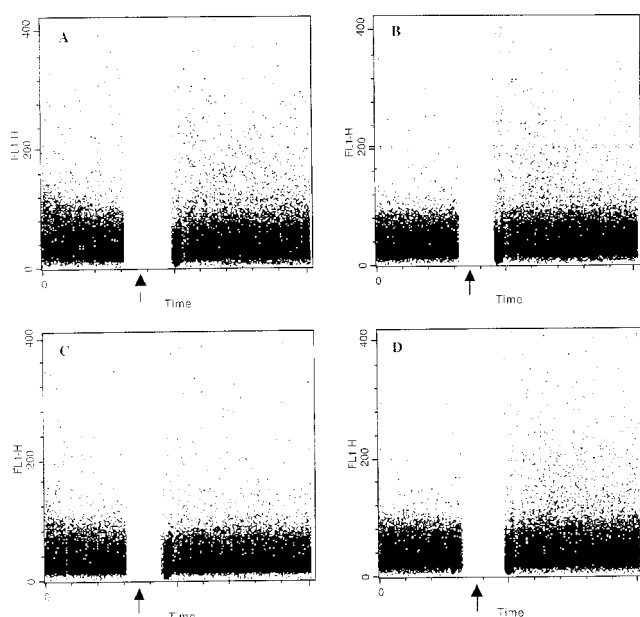
FIGURE 9. Effect of extracellular acidification on $[Ca^{2+}]_i$ in neutrophils incubated in bicarbonate-free PBS. Dot plots showing Fluo 3-AM fluorescence vs time from a single representative experiment ($n = 6$). Neutrophils were suspended in bicarbonate-free PBS (pH 7.4). Arrows indicate the addition of an isotonic HCl solution to each sample tube to adjust the pH_o to 6.5 (*A*), 6.7 (*B*), and 7.0 (*C*). *D*, Time-dependent dot plot represents Fluo 3-AM fluorescence of control neutrophils in which the HCl solution was replaced by bicarbonate-free PBS (pH 7.4) to maintain the pH_o at 7.4 throughout the time recorded.

The addition of isotonic-HCl solution to neutrophils suspended in RPMI 1640 medium induced an abrupt drop in their pH_i, which persisted over the recorded period. By contrast, previous reports have shown only slight changes in the pH_i of resting cells after extracellular acidification (19, 20, 46, 47). These disparate results can be attributed to differences in the experimental conditions of each study. Thus, our experiments were conducted in media containing bicarbonate to provide conditions as close as possible as those in vivo. Therefore, the immediate fall in the pH_i observed after extracellular acidification may be caused by the rapid diffusion into the cell of CO_2 originated from the reaction of protons with the bicarbonate present in the culture medium. This would in turn result in the overproduction of intracellular protons (37, 48). Conversely, the above mentioned studies were conducted using HEPES-buffered media without bicarbonate. In this case, the addition of HCl may lead to titration of the extracellular HEPES buffer without significantly affecting the pH_i. A role for bicarbonate in the drop of the pH_i after extracellular acidification is supported by the fact that the addition of HCl solution to neutrophil suspensions in bicarbonate-free PBS induced only a slight decrease in the pH_i (Fig. 8*B*), while external acidification of neutrophils suspended in bicarbonate-supplemented PBS caused an abrupt fall in the pH_i (Fig. 8*C*).

The biochemical basis of neutrophil activation in response to extracellular acidification has not been defined yet. However, our findings suggest a role for intracellular acidification in neutrophil activation induced by extracellular acidosis because only neutrophils that exhibited an abrupt drop in the pH_i (i.e., neutrophils suspended in bicarbonate-buffered media) underwent significant shape changes and rises in their $[Ca^{2+}]_i$. Other studies have also indicated that intracellular acidification plays a role in neutrophil activation. In this regard, it has been shown that the intracellular

acidification that occurs after the engagement of chemotactic factor-receptors is responsible for the induction of neutrophil shape change (49, 50). Moreover, a role for intracellular acidification as a second messenger for the initiation of chemotaxis, has also been postulated (49).

Our findings also suggest that extracellular acidic conditions not only trigger neutrophil activation, but also modulate the functional profile of neutrophils in response to stimulation with different agonists. In this regard, we have observed that either calcium mobilization, shape change response, and up-regulation of the CD18 expression induced by FMLP were all significantly increased when evaluated in acidic pH media. These results contrast with those reported by Leblebicioglu et al. (21), who found a moderate and nonsignificant reduction in calcium transients in pH 6.7 medium. These differences may be explained considering that those experiments were performed in bicarbonate-free medium and employing a high FMLP concentration (100 nM), conditions that could mask the ability of acidic pH_o to enhance this response.

We also found that acidic pH_o enhances H_2O_2 generation induced by conventional agonists. These results contrast with previous reports showing that extracellular acidic pH_o inhibits the generation of O_2^- triggered by different stimuli. In this regard, Gabig et al. observed that O_2^- production induced by Zy substantially declined as pH_o fell from 7.5 to 6.0, with values at pH 7.0, pH 6.5, and pH 6.0 being about 80%, 50% and 11% those at pH 7.5, respectively (5). Similar results were reported in separate works by Simchowitz, Ahlin et al., and Leblebicioglu et al., who found that acidic pH_o markedly reduces the amount of O_2^- generated by FMLP-activated neutrophils (19–21). In agreement with these studies, we also found that neutrophil O_2^- production in response to FMLP (10–100 nM) was markedly inhibited at acidic pH_o, even though the reaction took place in bicarbonate-buffered medium (our unpublished results). The apparent discrepancy between the effects of acidic pH_o on O_2^- production and H_2O_2 generation cannot be easily explained. However, an increased rate of dismutation of O_2^- at acidic pH_i values may account for these results. In support of this hypothesis, previous studies have demonstrated that spontaneous dismutation of O_2^- occurs more rapidly at acidic pH (51). Moreover, findings reported by Tonetti et al. (52) indicated that lowering pH_i in 0.1–0.3 U by the addition of short chain carboxylic acids to the culture medium induces not only a significant decrease in the O_2^- recovery but also an increase in the H_2O_2 generated by stimulated neutrophils. The fact that acidic pH_o favors H_2O_2 generation by stimulated neutrophils may also explain our previous findings indicating an enhancement of oxygen-dependent cytotoxic responses elicited by pIC, FMLP, and Zy under extracellular acidic conditions (22).

The current work has been developed under an experimental setting that resembles physiologic conditions, where the pairing CO_2/HCO_3^- is the most important extra- and intracellular buffering system (53). Taking into account the essential role that neutrophils play in acute inflammatory processes and considering that interstitial acidic pH characterizes most inflammatory microenvironments, our results support the notion that extracellular acidosis may intensify the acute inflammatory responses by enhancing neutrophil activity and extending its functional lifespan.

Acknowledgments

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