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**Article:**

Green, N.H., Huang, Q.Z., Corfe, B.M. et al. (2011) NF-kappa B is activated in oesophageal fibroblasts in response to a paracrine signal generated by acid-exposed primary oesophageal squamous cells. *International Journal of Experimental Pathology*, 92 (5). pp. 345-356. ISSN: 0959-9673

<https://doi.org/10.1111/j.1365-2613.2011.00778.x>

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This is the peer reviewed version of the following article: Green, N. H., Huang, Q., Corfe, B. M., Bury, J. P. and MacNeil, S. (2011), NF- $\kappa$ B is activated in oesophageal fibroblasts in response to a paracrine signal generated by acid-exposed primary oesophageal squamous cells. *International Journal of Experimental Pathology*, 92: 345–356, which has been published in final form at <http://dx.doi.org/10.1111/j.1365-2613.2011.00778.x>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving (<http://olabout.wiley.com/WileyCDA/Section/id-820227.html>).

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# **NF- $\kappa$ B is activated in oesophageal fibroblasts in response to a paracrine signal generated by acid-exposed primary oesophageal squamous cells**

Running title: Response of oesophageal cells to acid exposure

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## Summary

Oesophageal exposure to duodenogastroesophageal refluxate leads to reflux oesophagitis and is implicated in the development of Barrett's Metaplasia (BM). NF- $\kappa$ B signalling in epithelial cells is associated with the activation of transcription factors believed to be central to BM development, whilst NF- $\kappa$ B activation in fibroblasts plays a critical role in matrix remodelling. Our aim was to study the effects of acid exposure on NF- $\kappa$ B activation in primary human oesophageal fibroblasts and primary and immortalised oesophageal squames and to investigate any epithelial/stromal interactions in the response of these cells to acid. Primary human oesophageal fibroblasts, and primary and immortalised oesophageal epithelial cells were exposed to acid (pH 7 – pH 4  $\leq$  120 minutes) in single or pulsed treatments. Conditioned medium from epithelial cells following acid exposure was also applied to fibroblasts. Cell viability was determined by MTT-ESTA. NF- $\kappa$ B activation was determined by cellular localisation of NF- $\kappa$ B/p65 visualised by immunofluorescence. Conditioned medium from oesophageal epithelial cells subjected to pH 5 pulsatile exposure activated NF- $\kappa$ B in fibroblasts, with some inter-patient variability, but these conditions did not directly activate NF- $\kappa$ B in the epithelial cells themselves. Significant NF- $\kappa$ B activation was seen in the epithelial cells but only with greater acidity and exposure times (pH 4, 60 to 120 minutes). Our findings show that acid exposure can cause indirect activation of stromal cells by epithelial-stromal interactions. This may contribute to the pathogenesis of oesophageal diseases and the inter-patient variability may go some way to explaining why some patients with reflux oesophagitis develop BM and others do not.

**Key words:** acid; Barrett's Metaplasia; NF- $\kappa$ B; oesophageal adenocarcinoma; paracrine; reflux

## Introduction

Gastro-oesophageal reflux disease (GORD) is a key risk factor for the development of Barrett's Metaplasia (BM). In turn BM places an individual at risk of developing oesophageal adenocarcinoma (OAC), and the incidence of both OAC and BM is rising throughout the Western world. OAC typically has a poor prognosis and unlike some cancers there has been very little improvement in survival in recent years. Understanding the impact of duodenogastric reflux on oesophageal mucosa is therefore essential if we are to develop rational strategies to reduce mortality from OAC.

The histological manifestations of GORD include the presence of inflammatory cells within the epithelium, an increase in the thickness of the basal layers of the epithelium and elongation of the papillae. It has been thought that these reflect epithelial regeneration after caustic injury (e.g. erosion or ulceration), although this view has been challenged by recent data demonstrating the presence of these features very soon after acid/bile exposure, apparently without intervening ulceration (Souza *et al.* 2009). The mechanism by which GORD predisposes to the development of BM remains unclear, although aberrant activation of the intestine-specific *caudal*-related homeobox2 (CDX2) gene in oesophageal squamous cells seems critical (Eda *et al.* 2003; Lord *et al.* 2005; Moons *et al.* 2004). NF- $\kappa$ B is known to be upregulated in BM (Konturek *et al.* 2004) and activation of the NF- $\kappa$ B signalling pathway is also implicated in the aberrant expression of CDX2, putatively driving its expression via NF- $\kappa$ B binding sites in the CDX2 promoter region (Debruyne *et al.* 2006; Kazumori *et al.* 2006).

Several studies have examined the impact of acid exposure on different oesophageal cells. However the results obtained vary, with the differences presumably in part reflecting the variation in patterns of acid exposure (Fitzgerald *et al.* 1997) and cell lines employed. For example, two 3-minute exposures of a telomerase-immortalised Barrett's epithelial cell line to pH 4 causes a decrease in cell proliferation (Feagins *et al.* 2007); 1 hour exposure to pH 3.5 induces hyperproliferation in BM mucosal biopsies (Fitzgerald *et al.* 1998); 1 hour exposure to pH 4 causes an increase in proliferation for an immortalised normal epithelial cell line and an OAC cell line (Fu *et al.* 2006); and pH 4.5 exposure for 30 minutes induces DNA damage in two immortalised oesophageal epithelial cell lines (Jolly *et al.* 2004). In addition, exposure to acid induces OAC cell lines and immortalised squamous cells to express a number of genes involved in inflammation, proliferation, differentiation and the stress response (Duggan *et al.* 2006), while work with other OAC lines has shown that exposure to an increasingly acidic environment results in increased NF- $\kappa$ B activation, reaching a maximum at pH 4 (Abdel-latif *et al.* 2004). The relevance of this data from immortalised or established OAC cell lines to the initial development of BM is questionable, but published data from primary cells or other systems more closely reflecting the environment within the native oesophagus is limited.

Epithelial-stromal interactions are known to play important roles in many areas such as wound healing (Werner *et al.* 2007), organogenesis (Thesleff *et al.* 1995), morphogenesis and maintenance of differentiated epithelia (Sharpe and Ferguson 1988) and progression to cancer (Potter 2001). There has been only limited investigation of the role of such phenomena in the pathogenesis of oesophagitis and BM. However, the finding that histological manifestations of oesophagitis occur in the absence of preceding erosion or ulceration has led to the proposal that some

elements of the morphological appearances of oesophagitis, namely basal cell and papillary hyperplasia and inflammatory cell infiltration, represent a response to cytokines produced by oesophageal squamous cells following acid insult (Souza *et al.* 2009). Fibroblasts are the predominant cellular component of connective tissue and play a key role in tissue remodelling in response to injury, and it is likely that they would participate in the papillary elongation seen in GORD. Fibroblast activation would therefore seem to occur without direct exposure to refluxate, presumably through signalling from acid/bile exposed epithelial cells. NF- $\kappa$ B signalling has a distinctive role in governing fibroblast function, being specifically implicated in the regulation of matrix degradation and remodelling (Ferri *et al.* 2003; Xu *et al.* 1998), cell motility (Palumbo *et al.* 2007) and myofibroblastic differentiation (Ichikawa *et al.* 2008). Recent studies have demonstrated changes in the expression of inflammatory mediators by intra-lesional stromal cells during the evolution of Barrett's adenocarcinoma - underscoring the potential importance of such stromal-epithelial signalling (Saadi *et al.* 2010).

