Extracellular Acidosis Induces Neutrophil Activation by a Mechanism Dependent on Activation of Phosphatidylinositol 3-Kinase/Akt and ERK Pathways

Diego Martínez, Mónica Vermeulen, Analí Trevani, Ana Ceballos, Juan Sabatté, Romina Gamberale, María Eugenia Álvarez, Gabriela Salamone, Tamara Tanos, Omar A. Coso and Jorge Geffner

*J Immunol* 2006; 176:1163-1171; doi: 10.4049/jimmunol.176.2.1163

http://www.jimmunol.org/content/176/2/1163

### References
This article cites 61 articles, 30 of which you can access for free at:
[http://www.jimmunol.org/content/176/2/1163.full#ref-list-1](http://www.jimmunol.org/content/176/2/1163.full#ref-list-1)

### Subscription
Information about subscribing to *The Journal of Immunology* is online at:
[http://jimmunol.org/subscription](http://jimmunol.org/subscription)

### Permissions
Submit copyright permission requests at:
[http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

### Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
[http://jimmunol.org/alerts](http://jimmunol.org/alerts)
Extracellular Acidosis Induces Neutrophil Activation by a Mechanism Dependent on Activation of Phosphatidylinositol 3-Kinase/Akt and ERK Pathways

Diego Martínez,* Mónica Vermeulen,* Analía Trevani,* Ana Ceballos,* Juan Sabatté,* Romina Gamberale,* María Eugenia Álvarez,* Gabriela Salamone,* Tamara Tanos,† Omar A. Coso,‡ and Jorge Geffner2*

Inflammation in peripheral tissues is usually associated with the development of local acidosis; however, there are few studies aimed at analyzing the influence of acidosis on immune cells. We have shown previously that extracellular acidosis triggers human neutrophil activation, inducing a transient increase in intracellular Ca2+ concentration, a shape change response, the up-regulation of CD18 expression, and a delay of apoptosis. In this study, we analyzed the signaling pathways responsible for neutrophil activation. We found that acidosis triggers the phosphorylation of Akt (the main downstream target of PI3K) and ERK MAPK, but not that of p38 and JNK MAPK. No degradation of IκB was observed, supporting the hypothesis that NF-κB is not activated under acidosis. Inhibition of PI3K by wortmannin or LY294002 markedly decreased the shape change response and the induction of Ca2+ transients triggered by acidosis, whereas the inhibition of MEK by PD98059 or U0126 significantly inhibited the shape change response without affecting the induction of Ca2+ transients. We also found that acidosis not only induces a shape change response and the induction of Ca2+ transients in human neutrophils but also stimulates the endocytosis of FITC-OVA and FITC-dextran. Stimulation of endocytosis was partially prevented by inhibitors of PI3K and MEK. Together, our results support the notion that the stimulation of human neutrophils by extracellular acidosis is dependent on the activation of PI3K/Akt and ERK pathways. Of note, using mouse peritoneal neutrophils we observed that the enhancement of endocytosis induced by acidosis was associated with an improved ability to present extracellular Ags through a MHC class I-restricted pathway. The Journal of Immunology, 2006, 176:1163–1171.

Copyright © 2006 by The American Association of Immunologists, Inc. 0022-1767/06/$02.00

Received for publication June 1, 2005. Accepted for publication October 24, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*Institute of Hematologic Research, National Academy of Medicine and National Reference Center for AIDS, Department of Microbiology, Buenos Aires University School of Medicine, Buenos Aires, Argentina; and Department of Physiology and Molecular Biology, Faculty of Exact and Natural Sciences, Buenos Aires University, Buenos Aires, Argentina

1 This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires University School of Medicine, Fundación Anorchas, and Agencia Nacional de Promoción Científica y Tecnológica, Argentina.

