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Low pH and *Helicobacter pylori* Increase Nuclear Factor Kappa B Binding in Gastric Epithelial Cells: A Common Pathway for Epithelial Cell Injury?

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Abstract *Helicobacter pylori* infection results in peptic ulceration and chronic gastritis through mechanisms which are not fully elucidated. Live *H. pylori* activate the pro-inflammatory transcription factor NF-κB in gastric epithelial cells. Patients may have peptic ulcer disease in the absence of *H. pylori* infection; therefore other factors contribute to the inflammatory process. Maximal acid output in patients with *H. pylori* infection and duodenal ulceration is significantly increased indicating a role for acid in the pathogenesis of mucosal ulceration. The effect of low pH on NF-κB activation in gastric epithelial cells has not been studied. Human gastric epithelial cells (AGS) were exposed to a range of pH changes in the presence or absence of *H. pylori*. NF-κB DNA-binding and cytosolic IκB-α were measured using electrophoretic mobility shift assay and Western blotting. NF-κB DNA-binding in gastric epithelial cells dramatically increased when the pH of the culture medium decreased. Increases in NF-κB nuclear binding were paralleled by decreasing amounts of cytosolic IκB-α. These findings were similar but less potent than those observed when cells were exposed to *H. pylori*. Low pH resulted in enhancement of *H. pylori*-induced NF-κB nuclear binding. DNA binding of NF-κB activation secondary to low pH was attenuated by PD98059 but not by SB203580. Similar to *H. pylori*, low pH potently and independently augments NF-κB nuclear binding in AGS cells and such activation appears to be mediated through MEK1-dependant signaling pathways. J. Cell. Biochem. 96: 589–598, 2005.

Key words: low pH; *H. pylori*; NF-κB; inflammation; gastric cancer cells

Nuclear factor kappa B (NF-κB) is a transcription factor involved in regulating the expression of many genes of the inflammatory and immune systems [Baeuerle, 1991; Baeuerle and Henkel, 1994]. NF-κB also plays a role in the control of cell proliferation, apoptosis and in the regulation of adhesion molecule expression [Baldwin et al., 1991; Whelan et al., 1991; Moynagh et al., 1994; Beg and Baltimore, 1996]. NF-κB exists in an inactive state in the cytoplasm of unstimulated cells as a heterodimer of p50 and RelA (p65), bound to an inhibitory protein, IκB. Cell stimulation triggers the release of IκB resulting in the translocation of the p50/RelA heterodimer to the nucleus where it binds to DNA and regulates transcription of specific genes. The dissociation of IκB results from sequential phosphorylation, ubiquitination and subsequent degradation of the multiprotein IκB complex. This involves the recently discovered IκB kinases (IKK-1 and IKK-2) interacting with the NF-κB-inducing kinase, NIK [DiDonato et al., 1997; Maniatis, 1997; Woronicz et al., 1997]. NF-κB is activated in

Abbreviations used: *H. pylori*, *Helicobacter pylori*; EMSA, elctrophoretic mobility shift assay; NF-κB, nuclear factor kappa B; AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; MEK1, mitogen-activated ERK kinase 1; PMS/MTS, (phenazine methosulfate)/(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium, inner salt); ELISA, enzyme-linked immunosorbant assay; TNF-α, tumor necrosis factor-alpha; PMA, phorbol 12-myristate 13-acetate.

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response to a number of stimuli including bacterial lipopolysaccharide (LPS), phorbol esters, the pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), and oxidative stress [Brennan and O'Neill, 1995; Schulze-Osthoff et al., 1995].

Helicobacter pylori is a dominant factor in active chronic gastritis and peptic ulcer disease and is implicated in the pathogenesis of gastric carcinoma [Blaser, 1992; Moss and Calam, 1992]. Exposure of the gastric epithelial cell line AGS to live *H. pylori* resulted in NF-κB activation and subsequent increase in IL-8 mRNA [Keates et al., 1997; Munzenmaier et al., 1997]. Biopsies from patients infected with *H. pylori* were also found to have higher levels of NF-κB compared to normal biopsies [Keates et al., 1997]. The presence of *H. pylori* is however not a prerequisite for the development of mucosal ulceration as evidenced by duodenal ulceration in hyperacidic states such as the Zollinger–Ellison syndrome. Maximal acid output is increased in *H. pylori* infected patients with duodenal ulcers and conditions of low pH appear necessary for causing mucosal damage [Calam et al., 1997; Gillen et al., 1998]. However, the role of low pH on NF-κB regulation in gastric epithelial cells has not yet been examined. Given that gastric juice is markedly acidic, and that local tissue acidosis is prominent in inflammatory processes secondary to bacterial infection, we examined the effect of low pH on NF-κB DNA-binding in gastric epithelial cells.

