Lactic and hydrochloric acids induce different patterns of inflammatory response in LPS-stimulated RAW 264.7 cells

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Kellum, John A., Mingchen Song, and Jinyou Li. Lactic and hydrochloric acids induce different patterns of inflammatory response in LPS-stimulated RAW 264.7 cells. Am J Physiol Regul Integr Comp Physiol 286: R686–R692, 2004. First published December 24, 2003; 10.1152/ajpregu.00564.2003.—Metabolic acidosis frequently complicates sepsis and septic shock and may be deleterious to cellular function. Different types of metabolic acidosis (e.g., hyperchloremic and lactic acidosis) have been associated with different effects on the immune response, but direct comparative studies are lacking. Murine macrophage-like RAW 264.7 cells were cultured in complete medium and lactic acidosis) have been associated with different effects on the immune response, but direct comparative studies are lacking. Murine macrophage-like RAW 264.7 cells were cultured in complete medium with lactic acid or HCl to adjust the pH between 6.5 and 7.4 and then stimulated with LPS (Escherichia coli 0111:B4; 10 ng/ml). Nitric oxide (NO), IL-6, and IL-10 levels were measured in the supernatants. RNA was extracted from the cell pellets, and RT-PCR was performed to amplify corresponding mediators. Gel shift assay was also performed to assess NF-κB DNA binding. Increasing concentrations of acid caused increased acification of the media. Trypan blue exclusion and lactate dehydrogenase release demonstrated that acification did not reduce cell viability. HCI significantly increased LPS-induced NO release and NF-κB DNA binding at pH 7.0 but not at pH 6.5. IL-6 and IL-10 expression (RNA and protein) were reduced with HCl-induced acidification, but IL-10 was reduced much more than IL-6 at low pH. By contrast, acid significantly decreased LPS-induced NO, IL-6, and IL-10 expression in a dose-dependent manner. Lactic acid also inhibited LPS-induced NF-κB DNA binding. Two common forms of metabolic acidosis (hyperchloremic and lactic acidosis) are associated with dramatically different patterns of immune response in LPS-stimulated RAW 264.7 cells. HCl is essentially proinflammatory as assessed by NO release, IL-6-to-IL-10 ratios, and NF-κB DNA binding. By contrast, lactic acid is anti-inflammatory.

ACIDOSIS APPEARS TO BE COMMON in critically ill and injured patients. Although it remains uncertain whether there is a true cause-effect relation between acidosis and adverse clinical outcomes, acidosis is a powerful marker of poor prognosis in critically ill patients (4, 12, 36, 37). Acidosis may occur as a result of increases in arterial PCO2 (respiratory acidosis) or from a variety of organic or inorganic fixed acids (metabolic acidosis). There appears to be a difference in epidemiology between patients with respiratory acidosis and those with metabolic acidosis (20), leading some investigators to hypothesize that the cause of acidosis, rather than the acidosis per se, is driving the association with clinical outcomes. Metabolic acidosis results from a variety of common etiologies (7), including lactic acidosis (8, 19, 25, 35), hyperchloremic acidosis (17, 18, 32, 42), renal failure (30), and ketosis (23). Although the underlying disease process associated with each of these subtypes carries its own clinical consequences, acidosis itself might potentially contribute to, or even attenuate, the adverse effects of these conditions.

A potentially important consequence of acidosis is its effect on the immune response. Several studies have documented the effects of decreased extracellular pH (pHo) on the synthesis and release of inflammatory mediators, especially TNF and nitric oxide (NO). Most of these studies have been conducted in resident macrophages or RAW cells, and conflicting results have been found (2, 3, 10, 11). However, different studies have used different acids to lower pHo, and no studies have compared the effects of different forms of clinically relevant acidosis on the immune response. Because critically ill and injured patients may be adversely affected by even short-term alterations in the immune response (augmentation and attenuation) and because some forms of metabolic acidosis are largely iatrogenic (18, 27, 31, 32, 41, 42), we believe that it is necessary to characterize the effects of acidosis on the immune response. We further believe that it is imperative that the mechanisms responsible for these effects be elucidated. Accordingly, we have conducted a series of experiments using LPS-stimulated RAW 264.7 murine macrophage-like cells in which we have used different acids to decrease pHo.