To our knowledge no study has specifically investigated the impact of acid insult on NF- $\kappa$ B activation in oesophageal fibroblasts, nor sought evidence for crosstalk between acid exposed epithelial cells and fibroblasts. Our aims in this study are twofold. Firstly, we have sought to provide baseline data on the impact of acid exposure on primary human oesophageal fibroblasts (HOFs), and immortalised (Het-1A) and primary (HOS) human oesophageal squamous epithelial cells, focusing on cell viability and NF- $\kappa$ B activation. Secondly, we have sought evidence for paracrine interactions between epithelial cells and fibroblasts, which may be of relevance to the overall tissue response to acid exposure.

## Methods

All materials were purchased from Sigma Aldrich (Dorset, UK) unless otherwise stated.

### Human oesophageal squamous (HOS) cell isolation and culture

Oesophageal tissue samples were obtained with informed patient consent and appropriate ethical approval (SSREC 165/03 & 07/1309/138) from patients undergoing gastric or oesophageal surgery. Samples were taken from disease free background tissue proximal to any macroscopic pathology. Tissue was transported in sterile PBS containing 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.625 µg/mL amphotericin B and stored overnight at 4°C prior to cell isolation. This overnight storage step was necessary due to the surgical schedules; however during the optimisation of this protocol it was found that in all cases viable cells could be isolated from tissue that had been stored for 12 hours at 4 °C, indeed viable cells could very often be obtained following up to 60 hours storage.

The tissue was cut into 0.5 cm strips and incubated in 0.1% w/v trypsin (Gibco, Carlsbad, CA) for 1 hour at 37°C. Foetal Calf Serum (FCS) was then added to inhibit the trypsin and the epithelial cells removed by gentle scraping with a scalpel blade. Cells were collected into Greens medium (Rheinwald and Green 1975) (consisting of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 in 3:1 ratio, supplemented with 10 % FCS,  $2 \times 10^{-3}$  M glutamine, 10 ng/mL EGF, 0.4 µg/mL hydrocortisone,  $1.8 \times 10^{-4}$  M adenine, 5 µg/mL insulin, 5 µg/mL transferrin,  $1 \times 10^{-10}$  M cholera toxin,  $2 \times 10^{-9}$  M triiodothyronine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.625 µg/mL amphotericin B). The resulting cell suspension was centrifuged (200g, 5 min), the cells resuspended in 12 mL Green's medium and cultured in the presence of a feeder layer of lethally irradiated mouse 3T3 fibroblasts ( $1 \times 10^6$ /12 mL culture). Cell counts were performed using a haemocytometer and

trypan blue exclusion was used to assess cell viability. HOS cells were used between passage 1 and 4 since it is known that epithelial cells up to passage 4 retain most of the characteristics of the cells from which they were isolated but after this they rapidly differentiate and their functional biology changes significantly.

### **Human oesophageal fibroblast (HOF) cell isolation and culture**

Oesophageal stromal tissue resulting from HOS isolation was finely minced with a scalpel and incubated overnight at 37°C in 10 mL 0.5 % (w/v) collagenase A solution. The digest was then centrifuged (200g, 10 min) and the pellet resuspended in 12 mL fibroblast culture medium (DMEM, 10 % FCS,  $2 \times 10^{-3}$  M glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.625 µg/mL amphotericin B). Fibroblasts were used between passages 4 and 9, since this ensures the absence of contaminating HOS cells.

### **Het-1A culture**

An SV-40 immortalised human oesophageal epithelial cell line, Het-1A (ATCC-LGC, Middlesex, UK) was cultured in serum-free BRFF-EPM2 media (Axxora, Nottingham, UK). The medium was replaced every 3 to 4 days and cells were passaged once they had reached 80% confluency.

### **MTT-ESTA assay**

The MTT-ESTA assay was performed to determine the metabolic activity of cells following exposure to acidified medium. Cells were seeded at  $5 \times 10^4$ /well in 24 well tissue culture plates. After 24 hours they were exposed to fresh pH 7.4 medium for the control or HCl-acidified medium at pH 5, pH 4 or pH 3 for 3 to 60 minutes, washed in PBS and returned to pH 7.4 medium for a further 24 hours at 37°C. Cells were washed with PBS and incubated with MTT-ESTA ( $0.5 \text{ mg mL}^{-1}$  in PBS) for 40 minutes at 37°C. Acidified isopropanol (200 µL) was used to solubilise the formazan

and absorbance was measured at 540 nm with a protein reference of 630 nm subtracted. Activity was calculated as a percentage of the control response.

### **Acid treatment regimes**

#### *Direct exposure of primary human oesophageal cells to acid*

Monocultures of HOS and HOF cells were seeded in 24 well plates at  $5 \times 10^4$ /well. After 24 hours the medium was removed and replaced with fresh culture medium. In all cases the medium used was the normal culture medium for that cell type, i.e. DMEM 10% FCS for HOFs and Greens 10% FCS for HOS cells. The medium was used at pH 7.4, pH 6, pH 5 or pH 4. Cells were exposed for between 3 and 120 minutes. Positive controls were treated with lipopolysaccharide (LPS, 1  $\mu$ g/mL) for 2 hours. After exposure the medium was removed, the cells washed in PBS and fixed in formalin for 15 minutes. Fixed cells were washed x3 in PBS and stored in PBS at 4° C prior to immunostaining.

#### *Pulsatile exposure of primary human oesophageal cells to acid and the effect of the conditioned media produced*

Monocultures of HOS cells were seeded in 24 well plates at  $5 \times 10^4$ /well. After 24 hours the medium was removed and the cells exposed to Greens 10% FCS at pH 7.4, pH 5 or pH 4 for 30 minutes or treated by three pulses of acidified medium punctuated by periods of recovery in Greens 10% FCS at pH 7.4. An acid exposure time of 3 minutes (short pulse) and two intervals of 5 minutes (short recovery) or 30 minutes (long recovery) were employed. The duration of the acid exposure was selected to reflect that encountered by patients during an episode of gastro-oesophageal reflux (Dvorak *et al.* 2006; Richter *et al.* 1992). After the final acid exposure cells were washed in PBS and placed in fresh Greens 10% FCS for 2 hours to produce conditioned medium. After two hours the conditioned medium was

removed and placed on monocultures of the HOF cells (24 well plates seeded 24 hours previously at  $5 \times 10^4$ /well) and left for 3 hours. The negative control was untreated HOF cells which remained in DMEM 10% FCS and the positive control was HOF cells treated for 2 hours with LPS (1  $\mu\text{g}/\text{mL}$ ). The medium was then removed, the cells washed in PBS, fixed in formalin, washed x3 in PBS and stored in PBS at 4° C prior to immunostaining.

#### *Acid exposure of Het-1A cells*

Het-1A cells were seeded in 6 well plates at  $2 \times 10^5$ /well in BRFF-EPM2 serum free culture medium. After 48 hours the medium was removed and replaced with fresh BRFF-EPM2. The medium was either used without being first acidified (pH 7.4), or at pH 5 or pH 4 through the addition of 1M HCl. Cells were exposed for 30 minutes or pulsed for three 3 minute exposures separated by 5 minute recovery periods in pH 7.4 medium. Positive controls were treated with LPS (1  $\mu\text{g}/\text{mL}$ ) for 2 hours. After exposure the acidified medium was removed, fresh BRFF-EPM2 (pH 7.4) added and the cells cultured for a further two hours. The conditioned medium was collected, the Het-1A cells washed in PBS and fixed in formalin for 15 minutes. Fixed cells were washed x3 in PBS and stored in PBS at 4° C prior to immunostaining.