2 Address correspondence and reprint request to Dr. Jorge Geffner, Departamento de Inmunología, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Pacheco de Melo 3081, 1425 Buenos Aires, Argentina. E-mail address: geffnerj@fibertel.com.ar

induces an abrupt drop in the intracellular pH of neutrophils (20). This response appears to be triggered by the rapid diffusion into the cell of CO₂ originating from the reaction of protons with the bicarbonate present in the culture medium, which, in turn, results in the overproduction of intracellular protons. It is possible that the drop in the intracellular pH may be able to trigger signaling pathways leading to neutrophil activation. Supporting this hypothesis, previous studies focused on neutrophil chemotaxis have shown that cytosolic acidification acts as a second messenger for the induction of neutrophil activation (26, 27). Alternatively, as observed for conventional agonists, the ability of extracellular protons to activate neutrophils could be due to the interaction of protons with specific receptors expressed on the neutrophil surface. Supporting this possibility, recent reports have shown that extracellular protons can be effectively recognized by a subfamily of G protein-coupled receptors, namely GPR4 (28–30). This subfamily comprises four receptors that share significant sequence homology: GPR4, ovarian cancer G protein-coupled receptor 1 (OGR1), T cell death-associated gene 8 (TDAG8), and G2A (from G2 accumulation). Originally characterized by their ability to bind proinflammatory lipids such as lysophosphatidylcholine, sphingosylphosphorylcholine, and the lysosphingolipid psychosine, recent observations suggest that these receptors constitute a family of proton-sensing G protein-coupled receptors (28–30). The original observations related to the ability of these receptors to recognize protons were conducted in osteosarcoma cells and primary human osteoblast precursors and showed that acidosis triggered inositol phosphate formation (28). Subsequent studies performed in a variety of transfected cell lines cultured at low values of extracellular pH (6.0–7.0), found that OGR1 and G2A lead to the accumulation of inositolphosphate, whereas GPR4 and TDAG8 elicit camp formation (29, 30). The expression and function of these receptors in human leukocytes has not been defined.

In the present study, we show that the stimulation of human neutrophils by extracellular acidosis is dependent on the activation of P13K/Akt and ERK pathways. Moreover, using mouse peritoneal neutrophils, we found that acidosis not only stimulates endocytosis but also improves the presentation of extracellular Ags by neutrophils through MHC class I molecules (cross-presentation), a pathway of exogenous Ag presentation usually restricted to dendritic cells.

Materials and Methods

Reagents

Dextran T-500 and Ficoll-Hypaque were obtained from Amersham Biosciences. OVA and dextran (40,000 Da) (Sigma-Aldrich) were conjugated with FITC as described (31). The OVA₃₅₋₇₅₋₂₅₄ peptide was provided by Dr. S. Amigorena (Institut Curie, Paris, France). Sulfasalazine was from Sigma-Aldrich. The ERK inhibitor PD98059 and the JNK inhibitor SP600125 were then washed three times with cold PBS containing 1% FCS and were 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 an FL1 detector was measured. Measurements of fluorescence in samples suspended at pH 7.3 were used to establish a marker at fluorescence in samples suspended at pH 7.3 were used to establish a marker at FL1 fluorescence channel number greater than that exhibited by at least 95% of neutrophils as estimated by Giemsa staining.

Acidification of the culture medium

Extracellular acidification was achieved by suspending cell pellets in complete medium previously adjusted to the desired pH values or by the addition of a precalculated volume of isotonic HCl solution. Similar results were observed using both methods.

Flow cytometric measurements

Flow cytometric assays were performed in a FACSscan argon laser flow cytometer (BD Immunocytometry System). Data were analyzed by using CellQuest software (BD Biosciences).

Endocytosis of FITC-OVA and FITC-dextran

The analysis of the influence of extracellular acidosis on endocytosis was assessed by using two fluorescent markers that differ in their chemical composition: FITC-OVA, a protein with a molecular mass of ~45,000 Da, and FITC-dextran, a polymer of glucose with a molecular mass of ~40,000 Da. Cells were suspended in complete medium previously adjusted to pH 7.3 or 6.5. FITC-OVA and FITC-dextran were added at final concentrations of 10 and/or 100 µg/ml, and cells were incubated for 30 min at 37°C under 5 or 7% CO₂ for cultures performed at pH 7.3 or 6.5, respectively. The cells were then washed three times with cold PBS containing 1% FCS and 0.01% NaN₃, and then analyzed on a FACSDual III (BD Biosciences). The fluorescence background was determined by incubating cells with FITC-OVA or FITC-dextran at 4°C. In some experiments, we used the dye trypan blue to quench extracellular fluorescence as described (34, 35). In these experiments, endocytosis assays were performed as indicated previously, but the acquisition of samples was conducted in the presence of 200 µg/ml trypan blue. The efficacy of trypan blue to quench extracellular fluorescence was controlled in experiments in which neutrophils were stained with FITC-MAb directed to cell surface Ags (30 min at 4°C). Fluorescence intensity was diminished by >90% when the acquisition of the samples was performed in the presence of trypan blue.

