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Inducible nitric oxide synthase (iNOS) and nitric oxide (NO) are important mediators of reflux-induced cell signalling in esophageal cells

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Nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) has been implicated in both DNA damage induction and aberrant cell signalling in various tissue and cell backgrounds. We investigated here the role of iNOS and NO in DNA damage induction and nuclear factor-kappa B (NF-κB) signalling in esophageal cells *in vitro*. As esophageal adenocarcinoma develops in a background of Barrett's esophagus secondary to reflux disease, it is possible that inflammatory mediators like NO may be important in esophageal cancer development. We show that reflux components like stomach acid and bile acids [deoxycholic acid (DCA)] can induce iNOS gene and protein expression and produce NO generation in esophageal cells, using real-time PCR, western blotting and NO sensitive fluorescent probes, respectively. This up-regulation of iNOS expression was not dependent on NF-κB activity. DCA-induced DNA damage was independent of NF-κB and only partially dependent on iNOS and NO, as measured by the micronucleus assay. These same reflux constituents also activated the oncogenic transcription factor NF-κB, as measured by transcription factor enzyme-linked immunosorbent assay and gene expression studies with NF-κB linked genes (e.g. interleukin-8). Importantly, we show here for the first time that basal levels of NF-κB activity (and possibly acid and DCA-induced NF-κB) are dependent on iNOS/NO and this may lead to a positive feedback loop whereby induced iNOS is upstream of NF-κB, hence prolonging and potentially amplifying this signalling, presumably through NO activation of NF-κB. Furthermore, we confirm increased protein levels of iNOS in esophageal adenocarcinoma and, therefore, in neoplastic development in the esophagus.

Introduction

Barrett's esophagus (BE), a metaplastic condition predisposing patients to esophageal adenocarcinoma (EA), is caused by chronic exposure to gastroesophageal reflux (GER). GER causes sustained exposure of the esophageal mucosa to noxious luminal agents such as acid and bile. These agents are believed to drive the metaplastic process whereby the squamous epithelium is replaced by a columnar epithelium resembling the small intestine. Patients acquiring BE progress to adenocarcinoma at a rate between 0.4% per year (1) and 1% per year (2). As survival rates for patients with EA are atrocious, a better understanding of the biology of BE and the molecular pathways driving neoplastic progression are urgently needed. It is hoped that a better molecular understanding of the disease will lead to therapeutic targets for drug development and molecular markers to help identify patients at risk of developing cancer at an early stage.

Abbreviations: BE, Barrett's esophagus; DAF-2 DA, diaminofluorescein-2 diacetate; DCA, deoxycholic acid; EA, esophageal adenocarcinoma; ELISA, enzyme-linked immunosorbent assay; GER, gastroesophageal reflux; HBSS, Hanks balanced salt solution; IHC, immunohistochemistry; iNOS, inducible nitric oxide synthase; MN, micronucleus; NF-κB, nuclear factor-kappa B; NO, nitric oxide.

One molecule of particular interest in a context of BE is inducible nitric oxide synthase (iNOS). In contrast to endothelial nitric oxide synthase, or neuronal nitric oxide synthase (nNOS), iNOS is expressed only after cellular activation, i.e. it is not expressed constitutively. The iNOS protein is responsible for the production of cellular nitric oxide (NO) from the oxidation of L-arginine, with both iNOS and NO being implicated in a number of biological processes. In infectious diseases, human macrophages have been shown to express the iNOS protein, with the generated NO contributing to the killing of various microbes (3–5). Increased iNOS protein expression and NO level have been found in many chronic inflammatory and autoimmune diseases such as rheumatoid arthritis (6–7), multiple sclerosis (8), asthma (9) and type 1 diabetes (10,11). Hence, given that BE arises in a background of chronic inflammation, this link between iNOS/NO and inflammation further highlights a potential role for iNOS in BE.

An increased level of iNOS expression is also often seen in neoplastic tissue. This elevation has been observed in malignancies of the breast (12), lung (13), prostate (14) and colon (15). In the esophagus, increased iNOS mRNA has been found early in the neoplastic pathway, at the BE stage (16). Interestingly, neoplastic progression in the esophagus in rats has been halted on administration of the selective iNOS inhibitor *S*,*S*-1,4-phenylene-bis(1,2-ethanediyl) bis-isothiourea (17), indicating the potential of iNOS as a therapeutic target. In further support of this, thioproline (a nitrite scavenger) has also been shown to block duodenal reflux-driven EA in the rat (18).

One of the main routes by which NO and related reactive nitrogen species (e.g. peroxynitrite and nitrogen dioxide) promote carcinogenesis is by their ability to induce DNA damage via single- and double-stranded DNA breaks (19,20) and by increased levels of deamination (21). NO can also exacerbate this genotoxicity, by simultaneously inhibiting DNA repair enzyme activity through tyrosine and cysteine nitrosation (22–24). In terms of the transcriptional control of iNOS induction, nuclear factor-kappa B (NF-κB) has been heavily implicated, as multiple κB-binding sites are located in its promoter (25,26). As both NF-κB and iNOS are centrally involved in inflammatory processes, their association might well be expected. Other transcription factors have also been implicated in the regulation of this gene though, including AP-1 (27,28).

NO has also been shown to have the ability to be a potent cell signalling molecule. It has been shown that NO can induce NF-κB activation (29,30), with the mechanism of action postulated to be via tyrosine modification (31). In addition to this, NO has also been seen to activate the AP-1 transcription factor (32–34), which has been shown to be via ERK activation (31). Conversely, in some cell types it has been shown that NO can inhibit both iNOS expression (35) and NF-κB activity (34) representing a negative feedback system. Hence, cell context and choice of iNOS stimulant appear to be important determinants.

Several groups have published data implicating iNOS in neoplastic progression in BE. Wilson *et al*. (36) showed up-regulation of iNOS mRNA by real-time PCR in 76% of BE patients and in 80% of EA patients. This work has been recently confirmed by Vaninetti *et al*. (37) who showed up-regulation of intracellular iNOS at the RNA level in BE and EA patient tissues, although to a lesser extent than Wilson *et al.* (48% and 63%, respectively). As for the source of this up-regulation of iNOS, there have been several reports that reflux components such as the bile acids DCA (38) and GCDCA (39) can induce NO, presumably through increased iNOS expression. Indeed, duodenal reflux has been shown to induce esophageal iNOS in experimental rat models (40). In 2009, Jolly *et al.* (41) published data showing that the bile acid DCA caused DNA damage to esophageal cells *in vitro*, through an iNOS and NF-κB-dependent manner. As NF-κB is known to be activated by bile acids (42) and is known to be

increasingly activated during neoplastic progression in BE (43,44) it would make intuitive sense if it were shown to drive iNOS expression in BE. We have reported previously that iNOS inhibition blocks bile acid-driven interleukin-8 (IL-8) expression (45). As IL-8 is a known NF-κB target gene, this may reflect a NF-κB-mediated effect, but could also relate to other signalling pathways upstream of IL-8. Therefore, detailed knowledge of the biology of iNOS activation and its association with NF-κB, in the context of reflux exposure in the esophagus is lacking and hence this study aimed to address this.