MATERIALS AND METHODS

Gastric Epithelial Cell Culture

The gastric epithelial cell line AGS (derived from human gastric adenocarcinoma) was obtained from European Collection of Animal Cell Cultures. AGS cells were cultured in the presence of CO_2 (5%) and bicarbonate as a monolayer in RPMI 1640 medium (Gibco, Life Technology Ltd., Paisley, Scotland), supplemented with 100 U/ml penicillin, 10 μg/ml streptomycin, 20 mM L-glutamine, and 10% fetal calf serum. AGS cells were removed from flasks by trypsin/EDTA treatment and seeded into 6-well culture plates at 1×10^5 cells/ml. When the cells were grown to subconfluency (1×10^6 cells/well), the pH of the medium was adjusted from a resting pH of 7.4 to 6.0 by the addition of HCl (0.1 M). For controls, equal volumes of deionized water were added. Effects on cell viability were not noted within pH range 7.4–6.6. However, reduction of pH to levels below pH 6.6 resulted in loss of cell viability. To increase the resting pH of the culture medium, 0.1 M NaOH was added to adjust the pH to values between 7.4 and 9. In all experiments, the pH of the culture medium was measured at the start of the experiment and at the end of the incubation to ensure that the pH remained unaltered. The buffering capacity of the RPMI medium was provided partly by the bicarbonate ion (concentration of sodium bicarbonate = 24 mM) and partly by the presence of sodium phosphate (concentration of $Na_2HPO_4 = 11.4$ mM). Experiments were also performed by pre-treating AGS cells with either the MEK1 kinase inhibitor PD98059 (15 μM; Calbiochem, La Jolla, CA) or the p38 MAP kinase inhibitor SB203580 (3 μM; SmithKline Beecham Pharmaceuticals, King of Prussia, PA) for 30 min prior to reducing the extracellular pH. The effect of low pH in AGS cells pretreated for 1 h with the pro-inflammatory cytokine TNF-α (20 ng/ml) and the phorbol ester PMA (20 ng/ml) was similarly examined. As HuT 78 cells, a T-cell line derived from a human Sezary lymphoma (European Collection of Animal Cell Cultures), contain high levels of constitutive NF-κB [O'Connell et al., 1995], these cells (1×10^6) were used as positive controls in electrophoretic mobility shift assays. Cell viability was determined as greater than 90% as measured using acridine orange (AO) and ethidium bromide (EB) fluorescence staining [Hudson and Hay, 1989].

Determination of Intracellular pH (pHi) Using the pH-Sensitive Fluorescent Indicator BCECF

AGS cells were harvested and resuspended in PBS (5×10^6/ml) containing BCECF-AM ester (Molecular Probes, Leiden, The Netherlands) at a concentration of 2 μg/ml. Cells were then incubated for 30 min at 37°C. Excess dye was removed by centrifugation and resuspension of the cells in PBS. Fluorescence was measured in a Jasco FP750 spectrofluorimeter, maintained at 37°C, using dual excitation at 495 and 440 nm and emission at 535 nm. The ratiometric method was used to determine the pH. The BCECF fluorescence was calibrated by titrating BCECF-loaded cells in a high K^+ buffer (130 mM KCl, 20 mM HEPES, pH 7.28) with 1M MES (pH 4.8) in the presence of nigericin (2 mg/ml; Sigma-Aldrich, Dublin, Ireland). A calibration curve

with a regression coefficient of 0.995 was then calculated.

Cell Viability

AGS cells (1×10^5 cells/ml) were cultured in 96-well plates in triplicate overnight at 37°C. AGS cells were incubated in media of different pH values ranging from pH 7.4 to 6.4 for 1 h at 37°C. To the cultured cells, 20 μl of freshly prepared PMS/MTS (phenazine methosulfate)/(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2H-tetrazolium, inner salt) solution (Promega Corp., Madison, WI) was added to each well and the plates were incubated for 4 h at 37°C. The absorbance of these wells was read at 490 nm using an enzyme-linked immunosorbant assay (ELISA) plate reader (Labsystems Multiskanplus, type 314, Finland).

Bacterial Preparation

Two reference strains of *H. pylori* were used; NCTC 11638 and 11637 (both *VacA*+; *CagA*+). Bacteria were grown on 7% lysed blood columbia agar for 48–72 h at 37°C in a microaerobic environment. Cells were then harvested either in PBS (pH 7.4) or RPMI 1640 medium without antibiotics, then pelleted by centrifugation for 10 min, resuspended in either PBS or RPMI 1640 medium to an OD 600 nm of 1.5 (corresponding to 3.6×10^8 colony-forming units/ml), and used immediately. Serial dilutions of bacteria were also performed to estimate the number of live bacteria per ml of culture. Bacteria at a concentration of 1×10^8 (multiplicity of infection: 100:1), as determined following a dose-response experiment, were co-cultured with 1×10^6 AGS cells for various periods of time (as indicated in figure legends).

