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TUMOR NECROSIS FACTOR EXPRESSION IS AMELIORATED AFTER EXPOSURE TO AN ACIDIC ENVIRONMENT

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Abstract

Background—It has been well established that laparoscopic surgery presents several clinical benefits, including reduced pain and a shorter hospital stay. These effects have been associated with a decrease in the inflammatory response. Previous studies have demonstrated that reduced inflammation after laparoscopic surgery is the product of carbon dioxide insufflation, which decreases peritoneal pH. The objective of this study was to investigate the cellular and molecular mechanisms responsible for the reduced response after exposure to acidic environments.

Materials and methods—A murine macrophage line (J744) was incubated in culture medium at pH 6.0 or pH 7.4 for 3 hours at 37°C. Then, cells were stimulated with lipopolysaccharide (LPS) at pH 7.4, the expression of TNF- α (qRT-PCR or ELISA) and intracellular pH were measured. In addition, CD14 and Toll-like receptor 4 expression and NF- κ B nuclear translocation were analyzed.

Results—A significant decrease in LPS-induced TNF- α expression levels was observed in cells pre-incubated at pH 6.0 in comparison with cells at neutral pH conditions. This decrease in TNF- α levels was not associated with a reduction in cell surface expression of CD14 and Toll-like receptor 4. Exposure to an extracellular acidic environment resulted in a reduction of I κ B phosphorylation and NF- κ B nuclear translocation, secondary to a significant drop in cytosolic pH.

Conclusions—These observations provide a potential mechanism for the reduced expression of TNF- α after exposure to low extracellular pH, which may be related to acidification after CO₂ insufflation during laparoscopic surgery. In addition, extracellular acidic pH environments could emerge as an important regulator of macrophage function.

Keywords

cytokine; inflammation; macrophages; acidic environments; cellular pH

INTRODUCTION

Laparoscopic surgery has become an increasingly common procedure during the last two decades. This surgical approach has been extended to a growing number of clinical situations, which vary in complexity and time duration. The advantage of laparoscopic operations over open procedures is related to several clinical benefits, including reduced hospital stay, decreased post-operative pain, shorter post-operative ileus and improved cosmesis. Part of the benefits of laparoscopic surgery is associated with a reduced inflammatory response after the operative procedure (1,2,3). It was originally speculated that this reduced inflammatory response was due solely to the smaller incision size. However, studies in experimental animal models have challenged this assumption, and it has been shown that carbon dioxide (CO₂) insufflation is specifically responsible for the reduced inflammatory response observed during these procedures (4,5). These experimental findings are consistent with clinical observations demonstrating a significant benefit of the laparoscopic procedure using CO₂ with respect to helium or air insufflations (3) or in comparison with the wall-lift technique (2).

Previous studies from our laboratory have shown improved survival in response to intra-abdominal sepsis in animals that were insufflated with CO₂ in comparison with animals insufflated with helium (4). These results were correlated with reduced production of TNF- α and elevated plasma levels of IL-10 (6). In addition, exposure of aqueous solutions to CO₂ resulted in a decrease of the medium pH. Thus, we speculated that exposure of the peritoneal cavity of an animal to CO₂ pneumoperitoneum would produce a decrease in the pH of this compartment. Our studies have shown a significant local peritoneal acidosis during CO₂ pneumoperitoneum that is transient and reversible (7). These observations led us to hypothesize that the reduction in the inflammatory response was the product of peritoneal acidosis. This hypothesis was tested by chemical acidification of the peritoneum. We observed that, indeed, the inflammatory response triggered by LPS injection was reduced in these conditions with respect to match controls (8). West et al. have previously shown that peritoneal macrophages (M Φ s) exposed to CO₂ in culture conditions had a diminished LPS-induced inflammatory response compared to cells exposed to helium or air. Moreover, they showed that these cells underwent a cytosolic acidification following CO₂ exposure (5). The question that emerges is what is the mechanism of transduction of extracellular acidic pH? In the present study, we examined the cellular mechanism involved in the attenuated inflammatory response after exposure to acidic extracellular environments.

MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS) from E. Coli (026:B6) was obtained from Dibco Laboratories (Detroit, MI). Anti-mouse NF- κ B (p65), anti-rabbit I κ B and anti-rabbit phosphorylated IKK- α were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-mouse phosphorylated I κ B α , whole I κ B α , (L35A5) and secondary antibodies were purchased from Cell Signaling (Beverly, MA). Anti-mouse Ig-G conjugated with FITC, anti-rabbit Ig-G conjugated with Cy3, MTT (diphenyltetrazolium bromide), fatty-acid-free bovine serum, and Escherichia coli LPS (serotype 026:B6) were obtained from Sigma-Aldrich (St. Louis, MO). Purified anti-CD16/CD32 (clone 93), FITC-conjugated anti-mouse CD14 (clone Sa2-8) and APC-conjugated anti-mouse Toll-like receptor 4/MD-2 (clone MTS510) were all purchased from eBioscience (San Diego, CA). A pH sensitive dye, 2'-7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), nigericin, and TNF- α ELISA kit were purchased from Invitrogen (Carlsbad, CA). Complete EDTA-free protease inhibitor was obtained from Roche (Basel, Switzerland). The Supersignal West Pico Chemiluminescent Kit was purchased from Pierce (Rockford, IL).

Cell Culture

The murine MΦ line, J774A.1 (ATCC, Manassas, VA), was cultured in sodium bicarbonate buffered RPMI-1640 medium (RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum and 10 IU/mL penicillin and 10 µg/mL streptomycin) at 37° C with 5% CO₂ in a humidified incubator. Cells were plated at least 16 h prior to the experiments. Pretreatment was performed in a HEPES-buffered RPMI-1640 medium (containing 10% heat-inactivated fetal bovine serum, 10 IU/mL penicillin and 10 µg/mL streptomycin) titrated to pH 6.0 or pH 7.4 using hydrochloric acid. A calibrated pH probe (Dow Corning, Corning, NY) was utilized to measure the pH of the extracellular medium. Cellular viability was verified by an MTT assay or trypan blue exclusion when appropriate.

Cytokine Expression

Cells (2×10^5 cells/well) were plated in a 96-well plate and incubated for 1 or 3 h at pH 6.0 or 7.4 at 37°C. After 3 h, the pre-incubation medium was removed and its pH was measured. The cells were then stimulated with LPS (100 ng/mL) at a pH of 7.4 for 1.5 h. The supernatant was collected, and the amount of TNF-α was measured by ELISA and normalized by the number of viable cells using an MTT assay. Alternatively, cells (5×10^5 cells/well) were plated in a 6-well plate and incubated for 1 or 3 h at pH 6.0 or 7.4 at 37° C. After 3 h, the pre-incubation medium was removed and its pH was measured. The cells were then stimulated with LPS (100 ng/mL) at a pH of 7.4 for 1.5 h. Cells were lysed and total RNA was purified using the Trizol protocol. RNA was DNase treated to remove genomic DNA and a cDNA library was created using the Applied Biosystems high capacity cDNA synthesis kit. Standards for GAPDH and TNF-α were prepared after PCR amplification using primers from Applied Biosystems. PCR amplified fragments were then subcloned into a pGEM-Teasy vector from Promega. TNF-α mRNA levels were measured by quantitative real time PCR (qRT-PCR). Results were quantitated by comparison with a standard curve and expressed in mRNA copy numbers. Samples were normalized by content of GAPDH and results expressed in arbitrary units (AU).

Immunostaining

Cells (0.5×10^6) on a glass cover slide were pretreated at pH 6.0 or 7.4 for 3 h at 37° C. Cells were then returned to neutral pH (7.4) and stimulated with LPS (100 ng/mL) for 25 min. Cells were fixed with 4% paraformaldehyde and permeabilized with acetone (15 seconds, 4° C). Nonspecific binding was blocked by incubation with 20% goat serum and 20% sheep serum (30 min, 25° C). Fixed cells were incubated with primary antibodies (1:200) for 60 min at 4° C, washed thoroughly with phosphate buffered saline (PBS), and incubated with secondary antibodies (1:1000) to IκB and NFκB that were conjugated with Cy3 or FITC, respectively. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Cells were further washed with PBS and mounted with 3.9% 1,4 diazabicyclo(2,2,2)octane in PermaFluor aqueous mounting medium. Antibody binding was visualized using a fluorescent microscope.