Calcium measurements

Changes in intracellular free calcium concentrations ([Ca²⁺]i) were measured using fluo-3-AM as described previously (36). Neutrophils, suspended at a concentration of 5 × 10⁶ cells/ml in complete medium, were incubated with 4 µM fluo-3-AM for 30 min at 30°C. Then, cells were washed three times with RPMI 1640 medium and suspended at 5 × 10⁶ 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 7.3). The pretreated sample was immediately loaded onto the flow cytometer, and fluorescence was recorded for ~25 s. Then, the medium was acidified by the addition of a predetermined volume of isotonic solution of HCl to adjust its pH to 6.5, and the fluorescence was recorded during an additional 100 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 an FL1 detector was measured. Measurements of fluorescence in samples suspended at pH 7.3 were used to establish a marker at a 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 [Ca²⁺]i to higher levels than that shown by 97% of resting cells in response to pH 6.5 were considered to be activated.
Acidosis activates PI3K and ERK in human neutrophils. Neutrophils (3 × 10⁶ per 300 μl of complete medium) were prewarmed for 5 min at 37°C, and acidification was accomplished by the addition of a precalculated volume of isotonic HCl solution. Cells were incubated at pH 7.3 or 6.5 for different times at 37°C, and the samples were then analyzed by Western blotting as described in Materials and Methods. Pervanadate-treated neutrophils (with 0.1 mM orthovanadate plus 0.3 mM H₂O₂ for 10 min at 37°C (pH 7.3)) were used as positive controls. Western blots are representative of three to six experiments. The inhibitors of PI3K wortmannin and LY294002 were used at concentrations of 50 nM and 100 μM, respectively, whereas the inhibitors of MEK, PD98059, and U0126, were used at 100 and 25 μM, respectively (A and B).

**Neutrophil shape change**

Cell pellets containing 2.5 × 10⁶ neutrophils were suspended in 1 ml of RPMI 1640 medium with 1% FCS adjusted previously to the desired pH value and incubated in a shaking water bath for 5 min at 37°C. The cell shape change was then evaluated by flow cytometry as described (37). The shape change was measured as the shift in the forward light scatter parameter. Results were expressed as mean forward scatter (FSC) values.

**Ag presentation assay**

Presentation of OVA₂₅₇₋₂₆₄ epitope on Kb was detected using the T cell hybridoma B3Z, which carries a β-galactosidase construct driven by NF-AT elements from the IL-2 promoter (38). For Ag presentation assays, mouse peritoneal exudate neutrophils (>95% purity) were exposed to different concentrations of OVA at 37°C for 3 h at pH 7.3 or 6.5. Cells were then washed, suspended in complete medium at pH 7.3, and cultured in the presence of the T cell hybridoma B3Z. After 18 h of culture, the cells were washed with PBS, and a colorimetric assay using o-nitrophenyl-D-galactopyranoside (Sigma-Aldrich) as a substrate was used to detect LacZ activity in B3Z lysates. Western blotting