Cell Fractionation and Nuclear Extract Preparation

Nuclear extracts were prepared as described previously [Osborn et al., 1989; Stylianou et al., 1992]. Briefly, confluent AGS in 6-well plates were exposed to a range of pH changes. AGS cell experiments were also performed in the presence or absence of *H. pylori*. Experiments were terminated by the addition of 5 ml ice-cold PBS followed by centrifugation (1,400*g*, 5 min). The cells were then washed in 1 ml hypotonic buffer [10 mM HEPES, pH 7.9, containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.5 mM PMSF] and centrifuged at 10,000*g* for 10 min. Unstimulated HuT78 cells were prepared in a similar fashion. Cells were then lysed with hypotonic buffer containing 0.1% Nodinet P-40 on ice for 10 min. Lysates were then centrifuged at 10,000*g* for 10 min and the pellet was suspended in buffer (20 mM HEPES, pH 7.9, containing 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 0.5 mM PMSF) and left on ice for 15 min. Following centrifugation (10,000*g*, 10 min) the supernatant was added to 75 μl of storage buffer (10 mM HEPES, pH 7.9, containing 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM PMSF). The protein concentration of the crude nuclear extracts were determined using the method of Bradford and the preparations were immediately assayed for NF-κB activity or stored at −70°C until further use [Bradford, 1976].

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts, containing between 2 and 4 μg of protein of AGS and HuT 78 cells were incubated with 10,000 cpm of a 22-base pair oligonucleotide, containing the consensus sequence for the κB binding site (underlined) (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Promega Corp.) that had previously been labeled with [γ-^{32}P]ATP (10 mCi/mmol) by T4 polynucleotide kinase (Pharmacia Biosystems, Milton Keynes, UK). Similar experiments were also performed using the AP-1 (c-Jun) consensus motif (5′-CGCTTG ATGAGTCAGCCGGAA-3′) (Promega Corp.). Incubations were performed for 30 min at room temperature in the presence of 2 μg of poly (dI-dC), (Pharmacia Biosystems) as non-specific competitor, and binding buffer containing 10 mM Tris, pH 7.5, 40% glycerol, 1 mM EDTA, 100 mM NaCl and nuclease free bovine serum albumin (0.1 mg/ml). For competition studies, a 100-fold excess of unlabeled oligonucleotide containing the NF-κB consensus sequence was added to the extracts 30 min before incubation with labeled oligonucleotide. Supershift assays were also performed using rabbit antiserum to specific Rel protein species (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). This involved using 0.5 μl of a specific antiserum to anti-NF-κB p50 (sc-114X), anti-NF-κB p65 (sc-109X), or anti-NF-κB c-Rel (sc-70X). Each antibody was incubated with 4 μg nuclear extracts 30 min prior to the binding reaction. All samples were then resolved on a 4% native polyacrylamide gel and subsequently dried and subjected to autoradiography for

24–48 h at −70°C. All results are representative of experiments performed at least in triplicate.

Western Blotting for IκB-α Analysis

AGS cells were collected following co-culture in the presence or absence of *H. pylori* and at low pH by centrifugation (1,400 *g*, 5 min). The pellet of cells was resuspended in lysis buffer containing 50 mM Tris (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), 0.1 mM phenylmethylsulfonylfluoride (PMSF), leupeptin (10 mg/ml), 5% (v/v) 2-mercaptoethanol, 0.1% (v/v) bromophenol blue, and 10% (w/v) glycerol. Cells were then solubilized by boiling for 5 min. The protein concentration was determined on the cell extract by the method of Bradford. Whole cell extracts (50 μg of protein/lane) were resolved by electrophoresis through SDS–polyacrylamide gels using 10% separating gels. Proteins were electrotransfered onto PVDF membrane using a semidry blotting apparatus (Atto). Blots were blocked with 5% (w/v) dried skimmed milk in PBS for 1 h at room temperature and then incubated for 1 h at room temperature with anti-IκB-α antiserum (1/1,000). Blots were then incubated with anti-rabbit horseraddish peroxidase conjugated secondary antibody (1/1,000) for 1 h at room temperature. Immunodetection was performed by enhanced chemiluminescence.

RESULTS

Effect of Changes in pH on NF-κB DNA-Binding in AGS Cells

NF-κB DNA-binding in AGS cells strongly increased when the pH of the culture medium decreased from 7.4 to 6.6 (Fig. 1a). Time course experiments revealed enhanced NF-κB nuclear binding in response to conditions of low pH from 30 min to 24 h, and this effect appeared maximal at 1 h (data not shown). The increase in NF-κB DNA-binding was not due to alterations in cell viability as no change in cell viability was noted using EB/AO fluorescence staining between the pH range 7.4 and 6.6. Lowering the pH to below 6.6 resulted in rapid cell lysis with concomitant reduction in NF-κB activation (Fig. 1a). In addition, enhanced NF-κB DNA-binding was specific for acidic pH conditions, as increases in nuclear binding were not observed at pH above 7.4 (Fig. 1a). Indeed, at pH greater than 7.8, NF-κB binding appears to decrease. This effect was not due to alterations in cell viability. Supershift assays showed that NF-κB dimers binding to DNA predominantly consisted of p50 and RelA (p65) (Fig. 1b).