The conditioned medium was split into two aliquots. Half was stored at -20°C prior to bead array analysis and the remainder was placed on monocultures of the HOF cells (6 well plate seeded 48 hours previously at  $2 \times 10^5$ /well) and left for 3 hours. The medium was then removed from these cells, the cells washed in PBS, fixed in formalin, washed x3 in PBS and stored in PBS at 4° C prior to immunostaining. Positive controls were incubated with LPS (1  $\mu\text{g}/\text{ml}$ ) for 2 hours, negative controls remained in DMEM 10% FCS.

#### **Inhibition of NF- $\kappa$ B translocation**

HOS cells were treated with Greens 10% FCS medium supplemented with an NF- $\kappa$ B inhibitor pyrrolidine dithiocarbamate (PDTC, 10  $\mu$ M). After 1 hour cells were washed and treated with pH 7.4 or pH 4 medium, containing 10  $\mu$ M PDTC for 60 minutes. Cells were then washed in PBS, fixed in formalin for 15 minutes, washed x3 in PBS and stored in PBS at 4°C prior to immunostaining.

### **NF- $\kappa$ B/p65 immunostaining**

Fixed cells were permeabilised by incubating in 0.1% (v/v) Triton in PBS (20 minutes, room temperature). Cells were washed in PBS and blocked in 10% (w/v) milk protein for 1 hour before being incubated in anti- NF- $\kappa$ B/p65 rabbit polyclonal antibody (Autogen Bioclear, Wiltshire, UK, 1:100 in 5% milk protein, overnight, 4°C). After PBS washing, biotinylated secondary antibody (goat anti-rabbit, Vector Labs, Peterborough, UK, 1:1000 in 5% milk protein, 1 hour, room temperature) was added. After further washing cells were co-labelled with fluorescein-streptavidin (Vector Labs, 1:100 in PBS) and DAPI (1:1000) for 40 minutes at room temperature. Cells were washed in PBS and imaged using an epifluorescence microscope (ImageXpress, Molecular Devices, Union City, CA), at  $\lambda_{\text{ex}}495\text{nm}/\lambda_{\text{em}}515\text{nm}$  for FITC and  $\lambda_{\text{ex}}385\text{nm}/\lambda_{\text{em}}461\text{nm}$  for DAPI localisation or a confocal microscope (Zeiss LSM 510Meta, Welwyn Garden City, UK) at  $\lambda_{\text{ex}}488\text{nm}/\lambda_{\text{em}}500\text{-}550\text{nm}$  for FITC localisation and an HBO mercury lamp and Zeiss filter set 01 (excitation filter – bandpass (BP) 365/12 nm, emission filter – long pass (LP) 397 nm) for DAPI. Identical fields of view were captured and overlaid for analysis. NF- $\kappa$ B translocation was scored according to a previously published and validated protocol (Canton *et al.* 2010; Moustafa *et al.* 2002; Sun *et al.* 2004). Briefly, cells were scored 0 if NF- $\kappa$ B staining was stronger in the cytoplasm than the nucleus, 0.5 if there was no difference in staining intensity between cytoplasm and nucleus and 1 if the nucleus stained more

strongly than the cytoplasm. Samples were scored blind, the experiment performed in triplicate, a minimum of 100 cells was scored for each replicate and the percentage score determined.

### **Cytometric bead array**

Aliquots of conditioned medium (50  $\mu$ l) were analysed using the Cytometric Bead Array Flex Sets (BD Biosciences, Oxford, UK) to quantify the levels of specific soluble proteins. After incubation of the media with the appropriate capture beads followed by a phycoerythrin-tagged capture antibody, beads were distinguished and quantified using the BDFACSArray. Known standards were also quantified according to manufacturer's instructions. The proteins assayed in this way were FAS ligand (soluble), basic FGF, IFN- $\gamma$ , IFN- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-6, IL-8, IL-10, TNF, GM-CSF, CD14, IL12p70 and VEGF.

### **Statistical analysis**

Experiments were repeated a minimum of three times and the means determined and the data analysed by ANOVA and Dunnett's post test, with significant differences indicated by \*\*\* $P \leq 0.001$ , \*\*  $P \leq 0.01$ , \*  $P \leq 0.05$ . Where the use of different patient cells resulted in highly variable responses, Chi-squared test was used to compare the response of each patient after acid exposure to that observed at pH 7.4.

## **Results**

### **The effect of acid on primary oesophageal cell viability**

The MTT-ESTA data (Figure 1) reveals that neither HOS nor HOF cells show any change in viability following exposure to pH 5 for up to 120 minutes. Sixty minutes exposure to a pH 4 environment was necessary to induce a significant reduction in viability in HOS cells to  $19.5 \pm 21.5\%$  ( $P \leq 0.05$ ), whilst HOF cells were less resistant to increases in hydrogen ion concentrations, showing a significant loss of viability to

59.5 ± 22.5% after 30 minutes ( $P \leq 0.05$ ). At pH 3, a significant loss in viability was observed after 30 minutes (16.9 ± 1.6%,  $P \leq 0.001$ ) for HOS cells and 10 minutes for HOF cells (29.7 ± 18.6%,  $P \leq 0.001$ ). This slight increase in resistance to acid for the HOS cells is in keeping with the barrier role of the squamous epithelium, providing some protection for the underlying stromal tissue from the effects of acid exposure encountered during reflux.

### **The effect of acid on NF- $\kappa$ B translocation in primary oesophageal cells**

Since pH 3 exposure resulted in a rapid reduction in viability this was not used to study NF- $\kappa$ B translocation and instead HOS and HOF cells were exposed to less toxic acid environments (pH 7.4 to pH 4) for up to 120 minutes. Figure 2 shows NF- $\kappa$ B/p65 immunostained images of HOF and HOS cells that have undergone exposure to culture medium at pH 7.4, pH 6, pH 5 or pH 4 for 30 minutes together with LPS positive control images. It can be seen that NF- $\kappa$ B is located mainly in the cytoplasm of the cells in all cases, except for the pH 4 exposed HOS cells and the HOF and HOS LPS-treated positive controls where NF- $\kappa$ B is located in the nucleus for many of the cells. DAPI staining was used to confirm nuclear localisation (images not shown). Similar images were obtained for each pH at each time point and the extent of translocation determined. The images obtained from HOF cells following prolonged exposure to pH 4 and, to a much lesser extent, pH 5 showed some changes in morphology and reduced adhesion which reflect a reduction in cell viability. HOS cells when exposed to a pH 4 insult responded with NF- $\kappa$ B translocation (Figure 3). After 10 minutes exposure NF- $\kappa$ B activation (28.8 ± 8.9%) was significantly greater than the control ( $P \leq 0.05$ ), by 30 minutes exposure NF- $\kappa$ B activation increased to 32.5 ± 13.2%, however the larger standard deviation (possibly a reflection of inter-patient variability in the magnitude of the response) meant this was

not significant when compared to the control. Following 60 minutes exposure to pH 4 NF- $\kappa$ B activation rose to  $50.4 \pm 5.5\%$ , which was significantly greater than that observed at pH 7.4 ( $P \leq 0.01$ ) and remained significantly increased to the end of the experiment ( $52.2 \pm 7.9\%$ ,  $P \leq 0.01$ ). No effect was observed for up to 120 minutes exposure to either pH 5 or pH 6.