**Materials and Methods**

Abs directed to p38 MAPK (rabbit polyclonal), JNK-1 (rabbit polyclonal), phospho-JNK (T183/Y185, rabbit polyclonal), phospho-ERK1 and phospho-ERK2 (T202/Y204, mouse monoclonal), phospho-p38 MAPK (T180/Y182, rabbit polyclonal), phospho-Akt (S473, rabbit polyclonal) (Cell Signaling Technology), and IκB-α (BD Pharmingen) were also used. HRP-conjugated anti-mouse, anti-rabbit, or anti-goat IgG was from Santa Cruz Biotechnology. Neutrophils suspended in complete medium (3 × 10⁶ cells/300 μl) were prewarmed for 5 min at 37°C. The acidification of cells suspended in medium at pH 7.3 was accomplished by the addition of a precalculated volume of isotonic HCl solution. Cells were incubated at pH 7.3 or 6.5 for different times (0–60 min) at 37°C. The reactions were stopped at the times indicated by adding cold saline, and the samples were then centrifuged. Cell pellets were resuspended in loading buffer (60 mM Tris (pH 6.8), 2.3% SDS, 10% glycerol, 0.01% bromphenol blue, and 5% β-mercaptoethanol), boiled at 96°C for 5 min, and stored at –80°C. Samples were then separated by SDS-PAGE (10 or 12%), transferred to polyvinylidene difluoride membranes (Sigma-Aldrich), and then blocked with 5% skimmed milk in PBS containing 0.05% Tween 20. Membranes were then blotted with Abs against phospho-Akt, phospho-ERK, phospho-p38, phospho-JNK, or IκB-α, followed by HRP-conjugated anti-mouse or anti-rabbit IgG. Specific bands were developed by ECL (Amersham Biosciences). Membranes were stripped and reprobed with Abs against Akt-1, ERK 1/2, p38, and JNK-1 MAPK to confirm that equal amounts of protein were present in each lane of the gel. Western blotting assays were all performed in the absence of phosphatase inhibitors.

**Western blotting**

Abs directed to p38 MAPK (rabbit polyclonal), JNK-1 (rabbit polyclonal), phospho-JNK (T183/Y185, mouse monoclonal), and Akt-1 (goat polyclonal) - were obtained from Santa Cruz Biotechnology. Abs directed against ERK1/2 MAPK (rabbit polyclonal) (Promega), phospho-ERK1 and phospho-ERK2 (T202/Y204, mouse monoclonal), phospho-p38 MAPK (T180/Y182, rabbit polyclonal), phospho-Akt (S473, rabbit polyclonal) (Cell Signaling Technology), and IκB-α (BD Pharmingen) were also used. HRP-conjugated anti-mouse, anti-rabbit, or anti-goat IgG was from Santa Cruz Biotechnology. Neutrophils suspended in complete medium (3 × 10⁶ cells/300 μl) were prewarmed for 5 min at 37°C. The acidification of cells suspended in medium at pH 7.3 was accomplished by the addition of a precalculated volume of isotonic HCl solution. Cells were incubated at pH 7.3 or 6.5 for different times (0–60 min) at 37°C. The reactions were stopped at the times indicated by adding cold saline, and the samples were then centrifuged. Cell pellets were resuspended in loading buffer (60 mM Tris (pH 6.8), 2.3% SDS, 10% glycerol, 0.01% bromphenol blue, and 5% β-mercaptoethanol), boiled at 96°C for 5 min, and stored at –80°C. Samples were then separated by SDS-PAGE (10 or 12%), transferred to polyvinylidene difluoride membranes (Sigma-Aldrich), and then blocked with 5% skimmed milk in PBS containing 0.05% Tween 20. Membranes were then blotted with Abs against phospho-Akt, phospho-ERK, phospho-p38, phospho-JNK, or IκB-α, followed by HRP-conjugated anti-mouse or anti-rabbit IgG. Specific bands were developed by ECL (Amersham Biosciences). Membranes were stripped and reprobed with Abs against Akt-1, ERK 1/2, p38, and JNK-1 MAPK to confirm that equal amounts of protein were present in each lane of the gel. Western blotting assays were all performed in the absence of phosphatase inhibitors.

**Statistical analysis**

Student’s paired t test was used to determine the significance of differences between means, and p < 0.05 was taken as indicating statistical significance.