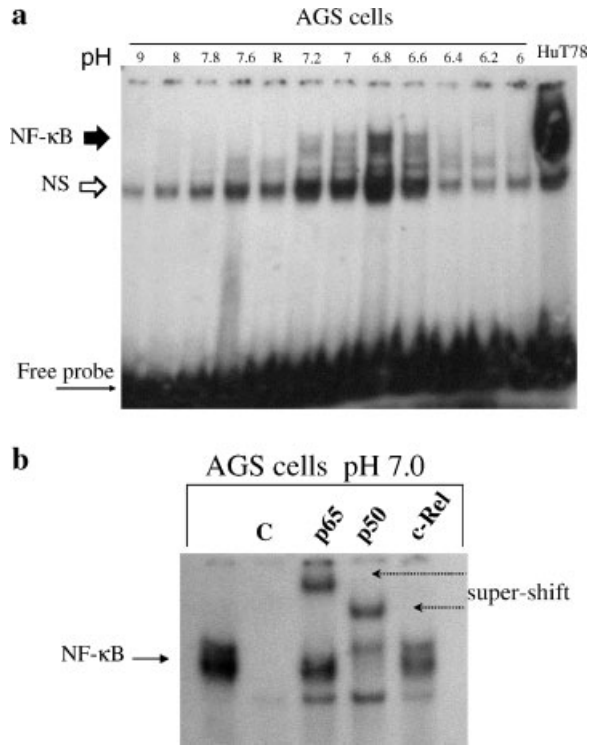

Fig. 1. **a**: Electrophoretic mobility shift assays (EMSA) of nuclear extracts from AGS cells which were cultured in media where the pH of the culture medium was adjusted using either 0.1–M HCl (pH 7.2–6.0) or 0.1M NaOH (pH 7.6–9.0) for 1h and incubated with 10,000 cpm of a radiolabeled probe containing the consensus sequence for NF-κB. Labeled extracts were subsequently electrophoresed on a 4% polyacrylamide gel. The resting pH of the culture medium (pH 7.4) is indicated, R. The solid arrow indicates activated NF-κB, the open arrow reveals a second non-specific (NS) band. Hut 78 T cells serve as positive controls. **b**: Competition assay for NF-κB in AGS cells treated at pH 7.0 in which an excess (100-fold) of unlabeled NF-κB was added to nuclear extracts 30 min prior to incubation with radiolabeled NF-κB abolishes NF-κB nuclear binding (C). Supershift assays were also performed using rabbit antisera to p65 (RelA), p50 and c-Rel in nuclear extracts of AGS cells at pH 7.0. This data reveals a further retardation of migration (''super-shift'') in the presence of p65 and p50 antisera but not c-Rel.

Measurement of the Intracellular pH

Measurement of the intracellular pH of AGS cells, using BCECF, over the extracellular pH range pH 7.3–6.8 demonstrated that the intracellular pH varied from 7.29 to 7.08 (Fig. 2) indicating that AGS cells can regulate their intracellular pH in response to alterations in the environmental pH.

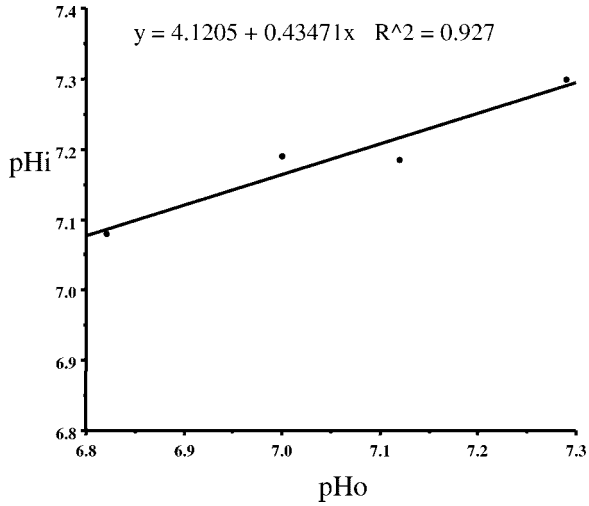

Fig. 2. Changes in intracellular pH (pHi) of AGS cells in response to changes in the extracellular pH (pHo) using the pH-sensitive fluorescent indicator BCECF. The experiment was performed as described in the ''Materials and Methods.''

Effect of MAPK Inhibitors on Low pH-Induced NF-κB Binding

As changes in osmolar conditions may occur in response to lowering the environmental pH, we also examined the potential role of the p38 MAP kinase (an important stress-responsive kinase) in stimulating NF-κB DNA-binding. Cells were cultured in the presence of the specific p38 MAP kinase inhibitor SB203580 prior to lowering the pH to 7.0. No difference in NF-κB nuclear-binding was detected by EMSA in the presence or absence of SB203580 (Fig. 3). On the other hand, pre-treatment of cells with the MEK1 kinase inhibitor PD98059 was found to potently attenuate NF-κB nuclear binding.