In contrast, no NF- $\kappa$ B activation was observed with the HOF cells exposed to acid for up to 120 minutes compared to cells exposed to pH 7.4 medium for the same time period (data not shown). This indicates that oesophageal fibroblasts do not respond to a short single acid insult by activating NF- $\kappa$ B.

Cells were also incubated in PDTC, an anti-oxidant which is a non-specific inhibitor of NF- $\kappa$ B activation, for one hour prior to acid exposure and throughout the acid exposure period. Figure 4 shows that although cells in the absence of inhibitor translocated NF- $\kappa$ B at pH 4 but not at pH 7.4, cells in PDTC showed no translocation at either pH, indicating that the increase in nuclear staining observed at pH 4 was a result of NF- $\kappa$ B activation.

#### **Pulsatile acid exposure of primary oesophageal cells and paracrine translocation of NF- $\kappa$ B in primary oesophageal fibroblasts**

These experiments aimed to mimic the clinical situation of pulsatile acid exposure during acid reflux, and also explored epithelial-stromal interactions as a response to acid exposure. Cells received either a single 30 minute exposure or three consecutive 3 minute exposures to pH 7.4, pH 5 or pH 4. The cells were allowed to recover in a pH 7.4 environment for either 5 or 30 minutes between treatments and cells were examined for evidence of NF- $\kappa$ B translocation. To eliminate any effects arising from the repeated media exchanges the results were calculated as a percentage of the response using the same treatment regime at pH 7.4.

Figure 5 shows that NF- $\kappa$ B translocation was significantly increased in these HOS cells exposed to pH 4 for 30 minutes compared to pH 5 and 7.4 ( $342.1 \pm 39.2$  % of the pH 7.4 response,  $P \leq 0.001$ ). In addition it was seen that HOS cells which encountered three short pulses of acid with either interval length did not show any significant increase in NF- $\kappa$ B translocation.

We then investigated paracrine signalling between the two cell types by exposing HOF cells to conditioned medium. In all cases the acid-exposed epithelial cells were maintained for a further 2 hours in a pH 7.4 environment following the final acid exposure to produce conditioned medium.

Exposure of HOF cells to the HOS conditioned medium revealed that the epithelial cells did, under certain conditions, produce a soluble factor capable of paracrine stimulation of NF- $\kappa$ B (Figure 6), although the degree of response varied considerably with cells derived from different patients. Again the results were calculated as a percentage of the response observed at pH 7.4 for the same treatment regime (Figure 7).

No significant NF- $\kappa$ B translocation was observed with any patient's cells when fibroblasts were exposed to conditioned medium from epithelial cells after 30 minutes at pH 5. However, brief pulsatile exposure of epithelial cells to acid did, in some circumstances, induce NF- $\kappa$ B activation in the fibroblasts (Table 1). This effect was statistically significant for epithelial cells from all 3 patients when exposed to pH 5 pulses (Figure 7), although the magnitude of the response varied from 119.5% to 224.1% of the response observed for the same patient at pH 7.4.

For patients 1 and 2, significant NF- $\kappa$ B translocation in HOFs was also detected after epithelial exposure to pH 4 pulses when the interval was restricted to five minutes (patient 1 - 134.7% of pH 7.4 response  $P < 0.0001$ , patient 2 - 155.8% of pH 7.4

response  $P = 0.0001$ ). However, in both cases this was significantly less than that observed at pH 5 (patient 1 -  $P < 0.0001$ , patient 2 -  $P = 0.035$ ). A small response was also observed for patient 1 after 30 minutes at pH 4 (116.2% of pH 7.4 response,  $P = 0.0116$ ) and patient 3 with pulsatile pH 4 treatment and a 30 minute interval (127.8% of pH 7.4 response,  $P = 0.0014$ ). In general, the cells from patient 1 appeared to show the greatest level of epithelial/stromal interactions following acid exposure while patient 3 was the least responsive.

### **The effect of acid exposure on Het-1A cells**

The Het-1A cell line is an SV-40 immortalised human oesophageal cell line commonly used as an experimental model, and we explored whether the results observed using primary oesophageal cells were reflected in these cells. NF- $\kappa$ B activation was assessed in Het-1A cells following 30 minutes or three 3 minute pulsatile exposures to an acidic environment (pH 4 - pH 7.4). Results were again calculated as a percentage of the equivalent regime at pH 7.4.

Significantly greater NF- $\kappa$ B activation in the epithelial cells was observed only when the Het-1A cells were exposed to 30 minutes at pH 4 ( $457.8 \pm 45.9\%$  of pH 7.4 response,  $P \leq 0.001$ ) or the positive control ( $427.4 \pm 114.1\%$  of pH 7.4 response,  $P \leq 0.001$ ). In all other cases no significant activation was observed (Figure 8a).

Following acid exposure the Het-1A cells produced conditioned media for 2 hours and primary oesophageal fibroblasts were exposed to this conditioned media for 3 hours. NF- $\kappa$ B translocation within the fibroblasts was then determined (Figure 8b).

HOF cells demonstrated relatively high NF- $\kappa$ B activation in the negative control. This may be the result of exposing the HOF cells, usually cultured in 10% FCS, to the BRFF-EPM2 serum free medium required for Het-1A culture. To account for this, activation was once again calculated as a percentage of the response observed for the

same treatment at pH 7.4. This demonstrated a significant increase in NF- $\kappa$ B activation in HOF cells exposed to conditioned media from Het-1A cells treated with pH 5 for 30 minutes ( $114.0 \pm 15.4\%$  of pH 7.4 response,  $P \leq 0.05$ ) or pulse treated with pH 5 media ( $114.0 \pm 17.2\%$  of pH 7.4 response,  $P \leq 0.05$ ).

We sought to identify the specific soluble mediator of this paracrine effect by screening the conditioned medium for a panel of 15 soluble proteins. We selected potential mediators based on previous reports of likely candidate molecules, however no significant differences were observed for any of the proteins assayed (results not shown).

## **Discussion**

The aim of this study was to examine how acidic conditions affect viability and NF- $\kappa$ B activation in oesophageal epithelial and stromal cells, and to investigate whether there is any evidence of paracrine signalling between these cell types in response to acid exposure. Our results suggest an acid induced paracrine activation of NF- $\kappa$ B in oesophageal stromal cells following exposure of the epithelial cells to acid. It is entirely plausible that such crosstalk would operate the oesophageal tissue response to GORD. Because the number of primary cell cultures employed was limited (due to the technical and logistical challenges associated with these cells), we sought to replicate the results in one of the commonly used oesophageal cell lines, Het-1A, and saw comparable results.

We demonstrated that acid insults affect the viability of primary HOS and HOF cells to a slightly different extent, with oesophageal epithelial cells proving a little more resistant to acid conditions than the oesophageal fibroblasts. It was the aim of the first part of this work to determine the extent of acid insult which cells could tolerate without a significant loss of viability. We considered environments that were directly

cytotoxic to be of less clinical relevance since cells exposed to such conditions are unlikely to be capable of transformation to a BM phenotype. We showed that primary oesophageal epithelial cells are capable of withstanding 30 minutes acid exposure at pH 4 or above without a significant loss in viability, and accordingly we consider that further studies should focus on the impact of exposure to acid in this range.