METHODS

Cell culture and reagents. All reagents were obtained from Sigma-Aldrich Chemical (St. Louis, MO) unless otherwise indicated. The RAW 264.7 mouse macrophage-like cell line (TIB-71, American Type Culture Collection, Manassas, VA) was grown in DMEM (BioWhittaker, Walkersville, MD) supplemented with 10% defined FBS (Hyclone, Logan, UT), 2 mM glutamine, and penicillin-streptomycin (1,000 U/ml) at 37°C in a humidified incubator containing 5% CO2. We subcultured these cells every 2–3 days when they achieved 70–80% confluence. For stimulation, we transferred cells (1 × 10^6/ml) to 24-well (1 ml/well), 12-well (2 ml/well), or 6-well (5 ml/well) polystyrene culture plates (Falcon, Franklin Lakes, NJ) in DMEM + 10% FBS. After overnight incubation, we removed the culture medium and incubated the cells in medium adjusted to various pH and with 5% FBS in the presence or absence of 10 ng/ml LPS (Escherichia coli 0111:B4) for different times. Variations in pH were accomplished by adjusting the medium with diluted HCl or lactic acid in the range 6.5–7.4. The concentrations of HCl were 2.5, 7.1, and 17.1 mM, yielding pH values of 7.4, 7.0, and 6.5, respectively. The concentrations of lactic acid were 0.5, 10, 15, 20, and 30 mM, and the corresponding pH values were 7.45, 7.41, 7.34, 7.18, 7.03, and 6.5, respectively. Cell viability was assessed by trypan blue exclusion and lactate dehydrogenase (LDH) assay (Promega, Madison, WI). Under the...
conditions employed in this study, neither HCl nor lactic acid appeared to have an effect on the viability of RAW 264.7 cells.

**NO production assay.** We measured total nitrite using cadmium-mediated reduction of NO\textsubscript{x} to NO\textsubscript{2} followed by the Griess reagent (40). To reduce NO\textsubscript{x} to NO\textsubscript{2} in supernatants from cell cultures or in plasma, cadmium filings (0.4–0.7 g/tube; Fluka Chemicals, Milwaukee, WI) were loaded into 1.5-mL microcentrifuge tubes. The filings were washed twice with 1.0 mL of deionized water, twice with 1.0 mL of 0.1 M HCl, and twice with 0.1 M NH\textsubscript{4}OH, and 10 µL of 30% (wt/vol) ZnSO\textsubscript{4} were added to 200 µL of culture supernatant or plasma, which was then vortexed, incubated at room temperature for 15 min, and centrifuged at 14,000 g for 5 min. The resulting supernatants were added to the cadmium-containing microcentrifuge tube and incubated at room temperature overnight with constant mixing. The samples were transferred to fresh microcentrifuge tubes and centrifuged again. The samples were subsequently measured for NO\textsubscript{2} content by the Griess reagent (9). The plates were read using an MRX microplate reader (Dynex Technologies, Chantilly, VA) at 550 nm.

**Cytokine assays.** The concentrations of IL-6 and IL-10 in culture supernatants were determined using ELISA kits (Pharmingen, San Diego, CA) according to the instructions provided by the manufacturer. Concentrations of IL-6 or IL-10 were calculated by comparison with standard curves.

**RT-PCR.** Total RNA was extracted from RAW 264.7 cells with RNA-Bee reagent (TEL-TEST, Friendswood, TX) according to the manufacturer’s instructions. The amount of RNA was determined by spectrophotometry. Two micrograms of RNA from each sample was reverse transcribed to cDNA by using 200 U/µL Moloney’s murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). The reaction was allowed to proceed for 10 min at 25°C followed by 60 min at 42°C. The reaction was terminated by heating to 95°C for 5 min. The PCR was performed in a total volume of 50 µL containing Taq polymerase buffer (Invitrogen), deoxynucleotide mixture (0.2 mM each), 1.5 mM MgCl\textsubscript{2}, 2.5 U of Taq polymerase (Invitrogen), oligonucleotide primers (0.5 µM each), and 2.5 µL of the RT product. The specific primers for TNF-α, IL-6, IL-10, and inducible NO synthase (iNOS) are indicated in Table 1. Amplification was performed for 33–35 cycles. Each cycle consisted of denaturation for 1 min at 95°C, annealing for 1 min at the appropriate primer-specific temperature (Table 1), and extension for 1.5 min at 72°C. An additional incubation at 72°C for 10 min was executed after the last cycle. To document equal loading of RNA, primers for 18S ribosomal RNA were used.