The reported observation by Jolly *et al.* (41) inspired us to ask more specific questions surrounding iNOS and NO, in particular the potential for establishing a cyclical positive feedback system, as NO has been shown to itself activate NF-κB (46,47). As the iNOS gene has been shown in other cell models to be induced by NF-κB activation, it was therefore considered possible that NO could induce the transcription of iNOS, through NF-κB activation, which would lead to the production of further NO. In theory, this positive feedback loop could amplify or prolong NF-κB activity in the esophagus after reflux exposure. Prolonging or amplifying intracellular signalling in this manner would be particularly pertinent in reflux-driven esophageal cancer, as exposures to reflux only occur in a pulsatile nature, each of short duration. Hence, prolonging the effect of each pulse may explain how these short exposures produce such a dramatic effect in terms of carcinogenesis in the esophagus.

We have assessed here the role of iNOS and NO in reflux-induced DNA damage and NF-κB activation in esophageal OE33 cells. We did not entirely confirm the data from Jolly *et al*. (2009), i.e. we did not observe reflux-induced DNA damage in an NF-κB-dependent manner, nor does our data support the role of NF-κB as the primary transcription factor driving iNOS expression in response to reflux exposure. We do, however, show here that reflux components including the bile acid DCA (deoxycholic acid) and acid (pH 5) can up-regulate the iNOS gene (43- and 12-fold). Furthermore, DCA led to intracellular NO production in an iNOS-dependent manner. iNOS was also shown to be upstream of basal and reflux-induced NF-κB activity, therefore potentially setting up a positive feedback system which may amplify or prolong DCA-induced NF-κB activity. Finally, we confirm increased iNOS abundance in esophageal cancer tissues, reinforcing the role of iNOS/NO in EA development.

Materials and methods

Micronucleus study

Freshly obtained OE33 cells (ECACC, Salisbury, UK) were cultured overnight in 25 cm^2 flasks prior to treatment with DCA. The following day, cells were exposed to 100 µM and 200 µM DCA in dimethyl sulfoxide for 4 h in serum-free media. The DCA was subsequently washed off and the cells cultured in fresh media containing 2 µg/ml cytochalasin B (Sigma–Aldrich, Poole, UK) for a further 20 h to allow the cells to divide. As cytochalasin B blocks cytokinesis producing binucleated cells, it is clear which cells have divided since exposure to DCA. Micronucleated OE33 cells were scored only in binucleate cells, with a minimum of 2000 cells per dose included. Duplicate cultures were produced and so ~4000 cells were studied per dose. In order to assess the contribution of NF-κB and/or iNOS to DCA-induced genotoxicity, inhibitors to both [10 µM IkK VII (IKK) and 10 µM 1400W (iNOS), both Merck, dissolved in dimethyl sulfoxide] were added to the cells for 30 min prior to the addition of DCA, with washing between DCA and inhibitor exposure. Parallel cultures exposed to the inhibitors alone or dimethyl sulfoxide (negative control) were established and scored concurrently. Lower doses of DCA (100 and 200 µM) were used in the MN assay compared with the later studies (300 µM) due to previously noted toxicity with 300 µM doses of DCA at the necessarily longer assay time required for the MN assay. Longer exposure times (4 h) were used here as compared with the later assays (60 min) as 4 h exposure is standard for the MN assay (OECD guideline 487).

Gene expression and NF-κB activation study

For gene expression studies, freshly obtained OE33 cells were seeded into six-well tissue culture plates at a density of 0.3×10^6 per well. After serum starvation (24 h), cells were treated for 60 min with (i) DCA (300 µM), or (ii) medium with a pH reduced to pH 5, or (iii) both. An untreated control was run concurrently. These studies were carried out in triplicate. For inhibitor studies, OE33 cells were seeded into six-well tissue culture plates at a density of 0.3×10^6 per well. After serum starvation (24 h), those cells to be treated with an inhibitor were pre-incubated with either 10 µM IKK inhibitor VII (IKK, Merck) or 10 µM 1400W (iNOS, Merck) for 30 min in serum-free medium. Following this, wells were washed once with phosphate-buffered saline prior to treatment with acid or DCA, in combination with the appropriate inhibitor, for 60 min. Untreated wells and wells where no inhibitor was added served as negative and positive controls.

For the pulsed exposures, OE33 cells were seeded as above and in some cases treated with the iNOS inhibitor for 30 min prior to DCA or pH 5 exposure. Exposure to either DCA (300 µM) or acid (pH 5) alone, or together was carried out for either 5 or 30 min. After the treatment time, the cells were washed once with phosphate-buffered saline and then fresh serum-free medium was added for the rest of the experimental time (or 'rest time'), which was either to a total of 60 or 120 min (DCA alone only). This meant cells were treated for (i) 5 min with 55 or 115 min rest time or (ii) 30 min with 30 min or 90 min rest time. Nuclear protein was subsequently extracted and nuclear NF-κB (p65) levels were assessed by enzyme-linked immunosorbent assay (ELISA) (see below).

Real-time PCR

Immediately following treatment, RNA was extracted from the cells and real-time PCR was performed to analyse expression of the IL-8 and iNOS genes (β-actin was used as a housekeeping gene). RNA extraction and real-time PCR details have been published previously (48,49). In brief, RNA was extracted using Qiagen reagents in conjunction with a Qiacube system (Qiagen, Crawley, UK). On-column DNA digestion was employed and cDNA synthesized using the Quantitect RT-PCR kit (Qiagen). Real-time PCR was performed using SYBR green (Bio-Rad, Hemel Hempstead, UK) with the standard curve method utilizing a diluted reference cDNA to generate a standard curve, off which the cDNA levels (starting quantity mean) were read for the unknown RNA samples. These were then normalized to the corresponding β-actin values. Primers for iNOS were as follows: forward: 5′-GCA GAA TGT GAC CAT CAT GG-3′; reverse: 5′-ACA ACC TTG GTG TTG AAG GC-3′ (50). For IL-8 the primers were as follows: forward: 5′-CAA TCC TAG TTT GAT ACT CCC-3′; reverse: 5′-AAT TAC TAA TAT TGA CTG TGG AG-3′. For β-actin the primers were as follows: forward: 5′-GAT GGC CAC GGC TGC TTC-3′; reverse: 5′-TGC CTC AGG GCA GCG GAA-3′.