**Results**

Extracellular acidification activates PI3K and ERK but not p38 MAPK, JNK, or NF-κB in human neutrophils

To determine the signaling mechanisms through which extracellular acidosis activates human neutrophils, Western blots of lysates...
from neutrophils cultured for different periods at pH 6.5 were probed with Abs against phosphorylated and total Akt, ERK, JNK, and p38 MAPK. Experiments were performed at pH 6.5, because, as described in the introduction (1–12), it represents a pH value frequently found at inflammatory sites. Control cells were cultured at pH 7.3 (neutral pH). The results (Fig. 1, A–D) show that acidosis triggers phosphorylation of Akt (a major target of PI3K) and ERK1/2 but not of JNK or p38 MAPK. As expected, the phosphorylation of Akt was prevented by the inhibitors of PI3K, wortmannin and LY294002, supporting the notion that it was dependent on PI3K activity, whereas phosphorylation of ERK1/2 was suppressed by the MEK inhibitors PD98059 and U0126 (Fig. 1, A and B). We also analyzed the possible involvement of NF-κB in the activation of neutrophils by acidosis. NF-κB activation usually requires IκB phosphorylation and degradation in the cytoplasm and the subsequent translocation of NF-κB to the nucleus (39). Fig. 1E shows that acidosis does not trigger the degradation of IκB, supporting the hypothesis that NF-κB is not activated under acidosis. Together, these results support the notion that extracellular acidosis activates neutrophils via two distinct signaling pathways.

**Blockade of PI3K and ERK pathways inhibits neutrophil activation triggered by extracellular acidosis**

After appropriate stimulation, neutrophils undergo transient shape changes that can be detected by analyzing variations in their light-scattering properties using flow cytometry or microscopic examination. We have shown previously that neutrophils suspended in culture medium adjusted to acidic pH values (6.5–7.0) underwent a rapid increase in the forward light-scattering properties (20). Fig. 2, A and B, shows a representative experiment in which the shape change response was evaluated by microscopic examination. To analyze the involvement of PI3K and ERK pathways in the induction of this response, we used the PI3K inhibitors wortmannin and LY294002 and the MEK inhibitors PD98059 and U0126. Cells were cultured in the absence or presence of these inhibitors at 37°C for 20 min at pH 7.3. Then, cell suspensions were acidified to pH 6.5 by the addition of a precalculated volume of an isotonic HCl solution. An equal volume of saline was added to control cells. After 5 min at 37°C, cells were analyzed by flow cytometry. Fig. 2, C–I, shows that inhibitors of both the PI3K pathway and the ERK pathway significantly decreased the shape change response triggered by acidosis, with the effect of the PI3K inhibitors being more pronounced. Consistent with the results depicted in Fig. 1 showing that acidosis does not trigger the activation of JNK, p38 MAPK, or NF-κB, we found that the specific inhibitors of these pathways, SB202190, SP600125, and sulfasalazine, used at concentrations able to suppress their activation in human neutrophils (40–42), did not inhibit the cell shape response triggered by pH 6.5.

We have also shown that acidosis triggers calcium mobilization in human neutrophils (20). Thus, we assessed the effect of inhibitors of PI3K and MEK on this response. To this aim,
Extracellular acidosis increases endocytosis by human neutrophils: its dependence on the PI3K and ERK pathways

We have shown previously that acidosis improves the endocytic capacity of mouse dendritic cells (21). Taking this finding into account, to further characterize the impact of extracellular acidosis on neutrophil function we performed another set of experiments to establish whether acidosis was also able to stimulate endocytosis by human neutrophils. To this aim, we used two markers, FITC-OVA and FITC-dextran. Cells were cultured in medium adjusted to pH 7.3, and analyzed by flow cytometry. In agreement with the observations made in dendritic cells (21), we found that the uptake of both markers by human neutrophils was markedly increased at pH 6.5 compared with pH 7.3 (Fig. 4), suggesting that the endocytosis of distinct compounds may be favored at acidosis irrespective of their chemical composition. To analyze whether the endocytic markers were actually internalized by neutrophils and not merely attached to the cell surface through nonspecific interactions, we performed additional assays in which endocytosis of FITC-OVA was conducted as described above, but the acquisition of samples was performed in the presence of trypan blue (200 µg/ml), a dye able to quench extracellular fluorescence (34, 35). The results obtained showed that trypan blue did not affect the increase in fluorescence of neutrophils cultured with FITC-OVA at pH 7.3 or 6.5, suggesting that the marker is actually internalized (percentage of increase was 596 ± 54, mean ± SEM, n = 7, p < 0.01 for pH 6.5 vs pH 7.3). Having shown that acidosis increases endocytosis by human neutrophils, we then analyzed the signaling pathways involved. Fig. 5 shows that the inhibitors of PI3K and MEK significantly diminished the stimulation of endocytosis triggered by acidosis, whereas the inhibitors of the JNK, p38 MAPK, and NF-κB pathways did not mediate any inhibitory effect.