Flow Cytometry

Cells (5×10^5 cells/well) were plated in 6-well plates and incubated for 1 or 3 h at pH 6.0 or 7.4 at 37° C. The cells were then washed twice with PBS and once with FACS staining buffer (DPBS without Ca²⁺/Mg²⁺ supplemented with 0.5% BSA). Thereafter, Fcγ III and II receptors were blocked using purified anti-CD16 and anti-CD32 antibodies respectively for 15 min at 4° C, and cells were stained with FITC-conjugated anti-mouse CD14 and APC-conjugated anti-mouse Toll-like receptor 4/MD-2 antibodies for 30 minutes at 4° C. Data acquisition was performed with a BD FACSCanto flow cytometer and analyzed using the FlowJo software (Tree Star, Ashland, OR).

Western Blotting

Cells (6×10^6) were pretreated at pH 6.0 or 7.4 for 3 h at 37° C. Cells were then returned to neutral pH (7.4) medium and incubated with LPS (100 ng/mL) for 10 min. Cells were washed twice with cold PBS (pH 7.4) and lysed with 500 μ L ice-cold lysis buffer (50 mM b-glycerophosphate, 1.5 EGTA, 1.0 EDTA, 1% Triton X-100, 1.0 mM DTT containing the complete EDTA-free protease inhibitor). Cell suspensions were sonicated (4° C, 1300 rpm, 5 min), and protein concentration was determined by the bicinchoninic acid (BCA) method (Pierce Chemical, Rockford, IL). Total protein extracts (30 μ g) were separated by SDS-PAGE using 8–16% tris-glycine polyacrylamide gradient gels and transferred to nitrocellulose membranes. The membranes were washed with TBS/Tween20 and incubated for 1 hour at room temperature with secondary antibody in 5% milk in TBS/Tween20, anti-rabbit IgG, HRP-linked or anti mouse, HRP-linked. The antibody complexes were detected using the Supersignal West Pico Chemiluminescent Kit (Pierce Chemical, Rockford, IL).

Internal pH Measurement

Cells (2×10^5 cells/well) were plated in a 96-well plate and incubated overnight (16 h) at 37° C in neutral pH (7.4) medium. The medium was removed and replaced with 300 μ L of HEPES-buffered RPMI at pH 6.0 or pH 7.4 containing 1 μ M of the acetoxymethyl ester form of the fluorescent pH-indicating dye 2'-7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF). Cytoplasmic esterases cleave the ester linkages in this compound, trapping it within the cell. The fluorescence of the internal BCECF was measured in a fluorometer at excitation 450 nm and 500 nm and emission 530 nm every 15 min for 60 min (8). The internal pH was determined by establishing a calibration curve. Cells (2×10^5 cells/well) were plated in a 96-well plate and incubated with medium of pH 5.5, 6.0, 6.5, 7.0, 7.5, or 8.0 supplemented with 1 μ M BCECF, 0.1 μ M nigericin, and 125 mM KCl (9). The ionophore, nigericin, exchanges K^+ and H^+ to equilibrium, resulting in cytosolic acidification at the same level as the extracellular medium. The internal pH was clamped with the addition of 5 mg/mL of fatty-acid-free serum albumin in medium. The fluorescence of the internal BCECF was measured in a fluorometer at excitation 450 nm and 500 nm and emission 530 nm. The calibration curve was established by plotting the excitation ratio of F_{500}/F_{450} vs. the known pH. A calibration curve was performed for each separate experiment. To measure the cytosolic pH during the recovery period after pretreatment, cells (2×10^5 cells/well) were plated in a 96-well plate incubated with 300 μ L of HEPES-buffered RPMI at pH 6.0 or 7.4 for 3 h at 37° C. After pretreatment, the cells were washed twice with neutral PBS, and the medium was replaced with neutral (pH 7.4) RPMI medium containing 1 μ M BCECF. The fluorescence of the internal BCECF was measured in a fluorometer at excitation 450 nm and 500 nm and emission 530 nm every 15 min for 60 min and compared to an established calibration curve (see above). To determine if the change in I κ B phosphorylation after acidic pretreatment was due to the reduction in the cytosolic pH, a pharmacologic approach was utilized to “clamp” the internal pH of the cells to our desired level before LPS stimulation. Cells (6×10^6) were plated and incubated with HEPES-supplemented RPMI medium at pH 6.0 or 7.4 with 0.1 μ M nigericin and 125 mM KCl for 30 min at which point 5 mg/mL fatty-acid-free serum albumin in medium was added. The cells were washed twice with sterile, neutral PBS, incubated with neutral (pH 7.4) RPMI for 30 min, and then stimulated with LPS (100 ng/mL) for 10 min. The cells were lysed, and a Western blot was performed as described above.