Western blotting

Following treatment with DCA, protein was extracted from scraped OE33 cells with 200 µl RIPA buffer (Sigma–Aldrich) for 5 min at 4°C. Lysates were centrifuged for 10 min at 10 000 × *g*. Total protein was quantified using the DC Protein Assay (Bio-Rad) according to the manufacturers instructions. Protein separation (5 µg, denatured at 95°C for 5 min) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out using 4% stacking gels (pH 6.8) and resolving gels of 8%. Protein blotting was carried out using the Mini-Trans Blot Cell (Bio-Rad) and Immuno-blot polyvinylidene fluoride (PVDF) membranes (Bio-Rad). Following blotting, membranes were rinsed with 1× tris-buffered saline with 0.1% Tween 20 (TBS/T) and primary antibodies [B-actin, Cell Signalling (#4970S), 1:1000 dilution, in blocking buffer and iNOS, Cell Signalling, (#2977S), 1:750 dilution, in blocking buffer] were added overnight at 4°C with gentle agitation. Following the primary antibody incubation, membranes were washed 4 × 5 min in TBS/T at room temperature with strong agitation. Horseradish peroxidase (HRP)-conjugated secondary antibodies [abcam (ab97080) 1:1000 dilution in blocking buffer] were added for 1 h at room temperature with gentle agitation. Following the secondary antibody incubation membranes were again washed 4 × 5 min in TBS/T at room temperature with strong agitation. Proteins were visualized by enhanced chemiluminescence using the Immun-Star™ HRP Chemiluminescent kit (Bio-Rad), according to the manufacturers instructions. Membranes were exposed using a ChemiDoc XRS gel documentation system (Bio-Rad).

NF-κB analysis

In the case of NF-κB analysis, cells were treated as above and nuclear NF-κB (p65) levels were assessed using a transcription factor ELISA kit based on binding of nuclear extracts to a plate-bound oligo containing the consensus κB-binding sequence (Active Motif, La Hulpe, Belgium). Nuclear extracts were obtained using the Active Motif kit following the manufacturers recommendations. The p65 transcription factor kit was used to assess nuclear levels of active (i.e. DNA binding) p65 in nuclear extracts following the manufacturers recommendations. Previous data (data not shown) have demonstrated consistent activation of NF-κB by DCA and acid and the potency of the NF-κB inhibitor used here.

Intracellular nitric oxide level assessment

OE33 cells were seeded into a black bottomed 96-well tissue culture plate (Greiner, Stonehouse, UK), at a density of 5000 cells per well in RPMI 1640

tissue culture medium overnight, then transferred to serum-free medium for 24 h. The cells were washed once with Hanks balanced salt solution (HBSS, Sigma) and following this, 50 µl of a 5 µM solution of diaminofluorescein-2 diacetate (DAF-2 DA, Invitrogen, Renfrew, Scotland), diluted in HBSS with 20mM HEPES and 5mM glucose was added to all cell containing wells. This was incubated at 37°C for 1 h to allow the chemical to penetrate into the cells and be hydrolyzed to DAF-2. After this time, the cells were washed twice with HBSS and then treated with DCA +/– the iNOS inhibitor 1400W (co-treatment, not pre-treatment). Cells treated for 5 min were washed once with HBSS after treatment, then incubated in HBSS plus HEPES and glucose for the remainder of the experimental time. The plate was transferred to a POLARstar Omega plate reader (BMG Labtech) and the fluorescence intensity measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm, every 10 min for a total of 180 min.

Immunohistochemistry

Suitable histological samples of Barrett's esophagus (with adjacent squamous, and/or adenocarcinoma tissue) were identified in the pathology tissue archive of ABMU Health Board. Identification of suitable tissues was aided through the use of a dedicated informatics platform that was able to interrogate the pathology database. This platform was designed and developed in conjunction with the Health Informatics Research Unit at Swansea Medical School. Ethical approval was obtained from the Dyfed Powys Local Research Ethics Committee prior to the start of the project and the R&D office at the Health Board also gave approval for the study. Tissue sections were stained with a Ventana automated immunostainer using an iNOS antibody (AbCam, Cambridge, UK) at 1:100 dilution. Scoring of the staining was undertaken by two independent members of the research team (GF and APG) using a H-score approach. Staining intensity was scored 0–3 with 0 equating to no staining and 1, 2 and 3 equating to low, moderate and strong staining. A distribution score of 0–3 was also employed with 0 equating to no staining, and 1, 2 and 3 equating to staining in up to 33% of cells, up to 66% of cells or up to 100% of cells in each section. The intensity and distribution scores were multiplied to obtain the final H-score.

Statistical analysis

Fishers Exact test was used to assess differences in micronucleus frequency in cells exposed to reflux agents and inhibitors. For the real-time PCR data, the immunohistochemistry (IHC) data and the p65 nuclear ELISA data, unpaired, two-tailed *t*-tests were used on the triplicate datasets comparing each treatment with the concurrent untreated control.

Results

DCA-induced chromosome damage is not NF-kB dependent

To further explore the data published by Jolly *et al.* (41), showing that bile acids induced DNA damage in a NF-κB and iNOS-dependent manner, we performed micronucleus (MN) studies in OE33 cells exposed to DCA (100 and 200 µM) with and without inhibitors for NF-κB (IKK VII) and iNOS (1400W). We have established previously that DCA can induce chromosome damage as measured by the MN assay and that this DNA damage is blocked by pre-treatment with antioxidants (42,44). This previous study also showed that other bile acids, or acid alone did not induce chromosome damage, hence only the bile acid DCA was used here. Figure 1A shows the results of these experiments. OE33 cells were treated with DCA (100 or 200 µM) for 4 h and the frequencies of micronuclei were subsequently quantified after 1 cell cycle (i.e. 4 + 20 h). Treatment with 100 µM DCA did not induce significantly increased levels of MN, whereas 200 µM DCA induced a 1.8-fold increase in MN frequency that was significant using Fishers Exact test ($P = 0.007$). When cells were pre-treated with a potent NF-κB inhibitor (IKK VII), no effect on DCA-induced DNA damage (200 µM) was observed and a significant increase in MN was still observed ($P = 0.024$). When cells were pre-treated with an iNOS inhibitor (1400W), there was no significant reduction in MN compared with treatment with DCA alone ($P = 0.16$). However, the significant increase in MN frequency seen with DCA (and DCA + NF-κB inhibitor) was lost when the iNOS inhibitor was included when compared with the untreated controls, suggesting a partial effect on the DNA damaging potential of DCA. There was no increased cellular toxicity induced by these treatments as evidenced by the fact that the binucleate frequency remained at similar levels to the untreated controls.