Extracellular acidosis improves MHC class I-restricted Ag presentation by mouse peritoneal neutrophils

We have shown previously that extracellular acidosis not only increases endocytosis but also improves acquisition of extracellular Ags by dendritic cells for MHC class I-restricted presentation (21). This pathway, called cross-presentation, allows the display of exogenous Ags in the context of MHC class I molecules (43, 44). To
analyze whether extracellular acidosis was able to enhance Ag delivery into the MHC class I pathway in neutrophils, we used inflammatory exudate cells (>95% of neutrophils) obtained from the peritoneal cavity of C57BL/6 mice 6 h after the injection of casein, as described in Materials and Methods. First, we analyzed whether acidosis was able to enhance endocytosis by mouse peritoneal neutrophils in a similar manner as that by human neutrophils. Cells were cultured in medium adjusted to pH 7.3 or 6.5 in the presence of FITC-OVA (100 μg/ml) for 30 min at 37°C. Then, cells were washed two times with saline, suspended in culture medium at pH 7.3, and analyzed by flow cytometry. In agreement with the results obtained in human neutrophils (Fig. 4A), we observed that the uptake of FITC-OVA by mouse peritoneal neutrophils in a similar manner as that by human neutrophils. Cells were cultured in medium adjusted to pH 7.3 or 6.5 in the presence of FITC-OVA (100 μg/ml) for 30 min at 37°C. Then, cells were washed two times with saline, suspended in culture medium at pH 7.3, and analyzed by flow cytometry. In agreement with the results obtained in human neutrophils (Fig. 4A), we observed that the uptake of FITC-OVA by mouse peritoneal neutrophils was markedly increased at pH 6.5 as compared with pH 7.3; percentage of increase was 378 ± 57 (mean ± SEM, n = 5). To analyze whether acidosis may improve the acquisition of Ags by neutrophils for MHC class I-restricted presentation, we studied presentation of OVA to a CD8⁺ T cell hybridoma called B3Z, which carries a β-galactosidase construct driven by NF-AT elements from the IL-2 promoter enabling the analysis of T cell activation by measuring β-galactosidase activity in cell lysates (38). Mouse peritoneal neutrophils were cultured with different concentrations of OVA for 3 h at 37°C at pH 7.3 or 6.5, and presentation of the OVA257–264-epitope/H-2Kb to B3Z cells was then evaluated. As shown in Fig. 6, extracellular acidosis enabled mouse peritoneal neutrophils to present OVA through a MHC class I-restricted pathway. In fact, cells pulsed with OVA under neutral pH do not display significant levels of Ag presentation. Consistent with the signaling pathways involved in the stimulation of endocytosis by acidosis, we found that both wortmannin (50 nM) and PD98059 (100 μM) significantly prevented the stimulation of cross-presentation triggered by acidosis (percentage of inhibition was 76 ± 16 and 48 ± 13, respectively, mean ± SEM, n = 4).

**FIGURE 4.** Acidosis enhances endocytosis of FITC-OVA and FITC-dextran. Neutrophils (2.5 × 10⁶/ml) were incubated for 30 min at 37°C with different concentrations of FITC-OVA (A) or FITC-dextran (B) at pH 7.3 or 6.5, and the amount of ligand accumulated was measured by flow cytometry. The uptake of either FITC-OVA or FITC-dextran (100 μg/ml) by neutrophils after incubation for 30 min at 4°C is also shown. Results are expressed as mean fluorescence intensity values and represent the mean ± SEM of seven experiments. *, p < 0.05; ***, p < 0.01; compared with neutrophils cultured at pH 7.3.