Statistical Analysis

Each experiment was performed in triplicate, and each assay was performed in duplicate. Data were expressed as means $\pm$ standard error of the mean (SEM). Differences between two groups were analyzed by the Student's t-test. Significance was accepted at $p < 0.05$.

RESULTS

The expression of TNF- α after pretreatment of macrophages in acidic conditions is reduced

Previous studies have shown that acidification of the peritoneal cavity by either CO₂ insufflation or acid buffers resulted in a reduction of the inflammatory response in rodents (6). We investigated whether exposure of MΦs to an acidic environment affects the expression of TNF- α after stimulation with LPS. MΦs (J774 cells) were incubated in culture medium at pH 6.0 or 7.4 for 1 or 3 h. There were no changes in medium pH during the incubation period. After the pretreatment period, cells were returned to neutral conditions and then immediately exposed to LPS (100 ng/mL) for 1.5 hours. TNF- α mRNA levels were measured by qRT-PCR (Fig. 1A) or protein levels in the extracellular medium by ELISA (Fig. 1B). Pretreatment in acidic conditions resulted in a significant reduction in the LPS-induced expression of TNF- α , both at the mRNA (57%) and protein (53%) levels ($p < 0.05$, Fig. 1A and B, respectively). There was no difference in cellular viability of the cells exposed to either pretreatment medium as determined by trypan blue exclusion method. Then, it was important to evaluate if the effect of low pH exposure was transient. MΦs were pretreated at pH 6.0 or 7.4 for 3 h and then returned to neutral conditions. Cells were stimulated with LPS (100 ng/mL) at different time points during the recovery period at neutral pH. A significant decrease in TNF- α production was observed up to 45 min during the recovery period at neutral pH in comparison with cells maintained at pH 7.4 during the whole experiment, returning to basal conditions within 1 h (Fig. 2). A possible explanation for the reduced expression of TNF- α after exposure to an acidic environment could be due to down-regulation of surface receptors involved in the response to LPS. Flow cytometry analysis of surface levels of CD14 and Tlr4/MD-2 in MΦs that were incubated at pH 6 for 1 or 3 h showed no difference from cells maintained at pH 7.4 (Fig. 3). Another possibility is that reduction of cytosolic pH alters the redox level of the cell, modifying the activation of NF- κ B (10). We did not observe any changes in glutathione levels after exposure of MΦs to a low external pH (not shown).

NF κ B nuclear translocation is impaired during low pH treatment

TNF- α production is mediated by the activation of NF κ B. In its inactive state, NF κ B resides in the cytosol associated with its inhibitory protein, I κ B. Stimulation of cells with LPS activates an intracellular signaling cascade, resulting in the phosphorylation of I κ B and the release of NF κ B, which is then free to translocate into the nucleus, promoting transcription of certain chemokines and cytokines, such as TNF- α . The translocation of NF κ B into the nucleus was monitored by indirect immunostaining. Macrophages were cultured on cover slides and pretreated at pH 6.0 or 7.4 for 3 h and were then returned to neutral conditions and incubated with LPS for 10 min. Then, cells were fixed, permeabilized and incubated with antibodies against I κ B and NF κ B as well as secondary antibodies against these two proteins conjugated to Cy3 (red) or FITC (green), respectively. In absence of LPS stimulation, there was co-staining of I κ B and NF κ B in the cytosol (yellow), suggesting the lack of NF κ B nuclear translocation in cells pretreated at a low or neutral pH level (Fig. 4A). After LPS stimulation, a clear translocation of NF κ B into the nucleus was observed as demonstrated by the visualization of a green fluorescent signal in the nucleus and a red signal (I κ B) in the cytosol. In contrast, cells that were pretreated at pH 6.0 and stimulated with LPS displayed reduced nuclear translocation of NF κ B (Fig. 4B).