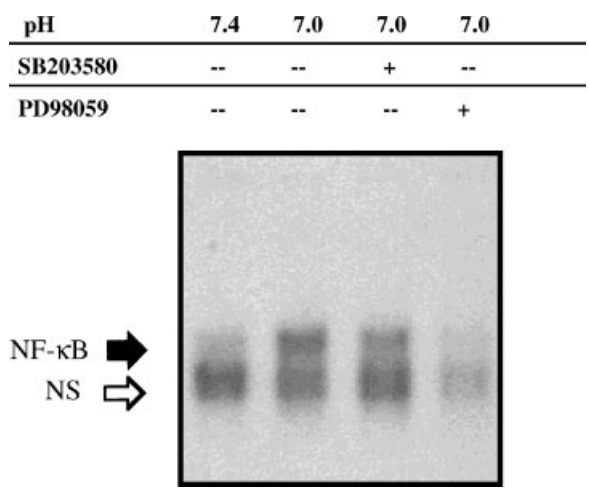

Fig. 3. The effect of inhibition of p38 MAP kinase and MEK1 kinase on NF-κB DNA-binding secondary to low pH in the gastric epithelial cell line, AGS. Cells were pre-incubated with the p38 MAP kinase inhibitor SB203580 (3 μM) or the MEKK-1 inhibitor PD98059 (15 μM) for 30 min prior to incubation in medium of pH 7.0 for 1 h. The solid arrow indicates activated NF-κB, the open arrow reveals a second non-specific (NS) band. Abbreviations: SB, SB203580; PD, PD98059.

Interestingly, NF-κB was not the sole transcription factor activated following exposure to low pH as decreases in the extracellular pH also increased the DNA-binding of the transcription factor AP-1 (Fig. 4a). PD98059, but not SB203580, inhibited nuclear binding of low pH-induced AP-1 (Fig. 4b), suggesting involvement of the MEK1/2 signaling pathway for this transcription factor. Finally, we examined whether low pH was associated with changes in cell viability by performing a colorimetric PMS/MTS proliferation assay (Fig. 5); no significant changes in cell viability were observed at 1 h incubation in response to the range of pH employed in our experiments, where the EMSAs were conducted in all experiments.

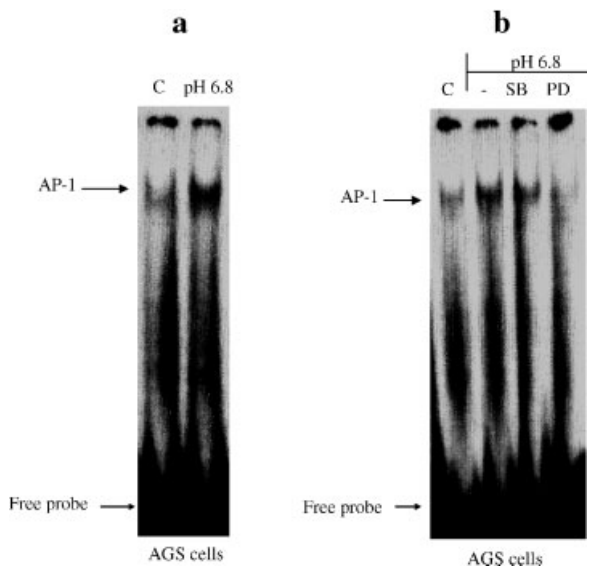

Fig. 4. **a**: EMSA of nuclear extracts from resting AGS cells (C) or AGS cells exposed to medium which was adjusted to pH 6.8 for 1 h using 0.1M HCl were incubated with 10,000 cpm of a radiolabeled probe containing the consensus sequence for AP-1. Labeled extracts were subsequently electrophoresed on a 4% polyacrylamide gel. Low levels of constitutive AP-1 were observed at resting pH (C), however strong nuclear DNA-binding is observed at pH 6.8. **b**: The effect of inhibition of p38 MAP kinase and MEK1 kinase on AP-1 DNA-binding secondary to low pH in the gastric epithelial cell line, AGS. Cells were pre-incubated with the p38 MAP kinase inhibitor SB203580 (3 μM) or the MEKK-1 inhibitor PD98059 (15 μM) for 30 min prior to incubation in media of pH 6.8 for 1 h. Abbreviations: SB, SB203580; PD, PD98059.

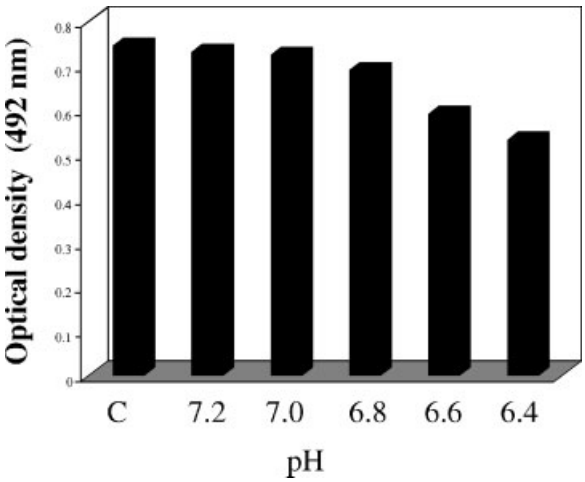

Fig. 5. Cell viability assessed using PMS/MTS Assay. AGS cells (1×10^5 cells/ml) were cultured in 96-well plates in triplicate overnight at 37°C. AGS cells were incubated in media of different pH values ranging from pH 7.4 to 6.4 for 1 h at 37°C. To the cultured cells, 20 µl/well of combined PMS/MTS solution were added. After 4 h at 37°C in a humidified, 5% CO_2 atmosphere, the absorbance was read at 492 nm using ELISA plate reader.