**FIGURE 5.** Involvement of PI3K and ERK signaling pathways in the stimulation of endocytosis induced by extracellular acidosis. Neutrophils were suspended in RPMI 1640 medium (2.5 × 10⁶/ml) and cultured for 20 min at 37°C at pH 7.3 with inhibitors of distinct signaling pathways. Then, cells were incubated with FITC-OVA (100 μg/ml) for 30 min at 37°C in culture medium adjusted to pH 6.5, and the amount of ligand accumulated was measured by flow cytometry. A, Histograms from a representative experiment are shown (n = 9). Wortmannin (W) was used at 50 nM and PD98059 (PD) at 100 μM. The uptake of FITC-OVA by neutrophils after incubation at pH 7.3 for 30 min at 4°C is also shown. B, Results are expressed as mean fluorescence intensity values and represent the mean ± SEM of 4–11 experiments. *, p < 0.05 compared with untreated neutrophils cultured at pH 6.5.
and cultured for 18 h at 37°C in the presence of B3Z cells (1 × 10^6). The cells were then washed, suspended in complete medium at pH 7.3,

A T cell hybridoma specific for OVA-Kb that carries a

The cells were then washed, suspended in complete medium at pH 7.3, and cultured for 18 h at 37°C in the presence of B3Z cells (1 × 10^6/ml), a T cell hybridoma specific for OVA-Kb that carries a β-galactosidase construct driven by NF-AT elements from the IL-2 promoter. T cell activation was measured using a colorimetric assay for LacZ activity with o-nitrophenyl-D-galactopyranoside as a substrate. Background absorbance values obtained for neutrophils cultured in the absence of OVA were subtracted. *, p < 0.05 compared with neutrophils cultured with OVA at pH 7.3.

Control experiments revealed that when neutrophils were fixed with glutaraldehyde before the addition of OVA, no presentation was observed (data not shown). We also determined whether acidosis could modulate direct presentation of the OVA257–264 peptide. To this aim, neutrophils were cultured with the peptide (10 ng/ml) for 3 h at 37°C at pH 7.3 or 6.5, and MHC class I presentation was assessed as described in Materials and Methods. We found no differences in the presentation of the peptide between neutrophils pulsed under neutral or acidic conditions; cell response measured as OD at 415 nm was 0.48 ± 0.12 vs 0.46 ± 0.09 (pH 7.3 and 6.5, respectively, mean ± SEM, n = 4).

Discussion

We have reported previously that acidosis triggers human neutrophil activation, inducing a transient increase in [Ca^{2+}], over the resting levels, a shape change response, the up-regulation of CD18 expression, and a delay in the rate of apoptosis (20). In the present study, we extend these observations and show that acidosis is also able to stimulate neutrophil endocytosis, enabling neutrophils to cross-present extracellular Ags.

Murine and human neutrophils express MHC class I molecules (45–47). These molecules usually present peptide Ags derived from endogenously synthesized proteins that are degraded in the cytosol by the proteosome. However, there is a specialized pathway that allows the acquisition of extracellular Ags facilitating the generation of an MHC class I-restricted immune response. This pathway is called cross-presentation and allows display of exogenous Ags in the context of MHC class I molecules to stimulate CD8 T cells (43, 44). Although cross-presentation in vivo has been localized mainly to dendritic cells, multiple types of endocytic cells, including macrophages, B cells, keratinocytes, and L cells, can cross-present exogenous Ags in vitro with a low degree of efficiency (43, 44, 48–51).

Our results support the notion that neutrophils, under the influence of an acidic microenvironment, may also be able to take up extracellular Ags and present them through a MHC class-I restricted pathway. Because acute inflammation may occur simultaneously with the recruitment of CD8^+ T cells during the course of the immune response against infectious agents, tumors, and allografts (52–54), our results support the notion that extracellular acidosis may influence the development of adaptive immunity, not only by activating dendritic cells (21) but also by stimulating CD8^+ T cell responses in peripheral tissues via Ag cross-presentation mediated by neutrophils.