Phosphorylation of I κ B is affected by pre-incubation in acidic conditions

Under resting conditions, the nuclear translocation of NF κ B is prevented by its inhibitory co-protein, I κ B. When cells are activated by LPS, IKK is phosphorylated and then this kinase phosphorylates I κ B, which separates from NF κ B and eventually is degraded by the

proteasome system. To investigate the effects of acidic conditions on the preceding signaling cascade, cells were pretreated for 3 h at pH 6.0 or 7.4 and then stimulated with LPS for 10 min. The presence of phosphorylated I κ B and IKK was visualized by Western blotting. Pretreatment of cells at pH 6.0 significantly reduced the LPS-stimulated phosphorylation of I κ B (Fig. 5A), but did not affect the phosphorylation of IKK (Fig. 5B) as compared with cells pretreated at neutral pH. Incubation with acidic conditions did not have any effect on the levels of non-phosphorylated I κ B (results not shown).

Cytosolic pH decreases during incubation with extracellular acidic conditions

Because intracellular signaling is affected by exposure to the acidic conditions of the extracellular medium, we hypothesized that the internal pH of the cell may change during the acidic pretreatment, as has been reported previously (5). To test this hypothesis, we incubated M Φ s with medium at pH 6.0 and 7.4 and monitored their intracellular pH using the fluorescent pH-indicating dye 2'-7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF). We found that M Φ s incubated at extracellular pH 6.0 displayed a rapid reduction of the intracellular pH, reaching pH 6.02 within 45 min. (Fig. 6A). In order to evaluate if the reduction of intracellular pH was reversible, cells were then pretreated for 3 h at extracellular pH 6.0, and internal pH was monitored after the cells were returned to neutral conditions. Cytosolic pH showed a rapid increase to pH 7.4 within 30 min of incubation at extracellular neutral pH (Fig. 6B).

A decrease in cytosolic pH correlates with inhibition of I κ B phosphorylation

To determine whether the effect of acidic pretreatment on I κ B phosphorylation is due to the reduction in cytosolic pH, the internal cellular pH was clamped to pH 6.0 or 7.4 by incubation with the ionophore nigericin in the presence of KCl (125 mM) at the respective extracellular pH (6.0 or 7.4) (9). This ionophore exchanges K⁺ and H⁺ to equilibrium, resulting in cytosolic acidification at the same level as the extracellular medium. Under these conditions, M Φ s were stimulated with LPS at neutral extracellular conditions. Cells that had an internal pH clamped at pH 6.0 had a significant reduction in the phosphorylation of I κ B. However, no difference in the phosphorylation of IKK was observed when compared to cells with an internal pH clamped at pH 7.4 (Figs. 7A and 7B).

DISCUSSION

It is well accepted that the inflammatory response associated with surgery is reduced after laparoscopic operations. Previous studies from our laboratory, as well as others, have demonstrated that CO₂ insufflation is a key component in reducing the inflammatory response during laparoscopic surgery. In an animal model of intra-abdominal sepsis induced by cecal ligation and puncture, the use of CO₂ insufflation decreased the systemic release of acute phase reactants and cytokines and decreased overall mortality compared with the same procedure using helium or air insufflation (4). The reduced inflammatory response after CO₂ in comparison with He insufflation was confirmed after injection of LPS (6). These observations are consistent with clinical studies demonstrating reduced release of acute phase proteins and pro-inflammatory cytokines after laparoscopic surgery compared with the equivalent open operations (2,3). Other investigators have shown that isolated peritoneal M Φ s had a diminished inflammatory response when exposed to acidic conditions produced by CO₂ in culture conditions (5). The “magic” associated with CO₂ insufflation has been demonstrated to be the product of local peritoneal acidosis (7,11). This observation was confirmed by chemical acidosis of the peritoneum (12).

In the current study, we used a cell culture model to investigate the mechanism involved in the reduction of the inflammatory response after exposure to acidic conditions. Using