**NF-κB DNA binding.** Nuclear extracts for EMSA were prepared by incubating 1 × 10\textsuperscript{6} RAW 264.7 cells on ice for 15 min with 1 mL of cell lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM MgCl\textsubscript{2}, 1 mM DTT, 0.1 mM Na\textsubscript{2}EDTA, 0.5 mM PMSF, 2 µg/ml aprotinin, and 2 µg/ml leupeptin]. After addition of 62.5 µL of 10% NP-40, the mixture was centrifuged at 5,000 g at 4°C for 10 min. The crude nuclear pellet was suspended in 200 µL of nuclear lysis buffer [50 mM HEPES (pH 7.9), 50 mM KCl, 300 mM NaCl, 0.1 mM Na\textsubscript{2}EDTA, 1 mM DTT, 0.1 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, and 10% glycerol] and incubated on ice for 30 min. The suspension was centrifuged at 16,000 g at 4°C for 30 min. The supernatant (nuclear proteins) was collected and stored at −80°C until it was assayed. Nuclear protein concentration was determined using a protein assay kit with BSA as a standard (Bio-Rad Laboratories, Hercules, CA). EMSA to assess NF-κB DNA binding was performed using an appropriate consensus oligonucleotide probe. The sequence of the double-stranded NF-κB oligonucleotide was as follows: 5′-agt tga ggg gag ttc ccc agg c-3′ (sense) and 3′-tca act ccc ctg aaa ggg tcc g-5′ (antisense; Promega). The oligonucleotides were end labeled with [γ-\textsuperscript{32}P]ATP (Du Pont-New England Nuclear, Boston, MA) using T4 polynucleotide kinase (Promega). Nuclear proteins (5 µg) were incubated with [γ-\textsuperscript{32}P]-labeled NF-κB probe at room temperature for 30 min in a binding buffer that consisted of 50 mM Tris (pH 7.5), 5 mM MgCl\textsubscript{2}, 250 mM NaCl, 2.5 mM DTT, 2.5 mM EDTA, 20% glycerol, and 0.25 mg/ml poly(dI-dC) to balance the volume of binding reaction mixture being 20 µL. The mixture was subjected to electrophoresis on a 4% nondenaturing polyacrylamide gel in 0.5× Tris-boric acid-EDTA buffer. After electrophoresis, the gels were vacuum dried and exposed to Kodak X-Omat AR film (Rochester, NY) between two intensifying screens at −80°C.

**Statistical analysis.** Differences within and between groups were analyzed using analysis of variance for repeated measures. Values are means ± SD. Statistical analysis was performed using MedCalc (version 4.2) and Stata (version 6.0) software. *P < 0.05* was considered statistically significant.

**RESULTS**

Dramatically different patterns of inflammatory mediator expression occurred with different acids, despite normalization to the same pH\textsubscript{0}. In our first set of experiments, in which HCl was used to acidify the cell culture medium, the acidic medium itself did not significantly affect the release of the inflammatory mediators NO, IL-6, and IL-10 in the absence of LPS. However, compared with pH\textsubscript{0} 7.4, acidification (pH\textsubscript{0} 7.0) was associated with a significantly increased NO release in response to LPS stimulation (40.23 vs. 68.35 µM). Interestingly, under more extreme acidic conditions (pH\textsubscript{0} 6.5), NO release decreased in response to LPS and was again similar to that at pH\textsubscript{0} 7.4 (40.08 µM; Fig. 1). At pH\textsubscript{0} 6.5, IL-6 and IL-10 release were significantly less than at pH\textsubscript{0} 7.0 or 7.4. However, IL-10 release was reduced far greater than IL-6 release, and thus the ratio of IL-6 to IL-10 increased significantly from 5:1 at pH\textsubscript{0} 7.4 to 55:1 at pH\textsubscript{0} 6.5 (Fig. 1). Under similar conditions, however, acidification induced by HCl had different effects on mRNA expression for iNOS, IL-6, and IL-10. Specifically, with a decrease in pH from 7.4 to 6.5, iNOS mRNA expression increased; IL-6, by contrast, decreased, while IL-10 did not change significantly (Fig. 2).

To clarify the mechanism by which HCl influenced the release of cytokines from LPS-stimulated cells, we measured NF-κB DNA binding using EMSA after exposure to different concentrations of HCl. Again, acidification (pH\textsubscript{0} 7.0) significantly increased LPS-induced NF-κB DNA binding compared with pH\textsubscript{0} 7.4, whereas more extreme acidification (pH\textsubscript{0} 6.5) actually attenuated NF-κB DNA binding (Fig. 3).