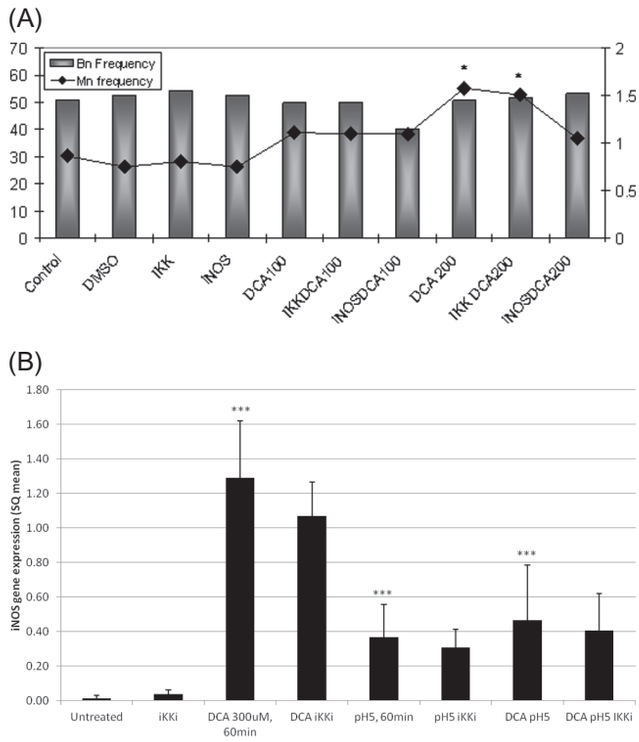

Fig. 1. (**A**) Micronucleus (Mn) data examining chromosome breakage, showing percentage of Mn induction (line graph, right-hand scale) by DCA (4 h exposure + 20 h recovery). DCA at 100 µM was insufficient to show significant induction of Mn. However, 200 µM DCA showed significant induction of Mn ($P = 0.007$). This induction was not abrogated by an inhibitor of NF-κB (IKK VII), but a (non-significant) effect was noted with an iNOS inhibitor (1400W). Bars show percentage of binucleate frequency (left-hand scale), as a measure of cytotoxicity—none was apparent. *$P < 0.05$, statistically significant relative to the untreated control . Note that iNOS inhibition led to loss of MN induction by DCA, however reduction was not significant relative to DCA (200 µM) alone ($P = 0.16$).(**B**) Real-time PCR data for iNOS induction by acid (pH 5) and DCA (300µM), as well as combined acidic DCA. All three treatments significantly up-regulated iNOS mRNA levels. Inhibition of NF-κB (IKK VII) had no significant effect on the level of iNOS induction observed by any of the three treatments.

iNOS mRNA and protein levels are up-regulated by reflux components

OE33 cells exposed to acid (pH 5) or DCA (300 µM) for 1 h showed strong up-regulation of iNOS mRNA by real-time PCR (Figure 1B). Slightly higher doses of DCA (300 µM) were used here relative to the DNA damage study above to compensate for shorter durations of exposure (1 h). Acid up-regulated iNOS by 12-fold with DCA up-regulating iNOS even more so, by 43-fold, combined treatment (acidic DCA) up-regulated iNOS less so, by 16-fold. The combined treatment (acid + DCA) resulted in some toxicity as evidenced by reduced MTS readings 24 h post-exposure (data not shown) and in cells lifting off the tissue culture flask. The changes in iNOS mRNA level were significant ($P = 6.8 \times 10^{-6}$ for acid, $P = 1.7 \times 10^{-5}$ for DCA, $P = 5.9 \times 10^{-4}$ for combined acid and DCA) when using the two-tailed *t*-test.

Interestingly and in support of the MN data above, the up-regulation of iNOS by DCA, acid or acidic DCA was not dependent on NF-κB, as the pre-treatment with a potent NF-kB inhibitor while reducing iNOS gene expression, did not do so significantly ($P = 0.371$, $P = 0.803$ and $P = 0.660$, respectively).

iNOS protein levels were measured in DCA-exposed OE33 cells by western blotting. The results of these experiments are shown in Figure 2. Treatment with 450 µM DCA, but not 300 µM DCA for up to 8 h led to increases in iNOS protein levels. This data confirms the mRNA data that DCA can up-regulate iNOS. The reason that 300 µM DCA did not

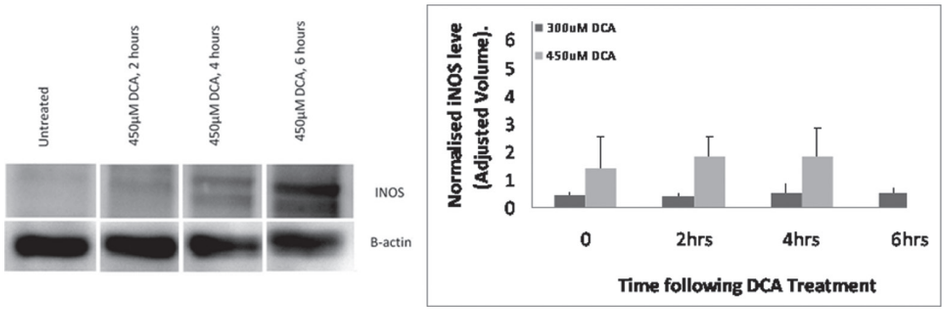

Fig. 2. Western blot data for iNOS protein levels in OE33 cells after exposure to DCA (300 and 450μM) for 2–6 h. iNOS protein levels across the time series increase (at 450 not 300 μM), whereas the housekeeping protein chosen as loading control (B-actin) does not. Left-hand image shows example of western blot (450 μM) and right-hand graph shows average densitometry of four separate westerns confirming increased iNOS protein levels after DCA exposure (450 μM). Due to variation in iNOS protein levels, the increases were not significant relative to the control ($P = 0.18$ for 6 h versus 0 h, at 450 μM).

up-regulate iNOS protein levels (but did up-regulate mRNA levels) is probably related to the lack of sensitivity with western blotting.