chemical, as opposed to gaseous, medium acidification, we found that exposure of MΦs to an extracellular low pH (6.0) environment resulted in reduced expression of TNF- α after stimulation with LPS. The transcriptional regulation of this gene is mediated by activation of the nuclear factor kappa B (NF- κ B). Thus, MΦs that are stimulated by an inflammatory signal trigger a complex intracellular signaling cascade, ultimately leading to the phosphorylation of several proteins, including I κ B kinase (IKK) and I κ B. Upon phosphorylation of I κ B, which is normally associated with NF- κ B, the transcriptional factor is released and translocates into the nucleus, whereas I κ B is degraded by the proteasome system. Since exposure to an acidic environment has been shown to modulate the release of several of the cytokines mediated by NF- κ B, we hypothesized that the signal transduction pathway associated with NF- κ B is affected by acidic extracellular conditions. In fact, we observed that phosphorylation of I κ B, which is a prime regulator of NF- κ B activation, was reduced after exposure to acidic extracellular conditions. Interestingly, phosphorylation of other kinases involved in the NF- κ B activation pathway, such as IKK, was not affected during acidic incubations. These observations were corroborated by the decrease in NF- κ B translocation into the nucleus following extracellular acidification. The effect of external low pH was not due to down regulation of surface receptors involved in the response to LPS, such as CD14 and Tlr4 as demonstrated by flow cytometry.

The apparent mechanism for the alteration in cell activation after exposure to external acidic environments was associated with a reduction in internal cellular pH. Cytosolic pH is well regulated by several membrane pumps that export protons outside the cell during metabolic events. Thus, it is possible that prolonged exposure to extracellular acidic conditions collapses this regulatory mechanism. It is very likely that exposure to acidic conditions affects the conformation of cytosolic proteins, affecting enzymatic activities, such as kinases, and modifying the cellular response. The precise mechanism involved in the change of kinase activity remains to be elucidated. An important observation is that cytosolic acidification is rapidly reversible when cells are switched to neutral environmental conditions. In fact, cellular viability was not compromised during exposure to acidic conditions as low as pH 6.0, at least during the time period of our experiments. Thus, it is unlikely that acidification during laparoscopic surgery would result in extensive cellular damage. These observations echo prior studies by Douvdevani et al. who reported that incubation of peritoneal MΦs in an acidic commercial dialysate resulted in a significant reduction in LPS-induced TNF- α release with a simultaneous reduction in NF- κ B DNA binding activity (13). West et al. also showed reduced production of LPS-induced TNF- α after exposure to a CO₂ environment in culture conditions. They also reported a decrease in cytosolic pH (5). Moreover, other studies have shown the ability of external pH to alter the internal pH of cells (5,13,14).

The finding that the NF- κ B pathway is modulated by an acidic environment may have widespread surgical implications given that NF- κ B activation is thought to contribute to the pathogenesis of many human diseases of chronic inflammation, including inflammatory bowel disease (15). Our findings suggest that CO₂ insufflation during laparoscopic surgery may be specifically beneficial for patients with chronic inflammatory conditions such as ulcerative colitis or Crohn's disease, since the acidic environment produced during laparoscopy may down-regulate the NF- κ B signaling cascade. Laparoscopic operations may also be particularly useful for individuals in whom there is an excessive, persistently-elevated inflammatory response, such as trauma patients. Carbon dioxide may, in these cases, be a therapeutic agent in reducing the negative effects of an exaggerated inflammatory response. Finally, the effect of exposure of MΦs to low extracellular pH emerges as a new potential regulator of the immune system. In fact, we have previously reported an increase in the phagocytic capacity of MΦs after exposure to an acidic environment (16). Thus, CO₂ insufflation may have a potential therapeutic application.