Table 1. Primers, annealing temperatures, and cycle numbers for semiquantitative PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Fragment Size, bp</th>
<th>Annealing Temp, °C</th>
<th>Cycles</th>
</tr>
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<tbody>
<tr>
<td>iNOS</td>
<td>426</td>
<td>60</td>
<td>28</td>
</tr>
<tr>
<td>S: CACCAACGGCCACATCGGATT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS: CGGACCTGATGTTGCAATTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>600</td>
<td>62</td>
<td>26</td>
</tr>
<tr>
<td>S: CTGGTGCAACACCAGCCTCCCCCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS: ATGCCTAGGCCAATAAGCCTAGGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>237</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>S: ACCTGCTAGAAGTGAGTCGCCAGCCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS: CTATGCTAGTTGAGAAGATGTCAAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18S</td>
<td>209</td>
<td>58</td>
<td>22</td>
</tr>
<tr>
<td>S: CCCCGGGAGGTAGTGACCAGAAAAAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS: CGCCCCGTCCTCAGATCACCTAC</td>
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iNOS, inducible nitric oxide synthase; S, sense; AS, antisense.
In a second set of experiments, we used increasing concentrations of lactic acid (0–30 mM) to increase acidification of the medium. However, unlike HCl, lactic acid significantly decreased LPS-induced NO, IL-6, and IL-10 expression at protein (Fig. 4) and mRNA (Fig. 5) levels in a dose-dependent manner. Furthermore, lactic acid inhibited LPS-induced NF-κB DNA binding, again in a dose-dependent fashion (Fig. 6).

DISCUSSION

To our surprise, dramatically different patterns of inflammatory mediator expression occurred with different acids, despite titration to nearly the same pHo. Our results suggest a proinflammatory effect of HCl, consistent with the existing literature on the effects of HCl on TNF synthesis (2, 10, 11). However, the paradox in which mild and severe acidosis induced by HCl results in opposite effects on NO has not been satisfactorily explained. Pedoto and colleagues (29) suggested that the optimal intracellular pH (pHi) for iNOS is near 7.0 and that the addition of acid would lower pHi toward the optimal value, thus increasing iNOS activity and NO production. Further addition of acid would cause pHi to fall below the optimal value, leading to a decrease in NO production (29). This hypothesis was recently tested by Huang et al. (14), who demonstrated that the optimal pHo for NO formation by iNOS was 7.2 in RAW 264.7 cells. However, they also noted that alkaline pHo favored expression of iNOS protein but that posttranscriptional mechanisms predominated, resulting in an increased NO release at slightly acidic pHo. Finally, it is worth noting that peroxynitrous acid increases with decreases in pH and that this is the active form of this oxidant (39).

Our results demonstrate that different degrees of hyperchloremic acidification have different effects on inflammatory mediator release as well as NF-κB DNA binding. Overall, the effects of HCl appear to be proinflammatory. These results agree with studies in resident peritoneal macrophages in which Bellocq and colleagues (2) found that these cells produced more NO when incubated in medium at pHo 7.0 that at pH 7.4, and this effect was associated with upregulation of iNOS mRNA as well as activation of NF-κB. However, the results with HCl are in marked contrast to our data using lactic acid, which appears to be anti-inflammatory to RAW 264.7 cells as assessed by decreased cytokine expression and NF-κB DNA binding.

Our results are in general agreement with previous studies. Most have been conducted in resident macrophages or RAW cells, and although they have found conflicting results (2, 3, 10, 11), our data provide potential explanations for these discrepancies. Studies using HCl have consistently shown proinflammatory effects at the level of NF-κB DNA binding or TNF synthesis provided pHo was not <6.0 (2, 10, 11), although TNF secretion was reduced, even at pHo as high as 7.0 (3, 10, 11). Studies of nonstimulated resident peritoneal macrophages (2) and LPS-stimulated RAW 264.7 cells (14) have shown increased NO formation at moderately reduced pHo (7.0–7.2). However, more severely acidic pHo reduces NO formation (2, 14), and there is an apparent dissociation between the pHo effects on iNOS mRNA, protein, and final NO release (14).
Indeed, our results confirm this dissociation, although timing could play a role. Thus HCl seems to affect inflammatory mediators differently at different stages in their synthesis and release. Until now, little has been known about the effects of HCl on other cytokines or on the kinetics of pHo-mediated effects.

Lactic acid has been studied in an even more limited way than HCl. Lactic acid (pH o 6.75) has been shown in one study (15) to result in increased TNF production and secretion in LPS-stimulated peritoneal macrophages. This finding is surprising in light of the growing evidence of a protective effect of lactic acid in certain scenarios, most notably neuronal injury (13, 33, 34). Several studies have sought to explore the effect of dialysis solutions on the immune response (5, 16). These acidic, lactate-based solutions have been shown to decrease various aspects of the immune response, including TNF synthesis and release (5, 16). Douvdevani et al. (5) also demonstrated a decrease in LPS-induced NF-κB DNA binding in human blood-derived macrophages incubated with lactated dialysis solution. Although these solutions are also hypotonic and have excessive glucose concentrations, variables known to influence immune function (1, 16), they provide additional evidence of a potential anti-inflammatory role of lactate and highlight potential differences between various acids and their effects on the immune response.