H. pylori and Low pH Combine to Activate NF-κB Nuclear Binding

Co-culture of AGS cells with *H. pylori* resulted in strong NF-κB DNA-binding which occurred over the time course of 30 min–24 h (Fig. 6a). Both NCTC11637 and 11638 reference strains of *H. pylori* induced NF-κB DNA-binding to similar extents in AGS cells (not shown) and this was paralleled by concomitant degradation of cytoplasmic IκB-α, as shown by immunoblotting (Fig. 6b). Supershift studies showed that the activated species of NF-κB consisted of p50/p65 heterodimers and p50 homodimers under these conditions (not shown). The combined effect of *H. pylori* and low pH on NF-κB activation in AGS cells was next examined. Cells were exposed to alterations in environmental pH in the presence or absence of *H. pylori* for 1 h. Low pH further increased *H. pylori*-induced NF-κB nuclear translocation (Fig. 7a) and this effect was paralleled by decreasing levels of cytoplasmic IκB-α (Fig. 7b). Similarly, the NF-κB p50/RelA heterodimer and a p50 homodimer were identified in cells exposed to both low pH and *H. pylori* (not shown). The combined effect of low pH in AGS cells treated with TNF-α (20 ng/ml) and the phorbol ester PMA (20 ng/ml) was also examined. Potent NF-κB DNA-binding occurred in response to the pro-inflammatory cyto-

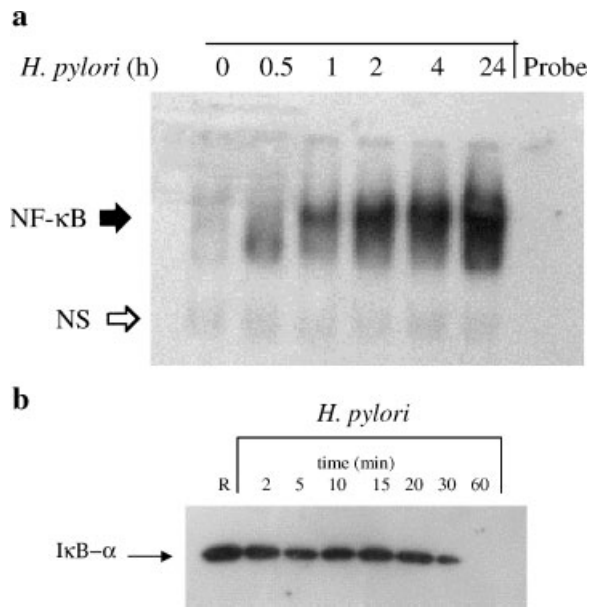

Fig. 6. **a**: EMSA of nuclear extracts of AGS cells incubated with *H. pylori* NCTC 11638 at a concentration of 10^8/ml bacteria over the times (in hours) as indicated. The solid arrow indicates activated NF-κB, the open arrow reveals a second non-specific band (NS). The lane to the extreme right (''probe'') is in the absence of nuclear extract protein and serves as control. **b**: Western blotting assays for IκB-α protein in cytosolic extracts of whole AGS cells co-cultured with *H. pylori* NCTC 11638 over various times (minutes) as indicated.

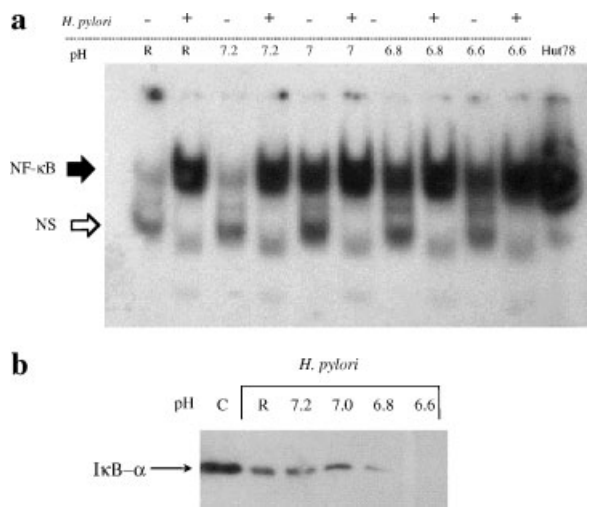

Fig. 7. **a**: EMSA of nuclear extracts from AGS cells in which the pH of the culture medium was adjusted from pH 7.4 (resting pH) to increasingly acidic pH ranges as indicated using 0.1–M HCl in the presence and absence of *H. pylori* (10^8) for 1 h. The solid arrow indicates activated NF-κB, the open arrow reveals a second non-specific band (NS). Hut78 cells serve as positive controls. **b**: Western blotting assay for IκB-α protein in cytosolic extracts in AGS cells co-cultured with *H. pylori* and increasingly acidic conditions from resting pH 7.4 (R) to pH 6.6. C refers to control AGS cells at resting pH in the absence of *H. pylori*.