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REFERENCES

1. Redmond HP, Watson RW, Houghton T, et al. Immune function in patients undergoing open vs. laparoscopic cholecystectomy. *Arch Surg.* 1994; 129:1240. [PubMed: 7986152]
2. Sietses C, von Blomberg ME, Eijssbouts QA, et al. The influence of CO₂ versus helium insufflation or the abdominal wall lifting technique on the systemic immune response. *Surg Endosc.* 2002; 16:525. [PubMed: 11928041]
3. Vittimberga FJ Jr, Foley DP, Meyers WC, Callery MP. Laparoscopic surgery and the systemic immune response. *Ann Surg.* 1998; 227:326. [PubMed: 9527054]
4. Hanly EJ, Mendoza-Sagaon M, Murata K, et al. CO₂ Pneumoperitoneum modifies the inflammatory response to. *Ann Surg.* 2003; 237:343. [PubMed: 12616117]
5. West MA, DJ Hackam DJ, Baker J, et al. Mechanism of decreased in vitro murine macrophage cytokine release after exposure to carbon dioxide: relevance to laparoscopic surgery. *Ann Surg.* 1997; 226:179. [PubMed: 9296512]
6. Hanly EJ, JM Fuentes JM, Aurora R, et al. Carbon dioxide pneumoperitoneum prevents mortality from sepsis. *Surg Endosc.* 2006; 20(1482)
7. Bergstrom M, Falk P, Park PO, Holmdahl L. Peritoneal and systemic pH during pneumoperitoneum with CO₂ and helium in a pig model. *Surg Endosc.* 2008; 22:359. [PubMed: 17522919]
8. Bergman JAMJ, Evan AP, Soleimani M. Use of the pH-sensitive dye BCECF to study pH regulation in cultured human proximal tubule cells. *J Tiss Cult Meth.* 1991; 13:205.
9. Thomas JA, Buchsbaum RN, Zimniak A, Racker E. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry.* 1979; 18:2210. [PubMed: 36128]
10. Rahman I, Yan S-R, Biswas SK. Current concepts of redox signaling in the lungs. *Antioxidants & Redox Signaling.* 2006; 8:681. [PubMed: 16677111]
11. Hanly EJ, Aurora AR, JM Fuentes JM. Abdominal insufflation with CO₂ causes peritoneal acidosis independent of systemic pH. *J Gastrointest Surg.* 2005; 9:1245. [PubMed: 16332480]
12. Hanly EJ, Aurora AA, Shih SP, et al. Peritoneal acidosis mediates immunoprotection in laparoscopic surgery. *Surgery.* 2007; 142:357. [PubMed: 17723887]
13. Douvdevani A, Abramson O, Tamir A, et al. Commercial dialysate inhibits TNF alpha mRNA expression and NF-kappa B DNA-binding activity in LPS-stimulated macrophages. *Kidney Int.* 1995; 47:1537. [PubMed: 7643522]
14. Kos M, Kuebler FJ, NK Jesch NK, et al. Carbon dioxide differentially affects the cytokine release of macrophage subpopulations exclusively via alteration of extracellular pH. *Surg Endosc.* 2006; 20:570. [PubMed: 16437285]
15. Yamamoto Y, Gaynor RB. Role of the NF-kappaB pathway in the pathogenesis of human disease states. *Curr Mol Med.* 2001; 1:287. [PubMed: 11899077]
16. Grabowski JE, Vega VL, Talamini MA, De Maio A. Acidification enhances peritoneal macrophage phagocytic activity. *J Surg Res.* 2008; 147:206. [PubMed: 18498871]

Figure 1. LPS-induced TNF- α expression is reduced in M Φ s by pretreatment at a pH of 6.0

Cells (5×10^5) were pretreated in HEPES-supplemented RPMI 1640 medium at pH 6.0 or 7.4 for 1 or 3 h at 37° C. After pretreatment, the cells were returned to neutral pH (7.4) and stimulated with LPS (100 ng/mL) for 1.5 h. TNF- α mRNA levels were measured by qRT-PCR (A) or protein in the extracellular medium by ELISA and normalized by the number of viable cells in each well measured by the MTT method (B). (A) All LPS-treated samples displayed higher TNF- α mRNA levels than their non-treated counterparts ($n=4$, * $p<0.05$). A reduction of 57% was observed in TNF- α mRNA levels after exposure to pH 6 for 3 h with respect to pH 7.4 ($n=4$, * $p<0.05$ pH 6, 3 h vs. pH 7.4). (B) Protein levels were reduced in 53% at pH 6.0 compared to those pretreated at pH 7.4 ($n=3$, * $p<0.05$).

Figure 2. The reduction in LPS-induced production of TNF- α in cells pretreated in an acidic medium persists after return to a neutral pH

Cells (2×10^5) were pretreated in HEPES-supplemented RPMI 1640 medium at pH 6.0 or 7.4 for 3 h at 37° C. After pretreatment, the cells were returned to neutral pH (7.4) medium. At different points in their recovery from pretreatment ($t=0$, 15, 30, 45, and 60 min), they were exposed to LPS (100 ng/mL) for 1.5 h. TNF- α levels were measured in the extracellular medium by ELISA and normalized by the number of viable cells in each well measured by the MTT method. There was a significant reduction in the amount of TNF- α by those cells pretreated at pH 6.0 until 45 min of recovery ($n=3$, * $p < 0.05$ as compared to control conditions, $t = 0$).