Our findings may have important clinical significance. Although common causes of metabolic acidosis, such as lactic acidosis and renal failure, may be unavoidable, often the source of metabolic acidosis is at least partly iatrogenic, because saline resuscitation is often used to treat shock. Large-volume saline infusion produces metabolic acidosis by increasing the plasma Cl⁻/HCO₃⁻ concentration relative to the plasma Na⁺ concentration (18, 27, 31, 32, 41, 42). The result is a reduction in the strong ion difference, the difference between positively and negatively charged electrolytes, which in turn produces an increase in free H⁺ to preserve electrical neutrality (38). Furthermore, the management of metabolic acidosis is controversial. Despite the association between metabolic acidosis and adverse clinical outcomes, clinical evidence that treating metabolic acidosis is beneficial has not been forthcoming. In their systematic review, Forsythe and Schmidt (6) could not find...
evidence to support the routine use of sodium bicarbonate in the treatment of lactic acidosis. Indeed, some authors have argued that some forms of acidosis, such as respiratory (22) or lactic (24) acidosis, may even have beneficial effects. In support of this point of view are data from experimental models of ischemia-reperfusion injury in animals that suggest that correcting respiratory acidosis with sodium bicarbonate may worsen inflammation and organ injury (21). Furthermore, even severe lactic acidosis commonly occurs as a result of exercise (26), and it would seem unlikely that short-term lactic acidosis would be harmful. However, these conditions may be quite distinct from hyperchloremia or organic acidosis of renal failure or even prolonged lactic acidosis. Our data suggest that different forms of metabolic acidosis have quite different effects on the immune response and could, therefore, have different effects on clinical outcomes.

Our study has several limitations. First, although RAW 264.7 cells are immunologically active and respond to LPS stimulation, they do not necessarily represent the full spectrum of immune effector cells. It is not known whether other cell types might behave differently when subjected to acidosis. Similarly, our model, 24-h LPS incubation, while being suitable for examination of NO, NF-κB DNA binding, and long-acting cytokines such as IL-6 and IL-10, is not ideal for study of other potentially important cytokines (e.g., TNF). Our methods also do not allow for the measurement of pHi. Thus it is not possible to discern whether our results are due to differences in the rates of intracellular acidification when different acids are added to the extracellular fluid. For example, lactate may penetrate the intracellular environment to a greater degree or more rapidly than Cl⁻. Indeed, lactate may arise from the intracellular compartment, whereas Cl⁻ is typically thought of as arising exogenously. Importantly, more lactic acid than HCl was needed to sustain a given pH, suggesting that these anions may be handled differently by the cells. Our study is further limited in scope as to mechanism by only examining one transcription factor (NF-κB). Although NF-κB DNA binding is integral to the expression of IL-6, IL-10 regulation appears to be considerably more complex (28).

Finally, our results raise the following hypotheses. First, because different fixed acids are associated with different patterns of effects on cytokine and NO release, we hypothesize...
that 1) different ions enter the cell at different rates, thus affecting pH differently for a given pHo, or 2) acids affect cytokine and NO release through pH-independent mechanisms. Second, given that different acids affect NO and cytokine release sometimes in the same direction and sometimes opposite to the effects on NF-κB DNA binding, we hypothesize that the effects of acids are mediated through NF-κB-dependent and other pathways. Third, because the effects of acids on inflammatory mediator expression occur at levels of pH2 and ion concentrations easily achievable under clinical conditions, we hypothesize that agents that specifically block or stimulate ion channels or mimic the action of specific ion channels (i.e., ionophores) should result in meaningful changes in the inflammatory mediator response.

Conclusion. We conclude that two common forms of metabolic acidosis (hyperchloremic and lactic acidosis) are associated with dramatically different patterns of immune response in LPS-stimulated RAW 264.7 cells. HCl is essentially proinflammatory as assessed by NO release, IL-6-to-IL-10 ratios, or LPS-stimulated RAW 264.7 cells. Ht is essentially proinflammatory mediator expression occurs at levels of pH2 and other pathways. Third, because the effects of acids on inflammatory mediator expression occur at levels of pH2 and ion concentrations easily achievable under clinical conditions, we hypothesize that agents that specifically block or stimulate ion channels or mimic the action of specific ion channels (i.e., ionophores) should result in meaningful changes in the inflammatory mediator response.

REFERENCES