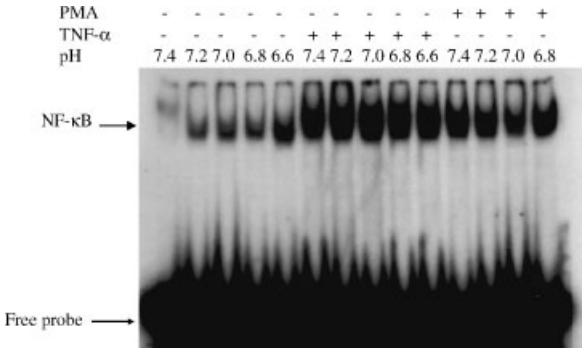

Fig. 8. NF-κB DNA binding activity in EMSA of nuclear extracts from AGS cells treated with TNF-α (20 ng/ml) or PMA (20 ng/ml) in which the pH of the culture medium was lowered (as indicated) for 1 h.

kine TNF-α and the phorbol ester PMA (both of which are known to strongly augment NF-κB nuclear binding) and lowering the extracellular pH did not further increase such binding (Fig. 8).

DISCUSSION

The importance of NF-κB in the pathogenesis of gastrointestinal inflammation has been previously demonstrated by the ability of antisense to the p65 (RelA) NF-κB subunit to control colitis in a TNBS mouse model [Neurath et al., 1996]. Nuclear levels of RelA (p65) have also recently been shown to be increased in patients with Crohns' disease [Schreiber et al., 1998]. The mechanisms of tissue injury as a result of high acid levels in patients with duodenal ulceration are not known. Recently, low pH has been shown to increase NFκB binding activity in macrophages [Bellocq et al., 1998]. Here we show that lowering the extracellular pH from 7.4 (resting pH of the medium) to 6.6 independently activates the pro-inflammatory transcription factors NF-κB in gastric epithelial cells, without affecting cell viability.

We acknowledge that experiments using a transformed cell line cannot mimic the physiological state however, despite much lower levels of pH in the human stomach; gastric epithelial cells in the fasted state are buffered from the harmful effects of acid due to the presence of bicarbonate and the mucus gel layer. A pH gradient from pH 1 to 2 at the gastric lumen to pH 6 to 7 at the cell surface is known to exist and acid was formerly thought to be secreted through pores in the mucus gel layer, the "viscous fingering" hypothesis [Bhaskar et al., 1992]. This theory has recently been questioned as have the overall protective mechanisms of gastric mucus and other protective factors are no doubt at play [Chu et al., 1999]. In hyperacidic states or when gastric or duodenal mucosal protective mechanisms are ineffective or compromised in the presence of exogenous agents such as non-steroidal anti-inflammatory drugs or *H. pylori*, tissue damage secondary to acid exposure may occur. Such injury may ensue even in response to small decreases in juxta-mucosal pH. Here, we provide a putative mechanism for such tissue injury in response to acidic conditions via activation of the pro-inflammatory mediators NF-κB. Further evidence supporting activation of this transcription factor to increasingly acidic conditions was provided by the concomitant decrease in cytosolic IκB protein. Induction of IL-8 in response to lowering the extracellular pH (7.4–6.7) in pancreatic cell lines was found to be dependent on activation of both NF-κB and AP-1 [Shi et al., 2000]. Here, gastric epithelial cells exposed to conditions of low pH also resulted in activation of the transcription factor AP-1. Acidic pH was previously shown in human glioblastoma cells to enhance VEGF transcription via AP-1 acticavtion [Xu et al., 2002].

AGS cells exposed to acidic conditions can regulate their intracellular pH. Changes in the ionic strength of the extracellular environment following intracellular pH regulation involving selective ion exchangers such as Na^+/H^+ and Cl^-/HCO_3 could play an important role in regulation of NF-κB in certain cells. Changes in osmolarity have previously been suggested to influence NF-κB DNA-binding [Taher et al., 2000], which may be mediated via stress-activated kinase pathways. The p38 MAP kinase, known to be activated in osmotic stress, has been shown to be involved in the activation of NF-κB-regulated genes in response to cytokine exposure [Raingeaud et al., 1995; Beyaert et al., 1996; Kim et al., 2000]. However, here we found that the specific p38 MAP kinase inhibitor SB203580 failed to inhibit NF-κB DNA-binding in response to low pH. Inhibition of NF-κB nuclear binding at pH 7.0 was observed in the presence of the MEK1 kinase inhibitor PD98059 indicating that low pH-induced NF-κB signaling may, in part be mediated via pathways involving the extracellular signal-regulated kinases, ERK1 and ERK2. Similar mechanisms were demonstrated in