Previous studies have shown that low extracellular pH is able to activate ERK2, JNK, and p38 MAPK in a variety of cell lines (55, 56). Moreover, decreasing extracellular pH from 7.4 to 6.1 has shown to be capable to activate phospholipase C, leading to Ca^{2+} mobilization and the production of inositol triphosphates in human fibroblast, endothelial, smooth muscle, and neuroblastoma cells (57). Although the mechanism responsible for the recognition of extracellular protons by neutrophils remains to be defined, our results demonstrate that neutrophil exposure to acidosis results in the activation of PI3K and ERK pathways. These pathways have shown to play a critical role in neutrophil function, because they are required for the activation of chemotaxis, phagocytosis, and the respiratory burst and are also involved in the control of neutrophil survival (58–60). Moreover, we observed that the blocking of these pathways by specific inhibitors prevented the activation of neutrophils by extracellular acidosis. Whereas the inhibitors of PI3K exerted a profound inhibitory effect on all the functions analyzed, the inhibition of MEK exerted a significant inhibition on the shape change response and endocytosis without affecting the induction of Ca^{2+} transients triggered by acidosis.

Interestingly, Owen at al. (61) have recently shown that exposure of human neutrophils to extracellular acidosis results in a marked increase in the synthesis of platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine). The maximum increase was found at pH 5.4, but a significant enhancement was also observed at pH 6.4, a value of extracellular pH similar to the one we used throughout our study (pH 6.5). When analyzing the mechanisms responsible for the stimulatory effect of acidosis, the authors found that pH 5.4 triggered the activation of ERK1, whereas pH 6.5 triggered the activation of both ERK1 and ERK2. Regarding p38 MAPK, the authors found that maximum activation was induced at pH 5.4, and low but significant levels of activation were observed at pH 6.5. This last result appears to be in disagreement with our findings, because we observed no phosphorylation of p38 MAPK in neutrophils cultured at pH 6.5. The reasons for these discrepant results are unknown. They could be related to the different experimental conditions used in each study. For example, Owen et al. (61) conducted their experiments using neutrophils suspended in a serum-free modified HBSS supplemented with Tris, histidine, acetic acid, and lactic acid, whereas all of our experiments were performed in RPMI 1640 medium supplemented with 1% heat-inactivated FCS.

It is widely appreciated that inflammatory responses in normal peripheral tissues as well as in tumors are usually associated with the development of acidic microenvironments (1–12). In fact, acidosis appears to be a hallmark of inflammatory processes. Surprisingly, there are few studies directed at analyzing the effect of extracellular acidosis on the immune response. We have reported previously that extracellular acidosis induces the activation of neutrophils (20) and dendritic cells (21), suggesting that acidosis acts...
as a danger signal able to stimulate both innate and adaptive im-
Mune responses. In the present study, we show that acidosis stim-
ulates neutrophil function by activating PI3K and ERK pathways.
Moreover, in agreement with our previous results in dendritic 
cells, we found that acidosis stimulates endocytosis, enabling neu-
rophils to cross-present extracellular Ags through a MHC class-I 
restricted pathway. Further studies are needed to evaluate in vivo 
the influence of extracellular acidosis on the function of both 
neutrophils and dendritic cells.

Acknowledgments

We thank Selma Tolosa and Nelly Villagira for their technical assistance 
and Maria Rita Furnkorn for her secretarial assistance.

Disclosures

The authors have no financial conflict of interest.

References


3. Abbot, N. C., V. A. Spence, J. Swanson-Beck, F. M. Carnochan, J. H. Gibbs, 
from transtranscutaneous measurements of pO2 and pCO2 potential for 
nominative mon-

Cytometry of response to tuberculin skin testing. 

toneal fluid, and drainage fluid in the presence or absence of bacterial infection 

P02, and PCO2 in draining fluid allows for rapid detection of infectious compli-


acidity correlates with radiological joint destruction in rheumatoid arthritis 
knees. J. Rheumatol. 16: 468–472.


acidity correlates with radiological joint destruction in rheumatoid arthritis 
knees. J. Rheumatol. 16: 468–472.

P02, and PCO2 in draining fluid allows for rapid detection of infectious compli-


acidity correlates with radiological joint destruction in rheumatoid arthritis 
knees. J. Rheumatol. 16: 468–472.

15. Hunt, J. F., K. Fang, R. Malik, A. Snyder, N. Malhotra, T. A. E. Platts-Mills, 


acidity correlates with radiological joint destruction in rheumatoid arthritis 
knees. J. Rheumatol. 16: 468–472.