We also demonstrated a clear difference in NF- $\kappa$ B activation between the two cell types in response to acid exposure. Both primary and immortalised epithelial oesophageal cells responded to even a relatively brief exposure to a pH 4 insult by translocating and thus activating NF- $\kappa$ B, whereas fibroblasts exposed to a similar regime showed no NF- $\kappa$ B translocation. NF- $\kappa$ B activation has a recognised anti-apoptotic effect in many circumstances, enabling cells to withstand exposure to stimuli that would normally induce cell death (Beg and Baltimore 1996) and it may be that the increased acid resistance of epithelial cells is due to NF- $\kappa$ B activation.

Our second key finding is that brief pulsatile exposure of primary or immortalised epithelial cells to a pH 5 acidified environment results in a paracrine signal which activates NF- $\kappa$ B in HOF cells, even though this exposure regime was insufficient to activate NF- $\kappa$ B in the epithelial cells themselves. This is in agreement with work performed recently in our laboratory (Canton *et al.* 2010) where certain environments induced the paracrine activation of NF- $\kappa$ B in human dermal fibroblasts exposed to dermal keratinocyte conditioned media while not directly activating NF- $\kappa$ B in the exposed keratinocytes.

NF- $\kappa$ B activity is regulated by interactions with inhibitory I $\kappa$ B proteins. When I $\kappa$ B $\alpha$  is bound to NF- $\kappa$ B DNA binding is prevented and the complex shuttles continuously between nucleus and cytoplasm. However, due to a strong nuclear export signal on I $\kappa$ B $\alpha$ , the complex is primarily located in the cytoplasm. Meanwhile, binding to an

alternative inhibitory protein I $\kappa$ B $\beta$ , retains NF- $\kappa$ B in the cytoplasm. Thus, in most cells, NF- $\kappa$ B is located in the cytoplasm as an inactive complex bound to I $\kappa$ B. It is only when the cell receives an NF- $\kappa$ B activation signal that I $\kappa$ B is unbound and NF- $\kappa$ B translocates to the nucleus to activate target gene expression. Consequently, most NF- $\kappa$ B activation assays use the translocation of NF- $\kappa$ B from the cytoplasm to the nucleus as the measure of NF- $\kappa$ B activation, although methods have been developed recently where cells are transfected with plasmid-based NF- $\kappa$ B reporter genes.

Unfortunately primary epithelial cells are particularly difficult to transfect, with the process frequently resulting in terminally differentiated cells, making this latter approach unsuitable for this study. Assays which require the production of nuclear or whole cell extracts to quantify NF- $\kappa$ B levels in specific subcellular locations typically require high volumes of material, and primary cells cannot be passaged sufficiently to generate such volumes.

For these reasons we used the immunocytochemical approach described to determine the subcellular location of the p65 subunit of NF- $\kappa$ B as a measure of NF- $\kappa$ B activation. The presence of a transactivating domain on the p65 subunit ensures that p65 nuclear localisation is a positive indicator of NF- $\kappa$ B transcriptional activation (Baeuerle and Henkel 1994) and previous work has confirmed that results obtained using the method described in this paper are comparable to those obtained by EMSA detection of nuclear NF- $\kappa$ B (Moustafa *et al.* 2002).

Our studies on the ability of oesophageal epithelial cells to induce paracrine NF- $\kappa$ B activation in fibroblasts used primary cells from three different patients. The benefits, in terms of biological applicability, of using primary cells are countered by the inherent practical difficulties around obtaining tissue samples from human volunteer donors. To broaden the applicability of our results we repeated our studies on the

immortalised oesophageal cell line Het-1A. Similar results were obtained from HOS and Het-1A cells for NF- $\kappa$ B activation following direct exposure to acid, with both requiring a pH 4 insult to activate NF- $\kappa$ B. However, although the paracrine response was broadly similar, there were some slight differences observed. Exposure of Het-1A cells to a pH 5 environment for 30 minutes induced NF- $\kappa$ B activation in HOF cells, but this was not observed with primary cells. In addition, Het-1A cells exposed to pH 5 pulses with a 5 minute recovery period resulted in paracrine NF- $\kappa$ B activation which was less than the mean response observed with primary oesophageal epithelial cells. It is worth noting that recent work in our laboratory has demonstrated that Het-1A cells grown in 3D organotypic cultures do not mature and differentiate in the same way as primary human oesophageal squames, retaining instead a proliferative phenotype (Green *et al.* 2010). Since NF- $\kappa$ B is known to play a role in many aspects of proliferation and cell differentiation it is perhaps not surprising that the response of acid-exposed Het-1A cells differs very slightly from that of primary HOS cells. However, for both primary and immortalised epithelial cells, the conditions which resulted in paracrine activation of NF- $\kappa$ B in HOF cells were also ones that did not result in significant NF- $\kappa$ B activation in the epithelial cells themselves. Nevertheless, further studies are obviously required to fully investigate the phenomenon we report. Attempts were made to determine the epithelial-derived factor that initiates the NF- $\kappa$ B response in the oesophageal fibroblasts. For this we used immortalised Het-1A cells, due to the limited availability of primary human oesophageal cells. When the conditioned media was screened for a bank of potential initiation factors no significant changes were detected and the factor or factors responsible for this effect remain undetermined.

Several cytokines known to activate NF- $\kappa$ B have been identified in Barrett's epithelia: these include TGF $\beta$ , IFN $\gamma$  and TNF $\alpha$  (Jankowski *et al.* 2000); IL-1 $\beta$  (Abdel-latif *et al.* 2005; Fitzgerald *et al.* 2002a; Jankowski *et al.* 2000); IL-8 (Abdel-latif *et al.* 2005; Fitzgerald *et al.* 2002a; Jenkins *et al.* 2007); IL-10 and IL-4 (Fitzgerald *et al.* 2002b). However, since they were all observed in epithelia that had already entered the metaplasia-dysplasia-carcinoma sequence this may not fully reflect events occurring in the initial stages of BM development. A more recent study demonstrated that 60 minutes exposure of primary HOS and Het-1A cells to pH 4.5 induced IL-6 and IL-8 production (Rafiee *et al.* 2009); however the acid exposure time was longer than in our study. Further research, ideally in the form of a full proteomic characterisation of acid exposed primary HOS cells from a larger number of patients is probably required to characterise the factor responsible for the paracrine NF- $\kappa$ B activation.

The inter-sample variability seen in the paracrine response when using primary cells derived from different patients is unsurprising. Our laboratory has previously reported a 50-fold difference in the response of epidermal keratinocytes to chromium when using cells isolated from different individuals (Little *et al.* 1996). This was attributed to differences in the enzymatic capabilities of these cells for dealing with oxidative stress. It remains unclear why some patients with GORD develop BM whilst others simply develop reactive changes in the squamous epithelium but our data raise the possibility that this clinical variation may be, in part, due to differences in the epithelial-stromal dialogue modulating the overall tissue response and we suggest that this warrants further investigation, alongside studies to identify the molecular mediators of this crosstalk

## **Conclusions**

These results show that human oesophageal epithelial cells respond to a pulsatile acid exposure with the paracrine activation of the NF- $\kappa$ B in the oesophageal fibroblasts.

The limited availability of primary human oesophageal cells restricted the sample size of the study and so further investigation into this phenomenon is required. However, these findings highlight the potential importance of epithelial-stromal interactions in the pathogenesis of oesophageal diseases. Given the role of NF- $\kappa$ B signalling in fibroblast functions such as matrix remodelling and cell migration, the secondary activation of NF- $\kappa$ B described here may well contribute to the morphological manifestations of GORD. Furthermore, the inter-patient variability observed may go some way to explaining why only a minority of patients with reflux oesophagitis develop BM. Further investigation into such epithelial-mesenchymal communications, specifically the stress-coping mechanisms of the cell, is likely to add to our understanding of the pathogenesis of both reflux oesophagitis and BM. This is a novel insight into the impact of GORD, which is amenable to further investigation and which may have therapeutic possibilities.