HUVEC cells where PD98059 blocked hypothermia-induced NF-κB activation but SB203580 had no effect on NF-κB [Roberts et al., 2002]. PD98059 was also found to block lipoteichoic acid-mediated formation of NF-κB-specific DNA-protein complexes in a pulmonary cell line [Lin et al., 2002]. Site-specific phosphorylation of IκB-α by MEK1 has been previously demonstrated in other cellular systems [Lee et al., 1999]. The complex signaling pathways leading to NF-κB activation was further highlighted by Kim et al. [2000] who demonstrated that activation of NF-κB in hepatocytes in response to both growth factors and hypoosmotic stress was dependent and independent, respectively, on pathways involving phosphoryation of p38 and ERK1/2. Thus, as blocking phosphorylation of ERK1/2 attenuates nuclear NF-κB binding in response to low pH in AGS cells, factors other than changes in ionic strength may be involved in its activation. Keates et al. [1999] recently demonstrated in AGS cells that while *H. pylori*-induced IL-8 production was dependent on both p38 and MEK1 kinase activities, these pathways were not essential for *H. pylori*-induced NF-κB activation. *H. pylori* was also found to activate cyclin D1 through the mitogen-activated protein kinase pathway and not through NF-κB activation in AGS cells [Hirata et al., 2001]. Thus, it is interesting that factors such as low pH may independently regulate NF-κB through ERK/MAP signaling pathways. Selective activation of the ERK/MAP kinase but not p38 kinase cascade was previously observed in *H. pylori*-mediated AP-1 activation in AGS cells using both specific blockers and immunoprecipitating experiments [Meyer-ter-Vehn et al., 2000]. Additionally, low pH-induced AP-1 activation was also found to be ERK1/2 MAPK kinase dependent pathways in glioblastoma cells [Xu et al., 2002].

We and others found that *H. pylori* enhanced NF-κB nuclear binding in gastric epithelial cells [Keates et al., 1997; Munzenmaier et al., 1997]. Bacterial strains also appear to be important in eliciting this response as shown by the ability of Cag-specific proteins within the Cag pathogenicity island of *H. pylori* to enhance NF-κB activation [Glocker et al., 1998]. In addition, *H. pylori* induces a potent immune response with increased production of the pro-inflammatory cytokines TNF-α and IL-1, both of which directly activate NF-κB [Crabtree et al., 1991; Fan et al., 1993]. Hence NF-κB activation in gastric epithelial cells may result from either direct contact with the bacterium or as a consequence of the immune response to the *H. pylori*. Our findings that low pH independently augmented NF-κB nuclear binding suggests the intriguing possibility that NF-κB induction could be a final common pathway for the major factors involved in gastric injury and peptic ulcer disease.

NF-κB is also known to protect against apoptosis and also plays a critical role in regulation of cell proliferation in certain cellular systems [Baldwin et al., 1991; Beg and Baltimore, 1996]. As patients with gastric cancer secondary to *H. pylori* gastritis have low levels of gastric acid secretion, it could be hypothesized that NF-κB would be induced to a lesser extent in these patients. This could have significant consequences for the regulation of cell proliferation. Patients with duodenal ulcers and *H. pylori* infection on the other hand are exposed to abnormally high levels of acid [Calam et al., 1997; Gillen et al., 1998]. In these individuals the mechanism of inflammation in the development of ulceration may result from NF-κB activation secondary to acid, either independently or in combination with *H. pylori* to augment induction of this transcription factor. This may also partly explain the mechanism underlying ulcer healing and reduction in gastritis following the use of proton pump inhibitors or histamine receptor antagonists. In addition, AP-1, which is known to play a crucial role in cell proliferation and is induced by *H. pylori* [Naumann et al., 1999; Mitsuno et al., 2001], was shown to be increased in response to conditions of low pH. Conditions of low pH can also lead to arrest of cell cycle in certain cellular systems [Bosticardo et al., 2001]. The complex mechanisms involved are also highlighted by the finding in lung cancer cell lines that H_2O_2 resulted in cell cycle arrest through activation of AP-1-dependent p21 which was mediated by ERK signaling pathway as demonstrated by the ability of the MEK inhibitor PD98059 to diminish H_2O_2-induced DNA AP-1-binding and decrease expression of p21(WAF1/CIP1) which released the cells from G_2/M arrest [Chung et al., 2002]. Pro-inflammatory effects of AP-1 may predominate in response to lowering the extracellular pH and further experiments will be required to elucidate the exact roles of these and perhaps other transcription factors.

In summary, we reveal for the first time that the pro-inflammatory transcription factor NF-κB can be induced by conditions of low pH in gastric epithelial cells; low pH also can increase AP-1 nuclear DNA-binding. Acid-induced NF-κB may independently contribute to gastric inflammatory responses in the presence or absence of *H. pylori* and this appears to be at least partially mediated through MEK1 kinase pathways.

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