Nitric oxide is induced by DCA exposure

To examine if iNOS mRNA induction led to functional iNOS protein activity, NO levels were measured after stimulation with DCA. OE33 cells exposed to DCA showed significant induction of NO, as measured by the NO sensitive DAF fluorescent probe (Figure 3). This induction of NO was dependent on iNOS and was blocked by co-incubation with an iNOS inhibitor (1400W, 10 μM). There was a significant increase in NO after 120 and 180 min ($P = 0.022$ and $P = 0.039$, respectively), with iNOS inhibition significantly reducing this increase after 120 min ($P = 0.003$), but this decrease was not quite statistically significant after 180 min ($P = 0.067$). Pulsed DCA exposure (5 min) representative of physiological exposure scenarios also led to elevated NO synthesis ($P = 0.050$ and 0.039 after 120 and 180 min, respectively), which was also significantly reduced on addition of the iNOS inhibitor after 180 min ($P = 0.005$) (results not shown). Measurement of NO after acid treatment of cells was not included here as technical issues with the fluorescence (presumably due to acid interference with the probe) were observed.

iNOS is not upstream of NF-κB after continuous exposure to DCA

In order to explore the possibility of a positive feedback system involving iNOS and NF-κB, we assessed p65 nuclear localization and IL-8 gene expression (a well-known NF-κB target gene) after continuous exposure to acid (pH 5) or DCA (300 μM) for 60 min, either in the presence or absence of the iNOS inhibitor (Figure 4). In both exposure scenarios, IL-8 expression was induced in an iNOS-dependent manner, with significant abrogation of expression in the presence of 1400W ($P = 0.005$ for DCA and $P = 0.008$ for acid). This confirmed our previous data (45). However, NF-κB activity induced by DCA was not affected by inhibition of iNOS ($P = 0.50$), suggesting that iNOS was not involved upstream of NF-κB in DCA-exposed cells. In the case of acid-exposed cells, significant abrogation of NF-κB activity was seen in the presence of the iNOS inhibitor (~20% reduction, $P = 0.02$), hence iNOS may be partly involved in acid-induced NF-κB activation. Interestingly in both sets of experiments (acid and DCA), the iNOS inhibitor alone led to a significant reduction in basal NF-κB activity ($P = 0.005$ for DCA and $P = 0.002$ for acid) highlighting the role for iNOS and NO in basal NF-κB signalling. Indeed, the reduction in NF-κB activity when cells were exposed to acid plus the iNOS inhibitor may be a reflection of the reduced background levels observed without acid.

iNOS may be upstream of NF-κB after a pulsed exposure to DCA

We considered that continuous exposure to DCA and acid (above) could mask any positive feedback system by supplying a steady stream of primary RO(N)S that would swamp lesser amounts of *de novo* iNOS-driven NO. Hence, in order to circumvent this possibility, we chose to perform pulsed exposures. In these cases, it was presumed that as the DCA is removed after 5 or 30 min and the NF-κB level measured some time later (60 or 120 min), that *de novo* NF-κB activity, secondary to iNOS activity would be more obvious. Figure 5A shows that pulsed DCA exposures of 5 and 30 min induce NF-κB activity when measured at 60 min ($P = 0.006$ and $P = 0.003$, respectively). Only a 30-min pulse activated NF-κB at 120 min ($P = 6.5 \times 10^{-4}$; $P = 0.09$ for the 5-min exposure). Interestingly, pulsed acid exposure (pH 5) did not activate NF-κB at all. Therefore, only DCA was considered in the following cyclical experiments. Figure 5B shows that inhibition of iNOS can significantly abrogate DCA-induced NF-κB activity after a 5 min pulse ($P = 0.007$), whereas the reduction in NF-κB activity by the iNOS inhibitor at 30 and 60 min was non-significant. Basal levels of nuclear NF-κB were again significantly reduced by the iNOS inhibitor ($P = 0.031$). As above, it is possible that the reduced basal level of NF-κB activity with the iNOS inhibitor is responsible (or at least partly responsible) for the drop in NF-κB activity when cells were exposed to pulsed DCA plus the iNOS inhibitor. Nonetheless, this data suggests a signalling role for iNOS/NO in NF-κB activity (basal or induced).

Immunohistochemistry for iNOS

In order to confirm the role of iNOS in patients with BE and EA, we studied iNOS protein levels by IHC in 39 tissues of squamous esophagus, BE and EA. Consistent with previous reports, we saw increased iNOS staining in the advanced histological grades (ADC). Figure 6 shows the staining scores for iNOS relative to the histology of the tissue. Here, a H-score approach was used with the intensity score (0–3) multiplied by a distribution score (0–3). As the tissue progressed from BE to adenocarcinoma, there was increased iNOS staining. This increase was significant using a two-tailed *t*-test ($P = 0.034$). Using intensity alone it was also obvious that iNOS expression was linked to histology. There were 2 out of 10 tissues with squamous histology with an intensity score of 2 or 3. This figure was 5 out of 17 for Barrett's tissues and 6 out of 12 for adenocarcinoma. Interestingly, iNOS staining was not different between squamous and Barrett's tissue, although it should be pointed out that here squamous tissue was adjacent to either Barrett's or adenocarcinoma tissue and hence may have some inflammation associated with it. Figure 6 shows examples of iNOS staining emphasizing the link to histology.