## **Competing interests**

The authors declare that they have no competing interests.

## **Acknowledgements**

We are grateful to Mr. Roger Ackroyd, Mr. Andrew Wyman and Mr. Chris Stoddard, Consultant Surgeons at Sheffield Teaching Hospitals NHS Foundation Trust, for their help in acquiring oesophageal tissue samples and their support of our work. We also thank Sue Newton from the Flow Cytometry Core Facility for performing the Cytometric Bead Array. This study was supported by grants from the Bardhan Research and Education Trust (BRET) and Yorkshire Cancer Research (YCR).

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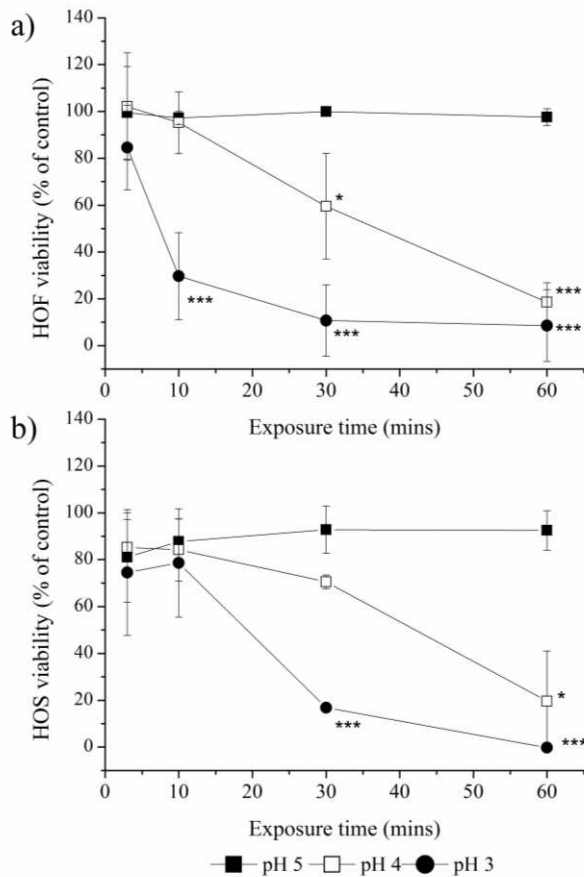
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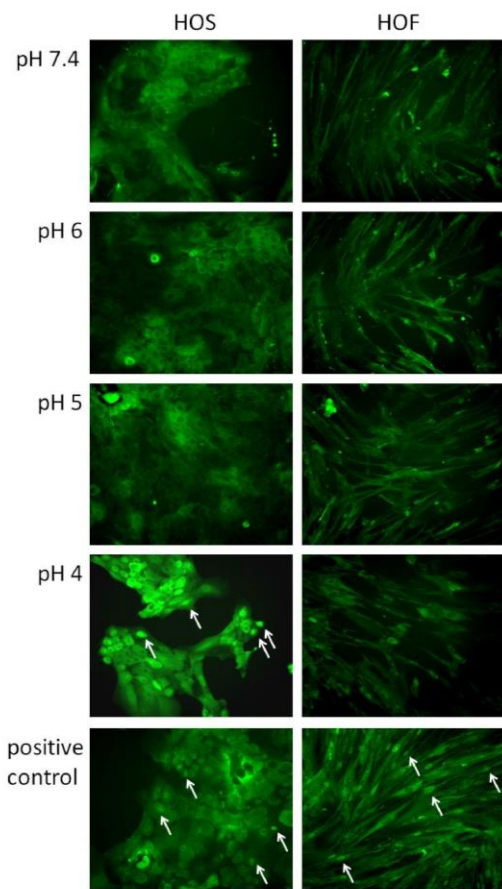
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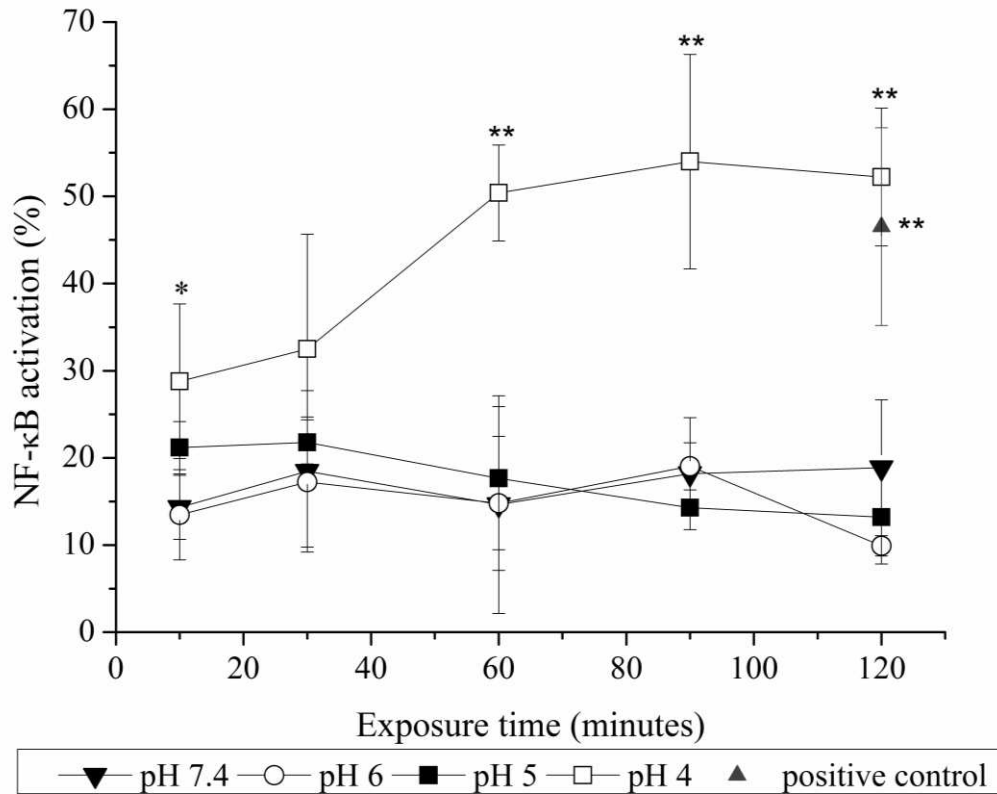
**Figure 1 The effect of exposure to acid on cell metabolic activity**

Cultures of HOF (a) or HOS (b) cells were directly exposed to control media (pH 7.4) or acidified medium at pH 5, pH 4 or pH 3 for 3 to 60 minutes followed by a 24 hour recovery period, prior to determination of metabolic activity by MTT-ESTA assay. 60 minutes exposure to pH 5 has no impact on cell viability for either HOF or HOS cells. HOF cultures show a significant loss of viability after exposure to pH 3 acidified medium for 10 minutes or pH 4 for 30 minutes, while HOS cultures require pH 4 for 60 minutes or pH 3 for 30 minutes before viability is significantly affected. Results were calculated as a percentage of the control and are presented as the mean  $\pm$  S.D. of 3 experiments. Significant differences from control are indicated by \*\*\*  $P \leq 0.001$ , \*\*  $P \leq 0.01$ , \*  $P \leq 0.05$