Figure 3. Cell surface levels of CD14 and Tlr4 did not change after exposure to low pH
 J774 cells were incubated for 1 or 3 h at pH 6.0 or 7.4 at 37° C. Cells were then harvested, washed, and Fcγ III and II receptors were blocked using purified anti-CD16/32 antibodies. Afterward, cells were stained with FITC-conjugated anti-mouse CD14 and APC-conjugated anti-mouse Toll-like receptor 4/MD-2 antibodies for 30 minutes at 4° C. Data acquisition was performed with a BD FACSCanto flow cytometer and analyzed using the FlowJo software. There was no change in the cell surface expression of CD14 and Tlr4/MD-2 after exposure to pH 6.0 for 1 h and 3 h compared to pH 7.4 (n=4).

Figure 4. Pretreatment at a pH of 6.0 inhibits NFκB nuclear translocation after incubation with LPS

Cells (0.5×10^6) on a glass cover slide were pretreated at pH 6.0 or 7.4 for 3 h at 37° C. Cells were then returned to neutral pH (7.4) and stimulated with LPS (100 ng/mL) for 25 min. Cells were fixed with 4% paraformaldehyde, permeabilized with acetone and immunostained for NFκB or IκB. Nuclei were stained with DAPI. (A) Control samples (without LPS stimulation); (B) Samples after LPS stimulation.

Figure 5. Pretreatment at pH 6.0 inhibits IκBα phosphorylation after incubation with LPS

Cells (6×10^6) were pretreated at pH 6.0 or 7.4 for 3 h at 37° C. Cells were then returned to neutral pH (7.4) medium and incubated with LPS (100 ng/mL) for 10 min. Then, cells were lysed for protein analysis. The phosphorylated form of IκBα (A) or IKKβ (B) was determined by Western blotting. Equal loading was determined by measuring actin levels by Western blotting. The histogram represents relative degree of LPS-stimulated phosphorylation. (A) There was a significant decrease in the phosphorylation of IκBα in those cells pretreated at pH 6.0 compared to pH 7.4. ($n=3$, * $p<0.05$). (B) There was no change in the phosphorylation of IKKβ in those cells pretreated at pH 6.0 compared to pH 7.4 ($n=3$). Representative Western blots of phosphorylated IκBα (A) or IKKβ (B) are presented.

Figure 6. Incubation in acidic medium reduces the internal pH of J774 MΦs

Cells (2×10^5 cells/well) were plated in a 96-well plate and incubated overnight (16 h) at 37°C in neutral pH (7.4) medium. (A) The medium was removed and replaced with HEPES-buffered RPMI at pH 6.0 or pH 7.4 containing $1\mu\text{M}$ BCECF. (B) The medium was removed and replaced with HEPES-supplemented RPMI 1640 medium at a pH of 6.0 or 7.4 for 3 h at 37°C . After pretreatment, the cells were returned to neutral pH (7.4) medium containing $1\mu\text{M}$ BCECF. The fluorescence of the internal BCECF was measured in a fluorometer at excitation 450 nm and 500 nm and emission 530 nm every 15 min for 60 min. The internal pH was determined by comparing to a calibration curve ($n=4$).

Figure 7. Pharmacologic reduction of the internal pH of J774 cells to pH 6.0 inhibits IκBα phosphorylation after incubation with LPS

Cells (6×10^6) were incubated with HEPES-supplemented RPMI 1640 medium at a pH of 6.0 or 7.4 containing 0.1 μM nigicerin, 125 mM KCl for 1 h 37° C. The internal pH was clamped with the addition of 5 mg/mL fatty-acid-free serum albumin in neutral RPMI medium. The cells were washed twice with neutral PBS, rested in neutral pH for 30 min, and then incubated with LPS (100 ng/mL) for 10 min. Then, cells were lysed for protein analysis. The phosphorylated form of IκBα (A) or IKKβ (B) was determined by Western blot. The histogram represents relative degree of LPS-stimulated phosphorylation. (A) There was a significant reduction in the phosphorylation of IκBα in those cells pretreated at pH 6.0 compared to pH 7.4. ($n=3$, * $p<0.05$). (B) There was no change in the phosphorylation of IKKβ in those cells pretreated at pH 6.0 compared to pH 7.4. Representative Western blots of phosphorylated IκBα (A) or IKKβ (B) are presented.