Discussion

We show here the central role that iNOS can play in intracellular signalling in esophageal cells. Not only is iNOS up-regulated by common reflux constituents, leading to NO production, but this axis also has the potential to amplify NF-kB activation. We show that iNOS up-regulation is not controlled by NF-κB after initial exposure to acid or the bile acid DCA in OE33 cells, nor is DCA-induced DNA damage NF-κB dependent in these cells. However, basal

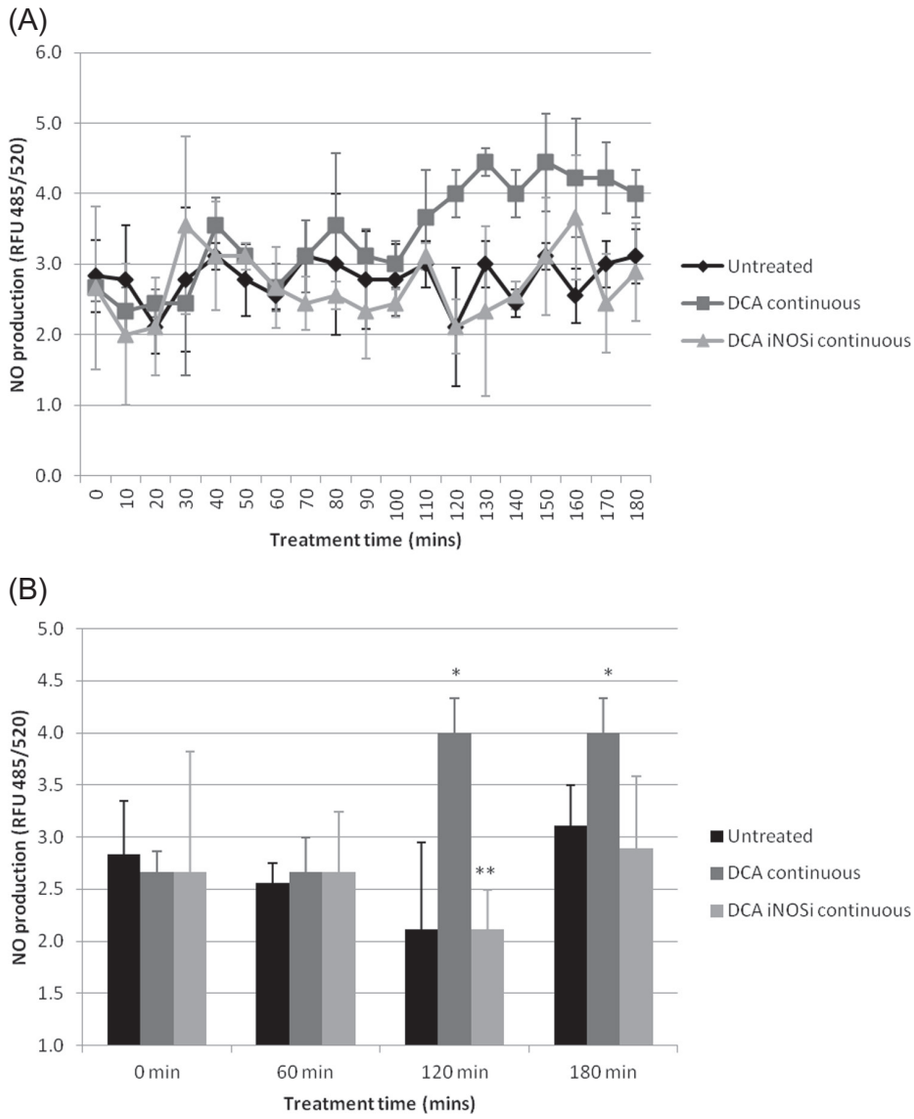

Fig. 3. Nitric oxide levels were assessed in DCA (300 μM)-treated OE33 cells by inclusion of a NO sensitive fluorescent probe (DAF-FM, Invitrogen). Fluorescence was measured in real-time with a plate reader. (**A**) Time series data graph (untreated, DCA treated and DCA + iNOS inhibitor treated). DCA induces elevated NO, and inhibition of iNOS prevented this. (**B**) Graph shows data for 60, 120 and 180 min only. $*P < 0.05$, $**P < 0.01$, respectively.

levels of NF-κB are dependent on iNOS (and presumably NO) and inhibition of iNOS significantly reduces basal NF-κB activity. Also in continuous acid exposure and pulsatile exposure to DCA, we show that iNOS can be located upstream of NF-κB, hence confirming the potential positive feedback system amplifying and prolonging NF-κB activation. However, the reduction in basal levels of NF-κB activity may also contribute to the drop in induced NF-κB activity after acid or DCA exposure. We also confirm previous reports that iNOS is up-regulated in adenocarcinoma tissue, reinforcing the notion that iNOS modulation is a physiological response to reflux exposure *in vivo.* Previous studies have highlighted the role of luminal NO at the gastroesophageal junction (51,52). In these studies it has been elegantly shown that dietary nitrates, after conversion to nitrite by saliva, can generate NO at the gastroesophageal junction due to combination with stomach acid. Hence, this luminal NO has been proposed to contribute to carcinogenesis in the esophagus due to the ability of NO to mutate DNA directly or to react with other dietary compounds to create mutagenic species. Our data here suggests that the burden of nitrosative stress in the esophagus is even further increased due to intracellular (as well as luminal) generation. However, in terms of NO-induced signalling effects at the gastroesophageal junction, both luminal and intracellular generated RO(N)S may be involved in carcinogenesis. Another recent report linking NO to carcinogenesis in the esophagus, demonstrated the role that NO plays in regulating intracellular pH. Goldman *et al.* (53) using intricate measures of intracellular pH, showed that bile acid and stomach acid-induced iNOS blocks activity of the sodium/hydrogen exchanger that can pump protons out of cells to maintain a neutral intracellular pH. By acidifying the intracellular environment, iNOS and NO also play an important role in acid-induced signalling, DNA damage, toxicity and neoplastic development.

Our data here showed that the DNA damage induced by DCA was not dependent on NF-κB, which somewhat contradicts the study by Jolly *et al.* (41). We only studied DCA in this series of experiments, because our previous data showed that only DCA and not the other commonly refluxed bile acids, nor acid alone, was chromosome damaging (42). The reason for the discrepancy between our data and that of Jolly *et al*. could be multifaceted. Not only did we use different cells in this

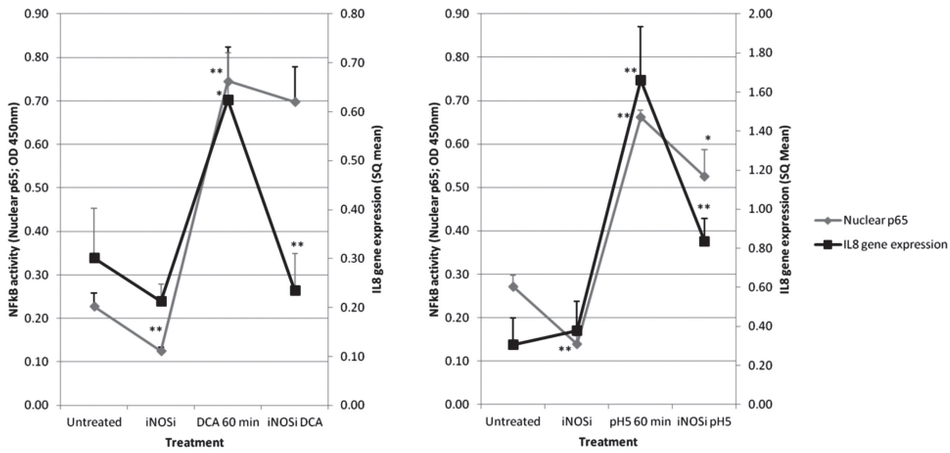

Fig. 4. NF-κB activity and IL8 mRNA levels are shown after continuous treatment with DCA (left hand graph) and acid (right hand graph) for 60 min. NF-κB is activated by DCA and acid. The iNOS inhibitor alone suppresses basal NF-κB activity in both cases and in combination with acid (but not DCA). IL-8 expression is also induced by DCA and acid and its induction is blocked by the iNOS inhibitor. $*P < 0.05$, $** P < 0.01$.