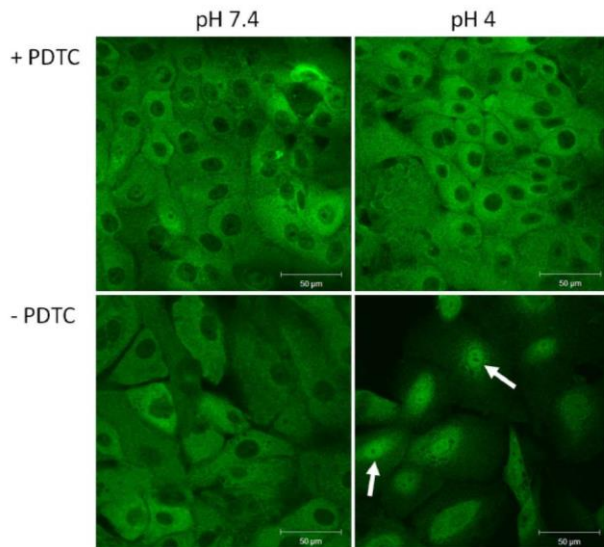
**Figure 2 Immunohistochemical localisation of NF- $\kappa$ B after exposure of cells to acid**

HOS and HOF cells were exposed to pH 7.4, pH 6, pH 5 or pH 4 medium for 30 minutes. LPS treated positive control cells (2 hour treatment) are also shown. Positive control HOS and HOF cells and pH 4 exposed HOS cells show nuclear localisation of NF- $\kappa$ B (arrows). Following all other treatments NF- $\kappa$ B is detected in the cytoplasm, demonstrating no NF- $\kappa$ B activation. The HOF cells at pH 4 show an abnormal morphology and reduced adhesion, which can be attributed to a reduction in cell viability.



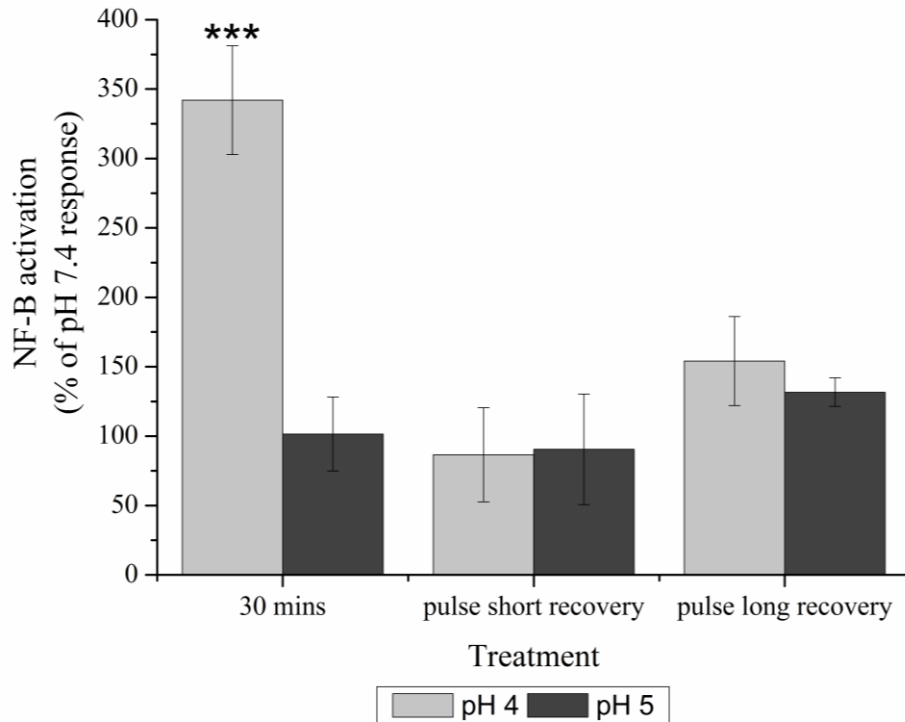
**Figure 3 Translocation of NF-κB after direct exposure of HOS cells to acidified medium**

Cells were exposed to medium between pH 7.4 and pH 4 for up to 120 minutes and NF-κB activation scored as described in the materials and methods section. No significant NF-κB activation was detected in HOS cells exposed to pH 7.4, pH 6 or pH 5 conditions for up to 120 minutes. However exposure to pH 4 resulted in significant NF-κB activation, which remained significantly higher to the end of the experiment. Results are presented as the mean ± S.D. of 3 experiments. Significant differences from pH 7.4 control are indicated by \*\*  $p \leq 0.01$ , \*  $p \leq 0.05$ .



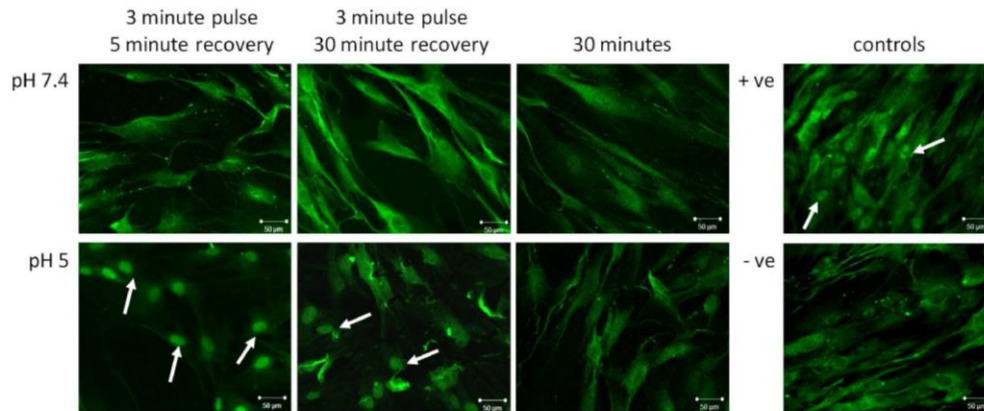
**Figure 4 Inhibition of NF- $\kappa$ B translocation**

NF- $\kappa$ B translocation in HOS cells after 60 minutes exposure to pH 7.4 or pH 4. The top panels show cells which were pre-treated for 1 hour with the NF- $\kappa$ B inhibitor PDTC and incubated with PDTC during acid exposure, while the bottom panels did not receive PDTC treatment. NF- $\kappa$ B is detected in the nucleus of cells exposed to pH 4 in the absence of PDTC (arrows) however in the presence of the inhibitor NF- $\kappa$ B is located in the cytoplasm. NF- $\kappa$ B is also located in the cytoplasm of cells exposed to pH 7.4 with or without PDTC.