study (OE33 versus Het1A), but we also used different DNA damage endpoints (micronucleus assay versus comet assay). Furthermore, different inhibitors of NF-κB were used by us (IKK VII) and by Jolly *et al.* (TPCK protease inhibitor). In our opinion the main difference to note is the latter, i.e the NF-κB inhibitor. We have extensive experience of studying NF-κB induction by oxidative stress and using direct measures of NF-κB activity, we are confident that the IKK VII inhibitor abolishes NF-κB activity specifically. The TPCK inhibitor is a non-specific inhibitor of proteases, which are important in preventing IkB degradation, but will also have multiple off-target effects. Hence, the difference in the results between the two studies is possibly due to specific versus non-specific NF-κB effects. Our iNOS expression data supports our DNA damage data in that neither DCA induced up-regulation of iNOS nor DCA-induced DNA damage appears to be NF-κB dependent. Our iNOS western data shows increased iNOS protein production at higher doses of DCA (450 μM) than those showing increased gene expression, which presumably reflects the lesser sensitivity of western blotting compared with real-time PCR.

The DNA damage induced by DCA, although not NF-κB dependent showed a possible association with iNOS and hence NO. However, the iNOS inhibitor did not significantly reduce the level of DCA-induced MN when compared with cells treated with DCA alone. If we accept that iNOS and NO were involved in some way in DCA-driven DNA damage then this would fit with the data from Jolly *et al*., who also showed that DCA-induced DNA damage was iNOS dependent (even though different iNOS inhibitors were used in the two studies; L-NAME and 1400W). This observation is certainly interesting and suggests that NO may be a key mediator of reflux-induced mutagenesis. It is well known that NO is a mutagen (20,54). Furthermore, NO is known to repress some DNA repair enzymes through tyrosine nitrosation (22,23), leaving cells susceptible to the mutagenic effects of nitrosative stress. Hence, the DNA damage induced in the cells here may have been the result of a two-pronged attack on both DNA and specific DNA repair proteins. It is also interesting to note that the induction of DNA damage by NO can be countered through an evolved pathway involving p53-mediated down-regulation of iNOS (55) following DNA damage induction. Hence, it is important to consider the antagonistic effects of iNOS stimulation by RONS and repression by DNA damage induced p53 activity. It is interesting to speculate that because mutations of the p53 gene are common in esophageal cancer development (56,57) the antagonistic balance in these cells could be altered. Clearly in esophageal tissues devoid of functional p53 proteins, nitrosative stress may prevail with minimal repression of iNOS occurring. Our IHC data here [and previous reports by Wilson *et al.* (36) and Vaninetti *et al*. (37)] clearly showed up-regulation of iNOS in EA, hence casting doubt on the ability of p53 in these tissues to negatively regulate iNOS expression. It is important to note that p53 and all other proteins can also be inactivated by nitrosative stress (58). In this context it is worth mentioning that in a previous study of EA tissues, there was a noted association between peroxynitrite staining (a result of NO and superoxide combination) and the presence of p53 mutations (37) further emphasizing the possibility that p53 negatively regulates nitrosative stress.

Our IHC data showed significant increases in iNOS protein staining in adenocarcinomas relative to both BE tissue and squamous tissue. However, we saw no difference between BE and squamous with regards to iNOS protein levels. Both Wilson *et al*. (36) and Vaninetti *et al.* (37) have reported previously a large induction of iNOS mRNA levels in BE tissues relative to gastric tissue and squamous tissue, respectively. Interestingly, GERD (squamous) tissue was noted to show iNOS induction also (37). Although the discrepancy between our IHC data and the previous mRNA data may be due to RNA not always necessarily being translated into protein, there is also another explanation. This may centre on our squamous tissue being sourced from adjacent material to BE and adenocarcinoma tissue. Hence, this material may have artificially higher levels of iNOS due to the concurrent reflux exposure in this squamous material. Indeed, Wilson *et al.* (36) who also carried out some IHC in parallel to mRNA analysis noted higher iNOS protein staining in squamous tissues adjacent to BE tissue. Nonetheless, the three reports all confirm up-regulation of iNOS mRNA and or protein in neoplastic development in BE.

Our data clearly shows that under certain circumstances, iNOS sits upstream of NF-κB, suggesting that NO can induce NF-κB activity (e.g. the basal NF-κB data). Hence, reflux-induced oxidative/nitrosative stress that activates NF-κB may be supplemented by secondary iNOS-induced NO production that is initially largely NF-κB independent. Interestingly, our real-time data on iNOS expression suggests non-significant reductions in iNOS expression when the NF-κB inhibitor is present in both continuous exposures to DCA and acid. Another interesting observation was that pulsed DCA, but not pulsed acid-induced NF-κB activity, therefore suggesting that the different reflux components may have differing NF-κB signalling potential. Bile acids like DCA may be more potent at inducing these effects due to better uptake into cells, through detergent-based mechanisms and active transport (59) coupled to membrane damage. Acid exposures may be buffered extra- and intracellularly and internal pH balanced by membrane pumps, leading to less acute effects. This is important in relation to which patients are at most risk of

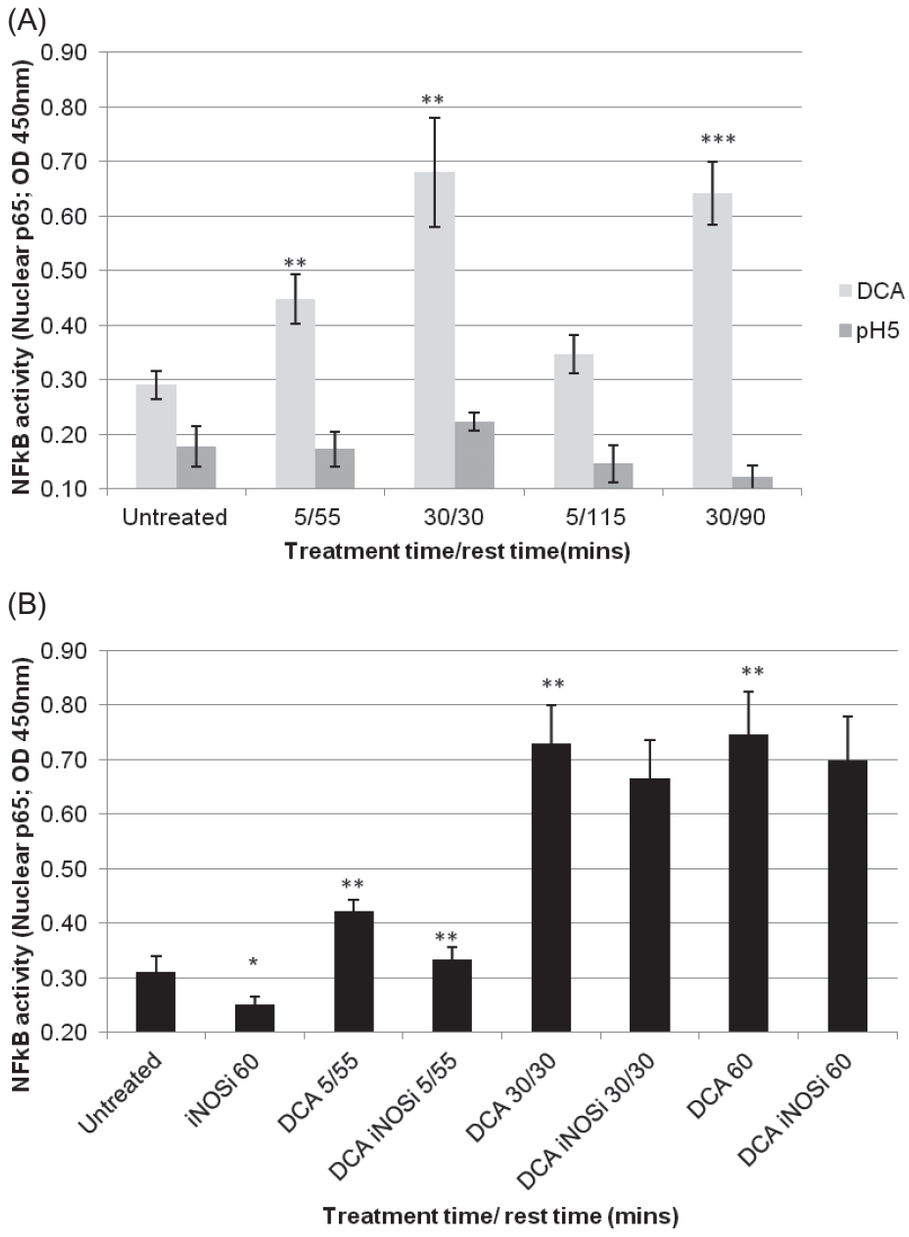

Fig. 5. (**A**) Pulsed DCA, but not pulsed acid, exposures activated NF-κB. For DCA, 5 or 30 min pulses are able to activate NF-κB when measured at 60 or 120 min (only at 120 min with a 30 min pulse). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (**B**) Using a pulsed exposure to DCA, at 5 min the iNOS inhibitor significantly abrogates DCA-driven NF-κB. This was not evident with a 30 min pulse or continual 60 min exposure. Inhibition of iNOS reduced background levels of NF-κB as seen previously in Figure 4 and this reduced basal NF-κB may contribute to the effect observed at 5 min (as mentioned above).

esophageal cancer formation and offers the possibility that this may be determined by their relative reflux constituents.

In conclusion, we show here that iNOS and NO can be upstream of basal NF-κB activity in OE33 cells and could theoretically play a role in the known NF-κB activity that is linked to esophageal cancer development *in vivo*. As NO is also known to lead to DNA damage and inactivate negative regulators of carcinogenesis (e.g. DNA repair and p53 proteins), new therapies aimed at suppressing excess iNOS/NO levels might represent effective treatment strategies for esophageal (and other) cancers.

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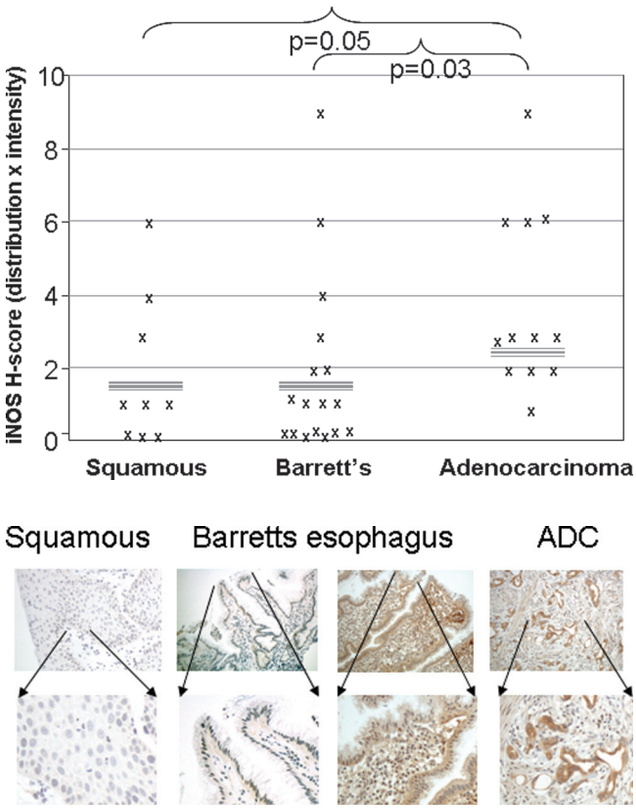

Fig. 6. iNOS IHC data showing increased expression of iNOS protein in adenocarcinoma tissue relative to BE or squamous esophagus. Graph shows individual H-score values for each histological grade (average data indicated by horizontal double line) highlighting the increasing level of iNOS with histological advancement. Representative IHC images are included for the three histological grades (2 BE figures are included showing both a weak and a moderately stained section). Lower images represent zoomed in regions of the upper images.

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