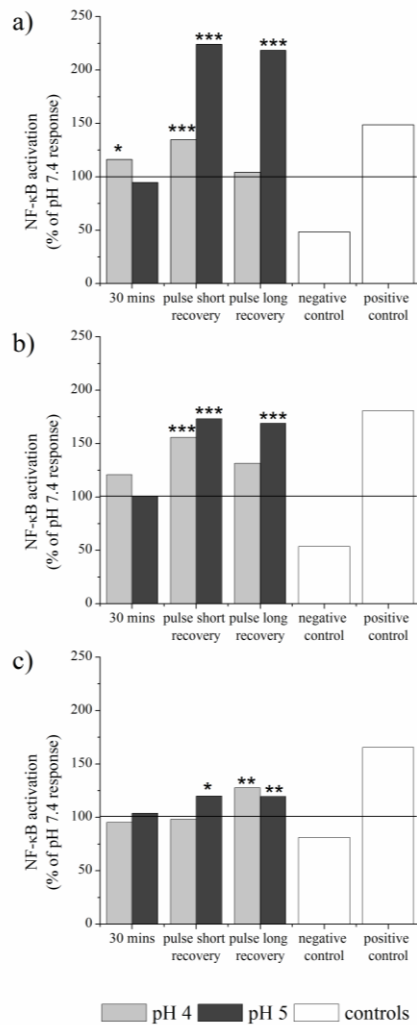
**Figure 5 The effects of pulsatile acid exposure on NF-κB translocation**

The activation of NF-κB in HOS cells following exposure to pH 5 or pH 4 for 30 minutes or three pulsed exposures with either a short (5 min) or long (30 min) recovery period. Results have been calculated as a percentage of the response after pH 7.4 treatment using the same regime. After treatment the cells were cultured in pH 7.4 media for 2 hours prior to fixing and immunostaining. NF-κB activation was detected in HOS cells exposed to pH 4 for 30 minutes, however no significant activation was detected for any other treatment regime. Results are presented as the mean ± S.D. of 3 experiments. Significant differences from pH 7.4 control are indicated by \*\*\*  
 $p \leq 0.001$



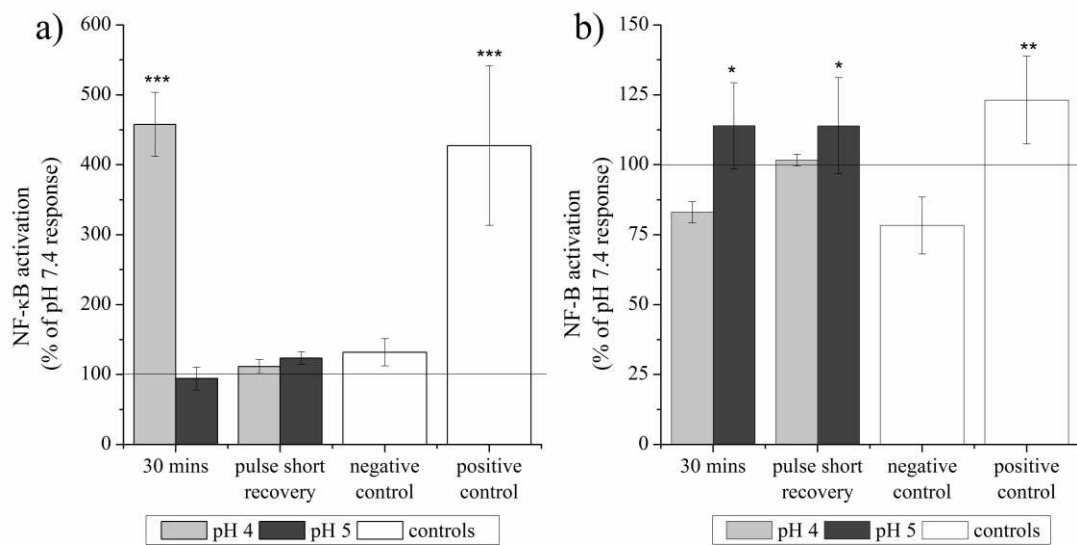
**Figure 6 The effect of HOS conditioned medium on HOF cells using patient set 1 cells**

NF- $\kappa$ B translocation in HOF cells following exposure to conditioned medium taken from HOS cells after treatment with medium at pH 7.4 or pH 5. Conditioned medium was taken after the following treatments:- three 3 minute treatments with a 5 minute interval, three 3 minute treatments with a 30 minute interval or 30 minutes. Positive and negative controls are also shown. NF- $\kappa$ B translocation to the nucleus is observed in the cells treated with conditioned medium from cells exposed to pH 5 for 3 minutes with either a 5 or 30 minute interval. Translocation is also observed in the positive control but in all other cells NF- $\kappa$ B remains in the cytoplasm.



### Figure 7 Activation of NF-κB in HOF cells after 3 hours exposure to HOS conditioned medium

NF-κB translocation in HOF cells following exposure to conditioned medium taken from acid treated HOS cells. Results are calculated as a percentage of the response at pH 7.4 using the same regime and are shown for cells from three different patients (a, b and c). The solid line at 100 % indicates the response seen for the pH 7.4 control. In all three experiments HOF cells show increased NF-κB activation over the pH 7.4 control after exposure to short pulses of pH 5 with both long (30 minutes) and short (5 minutes) intervals at pH 7.4, with higher levels of activation when the recovery period was reduced. Significant differences from pH 7.4 treatment are indicated by \*\*\*  $p \leq 0.001$ , \*\*  $p \leq 0.01$ , \*  $p \leq 0.05$ .



**Figure 8 Activation of NF-κB in Het-1A and HOF cells following Het-1A exposure to acid**

The activation of NF-κB in Het-1A cells after (a) a single 30 minute treatment or three pulsed exposures to pH 7.4, pH 5 or pH 4 followed by (b) the paracrine activation in HOF cells after exposure to Het-1A conditioned media. Results have been calculated as a percentage of the response following pH 7.4 treatment using the same regime. After acid treatment the Het-1A cells produced conditioned medium for 2 hours and HOF cells were then exposed to this for 3 hours. NF-κB activation was detected in Het-1A cells exposed to pH 4 for 30 minutes, and in HOF cells after Het-1A exposure to pH 5 (both single or pulsatile treatments). No significant activation was detected for any other treatment regime. Results are presented as the mean  $\pm$  S.D. of 3 experiments. Significant differences from pH 7.4 control are indicated by \*\*\*  $p \leq 0.001$ , \*\*  $p \leq 0.01$ , \*  $p \leq 0.05$ .

## Tables

HOS exposure regime		Paracrine NF-κB activation in HOF cells					
		Patient 1		Patient 2		Patient 3	
Insult	Interval (minutes)	% of pH 7.4 response	P-value	% of pH 7.4 response	P-value	% of pH 7.4 response	P-value
pH 5	0 (single treatment)	94.6	0.3286	100.6	0.2376	103.7	0.2776
	5	<b>224.1</b>	<b>&lt;0.0001***</b>	<b>173.1</b>	<b>&lt;0.0001***</b>	<b>119.9</b>	<b>0.0106*</b>
	30	<b>218.5</b>	<b>&lt;0.0001***</b>	<b>168.8</b>	<b>&lt;0.0001***</b>	<b>119.5</b>	<b>0.0034**</b>
pH 4	0 (single treatment)	<b>116.2</b>	<b>0.0116*</b>	120.8	0.0629	95.1	0.0893
	5	<b>134.7</b>	<b>&lt;0.0001***</b>	<b>155.8</b>	<b>0.0001***</b>	98.0	0.1243
	30	1.0	0.5162	131.4	0.0812	<b>127.8</b>	<b>0.0014**</b>

**Table 1 Paracrine activation of NF-κB in HOF cells following exposure to HOS conditioned medium**

Conditioned medium experiments demonstrated variable but statistically significant activation of NF-κB in fibroblasts in response to exposure to culture medium conditioned by HOS cells following an acidic insult. Acid treatments were either pulsatile, with three 3 minute exposures separated by 5 or 30 minute intervals at pH 7.4, or a single 30 minute exposure. The response and p-values are relative to the internal control experiment (HOS cells from the same patient exposed to pH 7.4 using the same treatment regime). Results in bold indicate those which are significantly greater (\*\*\*P ≤ 0.001, \*\* P ≤ 0.01, \* P ≤ 0.05).